Volume 259, number 1, 213–216 FEB 07985 December 1989

A single point mutation confers tetrodotoxin and saxitoxin insensitivity on the sodium channel II

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Received 13 November 1989

A single point mutation of the rat sodium channel II reduces its sensitivity to tetrodotoxin and saxitoxin by more than three orders of magnitude. The mutation replaces glutamic acid 387 with a glutamine and has only slight effects on the macroscopic current properties, as measured under voltage-clamp in *Xenopus* oocytes injected with the corresponding cDNA-derived mRNA.

Site-directed mutagenesis; cDNA expression; Sodium channel; Tetrodotoxin; Saxitoxin; (*Xenopus* oocyte)

1. INTRODUCTION

The voltage-gated sodium channel is a membrane protein that is essential for the generation of action potentials in excitable cells [1]. The primary structures of several types of sodium channels have been obtained by cDNA cloning and sequencing [2–7]. Injection of mRNAs derived from cloned cDNAs into *Xenopus* oocytes leads to functional expression of sodium channels. Sodium currents recorded from injected oocytes have been shown to be similar to those recorded from excitable cells [8–10].

It is well known that a large number of biological toxins modify the properties of sodium channels. Up to four toxin-binding sites have been postulated for the sodium channel [11], one of which is the binding site for TTX and STX. Occupancy of this site, thought to be located near the extracellular mouth of the channel, blocks the flow of sodium ions. It has been assumed that the TTX- and STX-binding site contains negatively charged functional groups because of the inhibition of toxin binding by carboxyl-modifying reagents [12–16], some monovalent cations, divalent metal ions and protons [17]. Here we report that a single point mutation of the rat sodium channel II which changes the glutamic acid residue 387 to glutamine (E387Q) renders the channel insensitive to concentrations of TTX and STX up to 10 μM, while it affects the macroscopic current properties only slightly.

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Abbreviations: TTX, tetrodotoxin; STX; saxitoxin; TMO, trimethyloxonium

2. MATERIALS AND METHODS

The codons for glutamine residues 86 and 88 in the β-lactamase gene [18] of pBSM13(+) and (−) (Stratagene) were converted to amber codons by replacing the 37-base pair *DdeI/HpaII* fragment with synthetic DNA to yield the vectors pKMN(+) and (−), respectively. pKMN(+) and (−) can express the ampicillin-resistant phenotype only when strains carrying *supE* suppressors are used as host bacteria. Thus, they are useful for oligonucleotide-directed mutagenesis by the gapped duplex DNA approach [19]. The *XbaI*(541)/*SmaI*(1898) fragment from pRII-2 [8] was subcloned into pBSM13(+) and pKMN(+) to yield pBSRIIXS and pKMNRIIXS, respectively. The single-stranded (+) DNA was prepared from pKMNRIIXS using the helper phage R408 [20]. The ~4.3-kilobase pair (kb) *Tth*111I(1038)/*NcoI*(1275) vector-containing fragment from pBSRIIXS was isolated as double-stranded DNA. The glutamic acid residue 387 of rat sodium channel II was mutated to glutamine (E387Q) using the synthetic oligonucleotide 5′-ATAAAGGTTTT-GCCAGAAGTCTTGAGTCATGAG-3′ as described in [19]. The *ApaI*(721)/*XmaI*(1896) fragment, isolated from a plasmid bearing this mutation, was substituted for the corresponding fragment of pRII-2A [21] to yield pRIICM-1. mRNAs specific for the wild type (pRII-2A) and the mutant sodium channel were synthesized in vitro using *SalI*-cleaved plasmids as templates [8,21].

