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Mapping the site of block by tetrodotoxin and saxitoxin of sodium channel II

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The SS2 and adjacent regions of the 4 internal repeats of sodium channel II were subjected to single mutations involving, mainly, charged amino acid residues. These sodium channel mutants, expressed in *Xenopus* oocytes by microinjection of cDNA-derived mRNAs, were tested for sensitivity to tetrodotoxin and saxitoxin and for single-channel conductance. The results obtained show that mutations involving 2 clusters of predominantly negatively charged residues, located at equivalent positions in the SS2 segment of the 4 repeats, strongly reduce toxin sensitivity, whereas mutations of adjacent residues exert much smaller or no effects. This suggests that the 2 clusters of residues, probably forming ring structures, take part in the extracellular mouth and/or the pore wall of the sodium channel. This view is further supported by our finding that all mutations reducing net negative charge in these amino acid clusters cause a marked decrease in single-channel conductance.

Sodium channel; Site-directed mutagenesis; cDNA expression; Tetrodotoxin; Saxitoxin; Single-channel conductance

1. INTRODUCTION

Tetrodotoxin (TTX) and saxitoxin (STX) are specific and potent blockers of the sodium channel, which is essential for the generation of action potentials in excitable cells. Several lines of evidence suggest that these positively charged toxins are bound close to the extracellular mouth of the channel [1] in a region containing negatively charged groups: the binding is inhibited by carboxyl-modifying agents [2–6], as well as by some monovalent cations, divalent metal ions and protons [7]. The primary structures of several types of sodium channel are known and they all contain 4 repeating units of homology (repeats I–IV) [8–13]. Recently Noda et al. [14] have shown that a single mutation (E387Q) in rat sodium channel II that alters the glutamic acid residue 387 (E387) to glutamine renders the channel insensitive to TTX and STX and reduces its single-channel conductance, whereas its macroscopic current properties are only slightly affected. In addition, Pusch et al. [15] have shown that another mutation (D384N) that neutralizes the aspartic acid residue 384 (D384) to asparagine almost completely eliminates ionic currents without preventing gating function as judged by gating currents. These results have suggested that the residues D384 and E387 are located at the extracellular mouth or inside the ion-conducting pore of the channel. D384 and E387 belong to the short segment SS2 in the region between the hydrophobic segments S5 and S6 in repeat I (see Fig. 1). In each repeat the S5–S6 region is thought to contain 2 short segments, SS1 and SS2, that may partly span the membrane as a hairpin and the SS2 segments have been postulated as forming part of the channel lining [16,17]. In line with this view is the finding that mutations in the SS1–SS2 region of potassium channels affect sensitivity to tetraethylammonium and/or ion permeation properties [18–21]. The present investigation deals with the effects on toxin sensitivity and single-channel conductance of site-directed mutations in the region encompassing the SS2 segment of each of the 4 repeats, focussing mainly on charged residues. The results obtained identify 2 clusters of predominantly negatively charged residues, comprising D384 and E387 of repeat I and the corresponding residues of the other repeats, as major determinants of toxin sensitivity.

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Abbreviations: TTX, tetrodotoxin; STX, saxitoxin.

2. MATERIALS AND METHODS

The recombinant plasmid pRII-2A [22], which carries the entire protein-coding sequence of the rat sodium channel II cDNA linked with the bacteriophage SP6 promotor [23], and its mutants were used for synthesis in vitro of specific mRNAs. The plasmids carrying mutants were constructed [24], using oligodeoxyribonucleotides prepared with an automatic DNA synthesizer (Applied Biosystems). The mutant plasmids differ from pRII-2A as follows (the substituted nucleotides with residue numbers [9] are given and the plasmids carrying