Xenopus laevis oocytes were injected with the wild type mRNA (0.2 μg/μl) or the mutant mRNA (0.3–0.5 μg/μl) and incubated for 4–7 days [22]. Measurements of macroscopic currents in cell-attached membrane patches were made as described previously [9] and analyzed as detailed in [21]. Toxin sensitivity was assayed by perfusing TTX- or STX-containing external solution and measuring whole-cell peak inward currents with a two-electrode voltage clamp [8,9]. The external bathing and the pipette solution had the following composition (in mM): NaCl 115, KCl 2.5, $CaCl_2$ 1.8, Hepes 10 (pH 7.2). TTX was from Sigma, and STX from Calbiochem.

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00145793/89/$3.50

3. RESULTS

Current records for the wild type sodium channel II (A) and the mutant E387Q (B) are shown in fig.1. Depolarizing pulses from a holding potential of −100 mV to test potentials between −60 and +90 mV elicited sodium currents with similar properties. The major difference between the wild type and mutant channels is the larger outward current relative to the inward current in the mutant. The scaled current-voltage relationships of the wild type and mutant allow a more detailed comparison (fig.2A). The voltage dependence of activation of the mutant is shifted to the left by about 15 mV relative to the wild type; however, the reversal potential for sodium currents is close to the expected Nernst equilibrium potential. This indicates that the selectivity for sodium ions is not greatly affected by the mutation. Fig.2B compares current-voltage relationships for tail currents from the wild type and mutant channels. The instantaneous tail current-voltage relation is less curved for the mutant than for the wild type, while the reversal potential is unaffected. In experiments such as those shown in fig.1B, biphasic current traces were observed near the reversal potential. We attribute this to a reduced ionic current which makes the gating current more evident. A rough estimate of the channel density can be derived from the size of gating currents measured near the reversal potential. On the basis of this estimate, an approximately four-fold decrease in inward current can be obtained. These results support the hypothesis that the inward conductance is reduced in the mutant E387Q.

The most dramatic difference between the wild type and the mutant E387Q is a virtual loss of sensitivity to TTX and STX resulting from the mutation. Fig.3 shows that 1 μM of TTX applied externally had no effect on the current records obtained from the mutant (even after 4 min). The TTX concentration that reduced the current by 50% (IC_{50}) in the wild type was 18 nM (fig.4A), in close agreement with values obtained previously [8,23,24]. The sensitivity to STX, which competes with TTX for the same binding site, was also essentially abolished in the mutant E387Q (fig.4B). The mutation reduced the sensitivity to both TTX and STX by more than three orders of magnitude.

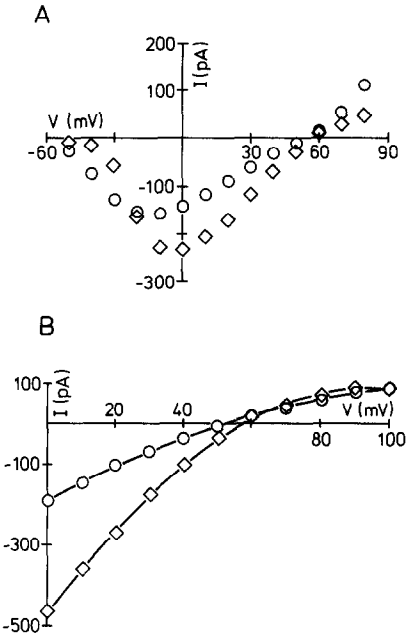

Fig.2. (A) Peak current-voltage relationships for the wild type sodium channel II (diamonds) and the mutant E387Q (circles). The current amplitudes for the wild type have been reduced by a factor of five to allow better comparison. (B) Current-voltage relationships for tail currents after a 0.5-ms depolarizing pulse to +30 mV. The wild type tail current amplitudes have been scaled down by a factor of 1.2 so that the outward currents become comparable. All the data were obtained from macro-patches.

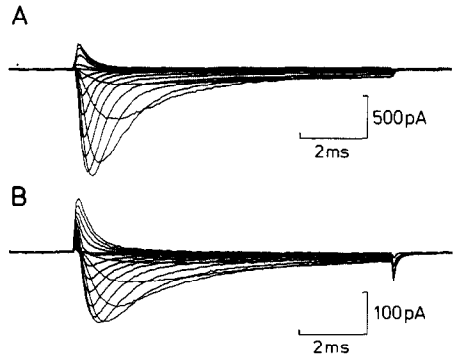

Fig.1. Current responses to depolarizing steps between −60 and +90 mV, in steps of 10 mV from a holding potential of −100 mV (A) Wild type sodium channel II (average of 4 records). (B) Mutant E387Q (average of 16 records). The currents shown were obtained from macro-patches in the cell-attached configuration after subtraction of linear leak and capacitive currents. Temperature = 15°C.

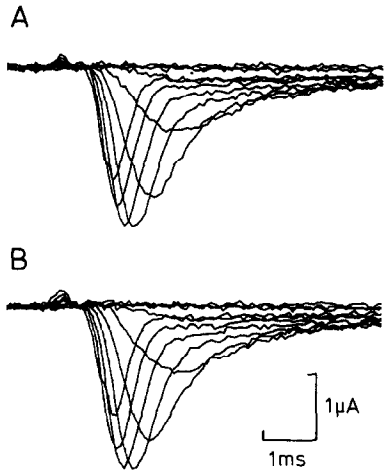

Fig.3. Whole-cell current responses of the mutant E387Q recorded with a two-electrode voltage clamp. Depolarizing steps were between −60 and +30 mV, in steps of 10 mV from a holding potential of −80 mV. Temperature = 23°C. (A) Control. (B) Four minutes after perfusion with extracellular solution containing 1 μM of TTX.

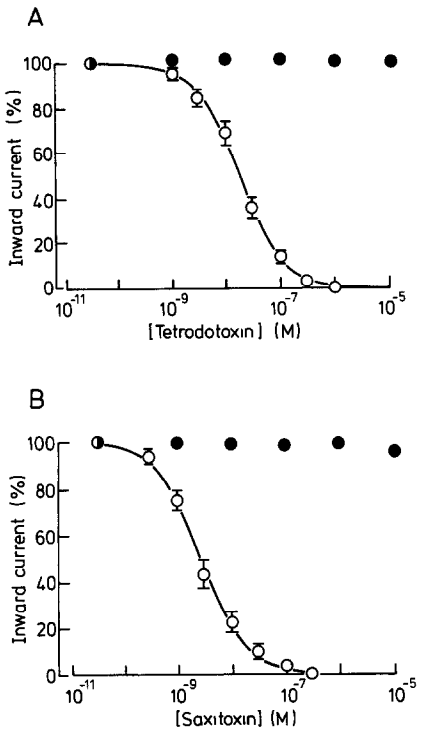

Fig.4. Dose-response curves for the wild type (open circles) and the mutant E387Q (filled circles) to TTX (A) and STX (B). The smooth lines are fitted to the open circles according to the equation $y = (1 + (T/IC_{50})^n)^{-1}$, where T is the toxin concentration and n the Hill coefficient. Error bars are ± SD and indicated only when larger than the symbol size. (A) For the wild type, the IC_{50} value for TTX is 18 nM with a Hill coefficient of 1.1. Data were averaged from 7 (wild type) and 8 (mutant) experiments. (B) For the wild type, the IC_{50} value for STX is 2.7 nM with a Hill coefficient of 1.1. Data were averaged from 4 (wild type) and 7 (mutant) experiments.

4. DISCUSSION

The results presented here show that replacing the glutamic acid residue 387 by glutamine greatly reduces the sensitivity of the rat sodium channel II to TTX and STX. This glutamic acid residue, which is concerved in all sodium channels with known sequence, is located between segments S5 and S6 of repeat I. According to current models for the transmembrane topology of the sodium channel [2,3,25], the region between segments S5 and S6 is assigned to the extracellular side of the membrane. Our results support this assignment since the binding site for TTX and STX is known to be located on the extracellular side [26]. In this context it has recently been reported that a glutamic acid residue located in the corresponding region of a voltage-gated potassium channel is involved in charybdotoxin binding [27].