mutant cDNAs are named after the mutant specification). pAM(D368N): A, 1102. pAM(D368K): A, 1102; G, 1104. pAM(R379Q): A, 1136, 1137. pAM(Q383E): G, 1147. pAM(Q383K): A, 1147. pAM(D384E): A, 1152. pAM(W386Y): A, 1157; T, 1158. pAM(E387S): T, 1159; C, 1160. pAM(E387Y): T, 1159; C, 1161. pAM(N388R): C, 1162; G, 1163. pAM(Q391K): A, 1171. pAM(G941E): A, 2822. pAM(E942Q): C, 2824. pAM(E942K): A, 2824. pAM(W943Y): A, 2828; T, 2829. pAM(E945Q): C, 2833. pAM(E945K): A, 2833. pAM(K1422E): G, 4264. pAM(M1425Q): C, 4273; A, 4274. pAM(M1425K): A, 4274. pAM(D1426N): A, 4276. pAM(D1426Q): C, 4276; G, 4278. pAM(D1426K): A, 4276; G, 4278. pAM(A1714E): A, 5141, 5142. pAM(D1717N): A, 5149. pAM(D1717Q): C, 5149; G 5151. pAM(D1717K): A, 5149; G, 5151. The plasmids carrying cDNAs encoding the mutant E387Q (pRIICM-1) or the mutant D384N (pRIICM-2) have been described previously [14,15]. mRNAs specific for the wild-type and mutant sodium channels were synthesized in vitro [23], using *Sal*I-cleaved plasmids as templates. Transcription was primed [25] with the cap dinucleotide G(5')ppp(5')G (1 mM).

Xenopus laevis oocytes were injected with the wild-type or a mutant mRNA and incubated as described in [14,26]. Toxin sensitivity was assayed by perfusing TTX- or STX-containing external solutions and measuring whole-cell peak inward currents with a 2-electrode voltage clamp [26] at room temperature. The external bathing solution had the following composition (in mM): NaCl 115, KCl 2.5, $CaCl_2$ 1.8, HEPES 10 (pH 7.2 with NaOH). Due to the fact that wild-type as well as mutated channels exhibited a use-dependent block for TTX and STX, care was taken to reduce the extent of depolarization to a minimum. Single-channel conductance was measured by non-stationary noise analysis [27,28]. Macroscopic currents from inside-out macro-patches were recorded at room temperature in a bath solution of the following composition (in mM): KCl 100, NaCl 16, EGTA 1.8, HEPES 10 (pH 7.2 with KOH). The pipettes contained (in mM): NaCl 100, KCl 16, $CaCl_2$ 1.8, HEPES 10 (pH 7.2 with NaOH). Estimates for single-channel conductance were obtained by at least 1 noise determination at −20 mV together with measurement of the reversal potential from macroscopic current-voltage relations. In case of the mutants D384N and E942Q the single-channel conductance was estimated by comparing the size of the small ionic currents with that of the gating currents [14,15].

3. RESULTS AND DISCUSSION

Table I lists the values of IC_{50} for TTX and STX (toxin concentration required for inhibiting peak current by 50%) and the single-channel conductances measured for the sodium channel mutants studied. In all 4 repeats, mutations were found that made the IC_{50} for TTX and/or STX more than 100 times larger than the

Table I

Effects of single mutations on TTX and STX sensitivities and single-channel conductance

Mutant	ΔQ	IC_{50}-TTX (nM)	n	IC_{50}-STX (nM)	n	Conductance (pS)
Wild-type	0	18 ± 4	6	1.2 ± 0.2	7	15.4
R379Q	−1	17 ± 2	3	1.5 ± 0.1	2	11.7
Q383E	−1	28 ± 3	5	1.9 ± 0.3	5	8.8
Q383K	+1	24 ± 13	4	1.9 ± 0.7	3	2.4
D384E	0	55 ± 30	4	1.8 ± 0.6	4	15.1
D384N	+1	>10000	2	>1000	3	<0.1
W386Y	0	270 ± 180	4	40 ± 10	3	8.0
E387Q	+1	>10000	8	>1000	7	3.1
E387S	+1	>10000	3	>1000	2	1.4
E387Y	+1	>10000	4	>1000	3	<0.1
N388R	+1	37 ± 11	5	7.1 ± 1.7	5	13.1
Q391K	+1	28 ± 8	4	5.7 ± 0.5	4	12.8
E942Q	+1	>10000	5	>1000	5	<0.5
W943Y	0	25 ± 11	4	2.1 ± 0.6	4	14.4
E945Q	+1	2800 ± 180	4	>1000	5	8.2
E945K	+2	>10000	5	>1000	5	1.4
K1422E	−2	>10000	4	>1000	4	14.2
M1425Q	0	240 ± 90	5	14 ± 3	4	14.4
M1425K	+1	>10000	5	1000	5	4.1
D1426N	+1	30 ± 11	4	8.9 ± 0.4	3	14.0
D1426Q	+1	9.8 ± 2.3	3	2.7 ± 0.6	2	15.1
D1426K	+2	7.1 ± 3.0	3	38 ± 16	3	13.4
A1714E	−1	990 ± 220	7	450 ± 60	3	12.8
D1717N	+1	350 ± 90	5	>1000	4	8.8
D1717Q	+1	>10000	2	>1000	2	6.6
D1717K	+2	4100 ± 1900	4	>1000	5	<5