It has been suggested that a negatively charged carboxyl oxygen is essential for the binding of TTX and STX to the sodium channel, partly because the *O*-methylation of such a group by TMO markedly reduces toxin sensitivity [15,16]. TMO-modified, TTX-resistant sodium channels have a small single-channel conductance with a more linear instantaneous current–voltage relationship than that of unmodified sodium channels [28]. However, the chemically modified channels retain their selective permeability to sodium ions [15]. Our results resemble these findings and thus suggest that the inhibitory effect of carboxyl-modifying reagents on toxin binding may involve modification of the glutamic acid residue 387.

TTX-insensitive sodium channels in some neuroblastoma cell lines [29] or denervated rat muscle cells [30] have the same high selectivity for sodium ions as TTX-sensitive sodium channels. It has been shown that TTX-insensitive sodium channels in myoblasts have a lower single-channel conductance than TTX-sensitive sodium channels and can be activated at more hyperpolarized potentials [31], as is the case for the mutant E387Q. Interestingly, the putative TTX-resistant rat heart sodium channel isoform, whose amino acid sequence has recently been reported [7], shows substitution of an arginine residue for the conserved asparagine next to the glutamic acid corresponding to that neutralized in the mutant E387Q. This substitution may cause an effect similar to that of neutralizing the negative charge of the glutamic acid residue 387 in rat sodium channel II.

The binding site for TTX and STX is thought to be located close to the mouth of the sodium channel [26]. Since the affinity for toxin binding can be modified without affecting ion selectivity, it is unlikely that the same negative charges are involved both in the binding of these toxins and in the ion selectivity of the channel. Nevertheless, the sites for toxin binding and for selectivity are thought to be close since chemical modifications which affect toxin binding reduce the inward current [28]. This reduction in inward current may be attributed to the lower extracellular concentration of sodium ions near the ion pore which would result from the localized decrease in negative charge. The outward current would then be less affected than the inward current. This is the case for the mutant E387Q, in which the outward current seemingly increases relative to the inward current. The data thus suggest that the region between segments S5 and S6 in repeat I of sodium channel II is in close proximity to the channel mouth.

Acknowledgements: We thank Drs Keiji Imoto and Michael Cahalan for critically reading the manuscript. This investigation was supported in part by research grants from the Ministry of Ecucation, Science and Culture and the Science and Technology Agency of Japan, the Mitsubishi Foundation and the Japanese Foundation of Metabolism and Diseases.

REFERENCES

[1] Hille, B. (1984) in: Ionic Channels of Excitable Membranes, Sinauer, Sunderland, MA.

[2] Noda, M., Shimizu, S., Tanabe, T., Takai, T., Kayano, T., Ikeda, T., Takahashi, H., Nakayama, H., Kanaoka, Y., Minamino, N., Kangawa, K., Matsuo, H., Raftery, M.A., Hirose, T., Inayama, S., Hayashida, H., Miyata, T. and Numa, S. (1984) Nature 312, 121-127.
[3] Noda, M., Ikeda, T., Kayano, T., Suzuki, H., Takeshima, H., Kurasaki, M., Takahashi, H. and Numa, S. (1986) Nature 320, 188-192.
[4] Kayano, T., Noda, M., Flockerzi, V., Takahashi, H. and Numa, S. (1988) FEBS Lett. 228, 187-194.
[5] Auld, V.J., Goldin, A.L., Krafte, D.S., Marshall, J., Dunn, J.M., Catterall, W.A., Lester, H.A., Davidson, N. and Dunn, R.J. (1988) Neuron 1, 449-461.
[6] Trimmer, J.S., Cooperman, S.S., Tomiko, S.A., Zhou, J., Crean, S.M., Boyle, M.B., Kallen, R.G., Sheng, Z., Barchi, R.L., Sigworth, F.J., Goodman, R.H., Agnew, W.S. and Mandel, G. (1989) NEuron 3, 33-49.
[7] Rogart, R.B., Cribbs, L.L., Muglia, L.K., Kephart, D.D. and Kaiser, M.W. (1989) Proc. Natl. Acad. Sci. USA 86, 8170-8174.
[8] Noda, M., Ikeda, T., Suzuki, H., Takeshima, H., Takahashi, T., Kuno, M. and Numa, S. (1986) Nature 322, 826-828.
[9] Stühmer, W., Methfessel, C., Sakmann, B., Noda, M. and Numa, S. (1987) Eur. Biophys. J. 14, 131-138.
[10] Suzuki, H., Beckh, S., Kubo, H., Yahagi, N., Ishida, H., Kayano, T., Noda, M. and Numa, S. (1988) FEBS Lett. 228, 195-200.
[11] Catterall, W.A. (1986) Annu. Rev. Biochem. 55, 953-985.
[12] Shrager, P. and Profera, C. (1973) Biochem. Biophys. Acta 318, 141-146.
[13] Reed, J.K. and Raftery, M.A. (1976) Biochemistry 15, 944-953.
[14] Baker, P.F. and Rubinson, K.A. (1975) Nature 257, 412-414.
[15] Spalding, B.C. (1980) J. Physiol. 305, 485-500.
[16] Worley, J.F. iii, French, R.J. and Krueger, B.K. (1986) J. Gen. Physiol. 87, 327-349.
[17] Henderson, R., Ritchie, J.M. and Strichartz, G.R. (1974) Proc. Natl. Acad. Sci. USA 71, 3936-3940.
[18] Sutcliffe, J.G. (1979) Cold Spring Harbor Symp. Quant. Biol. 43, 77-90.
[19] Kramer, W., Drutsa, V., Jansen, H.-W. Kramer, B., Pflugfelder, M. and Fritz, H.-J. (1984) Nucleic Acids Res. 12, 9441-9456.
[20] Russel, M., Kidd, S. and Kelley, M.R. (1986) Gene 45, 333-338.
[21] Stühmer, W., Conti, F., Suzuki, H., Wang, X., Noda, M., Yahagi, N., Kubo, H. and Numa, S. (1989) Nature 339, 597-603.
[22] Methfessel, C., Witzemann, V., Takahashi, T., Mishina, M., Numa, S. and Sakmann, B. (1986) Pflügers Arch. Ges. Physiol. 407, 577-588.
[23] Gundersen, C.B., Miledi, R. and Parker, I. (1984) Nature 308, 421-424.
[24] Stuhmer, W., Methfessel, C., Witzemann, V. and Sakmann, B. (1986) in: Membrane Control of Cellular Activity, vol. 33, Fortschritte der Zoologie (Lüttgau, H.C. ed.) pp. 155-159, Gustav Fischer, Stuttgart.
[25] Guy, R.H. (1988) in: Molecular Biology of Ionic Channels, vol. 33, Current Topics in Membranes and Transport (Agnew, W.S., Claudio, T. and Sigworth, F.J. eds), pp. 289-308, Academic Press, San Diego.
[26] Hille, B. (1975) Biphysical J. 15, 615-619.
[27] MacKinnon, R. and Miller, C. (1989) Science 245, 1382-1385.
[28] Sigworth, F.J. and Spalding, B.C. (1980) Nature 283, 293-295.
[29] Huang, L.Y.M., Catterall, W.A. and Ehrenstein, G. (1979) J. Gen. Physiol. 73, 839-854.
[30] Pappone, P.A. (1980) J. Physiol. (Lond.) 306, 377-410.
[31] Weiss, R.E. and Horn, R. (1986) Science 233, 361-364.