The IC_{50}-values are given as means ± SD. ΔQ is the change in net charge caused by the mutation. *n*, Number of oocytes used, taken from at least 2 different series of successful injections. The data for toxin sensitivities of the mutant E387Q have been taken from Noda et al. [14]. Four other mutants, D368N, D368K, G941E and E942K, were tested which are not listed in the table. The mutants D368N and D368K yielded no detectable currents. The mutant E942K had ionic currents smaller than gating currents; TTX and STX sensitivities of the ionic currents of this mutant were not measured. For the mutant G941E, 2 out of more than 90 oocytes tested showed small ionic currents (less than 250 nA) with an IC_{50} value lower than 80 nM for TTX and lower than 2 nM for STX.

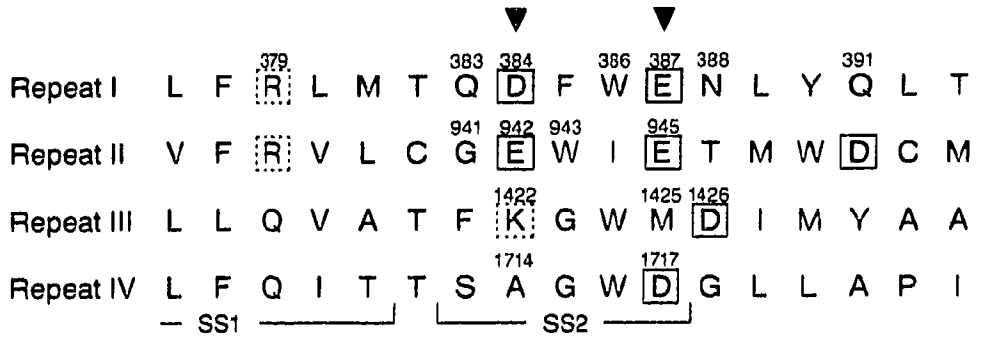

Fig. 1. Regions encompassing the SS2 segment [17] of the 4 repeats of rat sodium channel II. The relevant amino acid sequences (1-letter code) [9] are aligned as in [17]. The positions of the SS1 and SS2 segments and the numbers of the amino acid residues mutated are indicated. Negatively charged residues are boxed with solid lines, and positively charged residues with broken lines. The positions of the clusters of residues that have been identified as major determinants of toxin sensitivity are indicated by arrowheads.

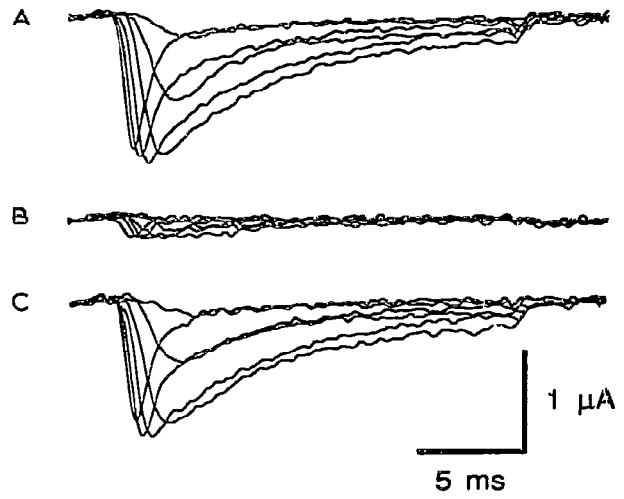

Fig. 2. Whole-cell current responses recorded from a *Xenopus* oocyte expressing the mutant D1717N before (A) and after exposure to 1 μM TTX (B) and after subsequent exposure to 1 μM STX (C). Records were obtained with a 2-electrode voltage clamp, using depolarizing steps between −25 mV and +25 mV in steps of 10 mV. Holding potential −80 mV. Filter frequency 1.5 kHz. The current signals have been corrected for leakage and capacitive transients. Note the increase in current on switching from TTX to STX.

wild-type values. These mutations involve changes in the charge of the residues D384, E387, E942, E945, K1422, M1425, A1714 and D1717 (Fig. 1). Charge mutations at other positions (R379Q, Q383E, Q383K, N388R, Q391K, G941E, D1426N, D1426Q, D1426K) produced only minor or insignificant changes in toxin sensitivity. Mutations without a change in the net charge at positions 384 (D384E) and 1425 (M1425Q) and at other positions of the SS2 segment (W386Y and W943Y) also had minor or insignificant effects.

Some mutations affected TTX and STX sensitivity to different degrees, as exemplified by the mutant D1717N, which is essentially insensitive to STX but has an IC_{50} for TTX increased only 20-fold (Fig. 2). TTX and STX are thought to bind to the same toxin binding site [1], although they have different structures and carry 1 and 2 positive charges, respectively. Thus, it is not surprising that modifications of the common binding site affect their binding affinity differentially. Interestingly, changes in toxin sensitivity were not solely or directly related to the decrease in net negative charge resulting from the mutations, as observed for K1422E and A1714E. The mutant N388R, which like TTX-resistant sodium channels from heart and skeletal muscle [13,29] has an arginine at position 388, showed only a slight decrease in TTX and STX sensitivity.

All of the mutations involving a decrease in net negative charge that strongly reduced toxin sensitivity also caused a marked decrease in single-channel conductance. The mutants K1422E and A1714E, which have an increased net negative charge, showed nearly no reduction in single-channel conductance, whereas their toxin sensitivity was strongly reduced. In some mutants (Q383E and Q383K), single-channel conductance was affected without significant alterations in toxin sensitivity.

Our results show that the sensitivity to TTX and STX of the sodium channel is strongly reduced by mutations of specific amino acid residues in the SS2 segment of each of the 4 internal repeats. These residues are found in 2 clusters (D384, E942, K1422 and A1714 in 1 cluster and E387, E945, M1425 and D1717 in the other), the residues in each cluster being equivalently positioned in the 4 repeats (Fig. 1). Mutations of other amino acid residues in the SS2 and adjacent regions cause minor or insignificant changes in toxin sensitivity. These results, together with our observation that the gating kinetics of all the mutants listed in Table I appeared normal, provide evidence that the amino acid residues in the 2 clusters we have identified interact specifically and directly with the toxins. This suggests that these 2 clusters of predominantly negatively charged residues, probably forming ring structures as in the case of the nicotinic acetylcholine receptor channel [30,31], line part of the extracellular mouth and/or the pore wall. This view is further supported by our finding that all mutations reducing net negative charge in these amino acid clusters markedly diminish single-channel conductance.

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REFERENCES

[1] Hille, B. (1984) Ionic Channels of Excitable Membranes, Sinauer, Sunderland, MA.
[2] Shrager, P. and Profera, C. (1973) Biochim. Biophys. Acta 318, 141–146.
[3] Baker, P.F. and Rubinson, K.A. (1975) Nature 257, 412–414.
[4] Reed, J.K. and Raftery, M.A. (1976) Biochemistry 15, 944–953.
[5] Spalding, B.C. (1980) J. Physiol. 305, 485–500.
[6] Worley III, J.F., French, R.J. and Krueger, B.K. (1986) J. Gen. Physiol. 87, 327–349.
[7] Henderson, R., Ritchie, J.M. and Strichartz, G.R. (1974) Proc. Natl. Acad. Sci. USA 71, 3936–3940.
[8] 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.
[9] Noda, M., Ikeda, T., Kayano, T., Suzuki, H., Takeshima, H., Kurasaki, M., Takahashi, H. and Numa, S. (1986) Nature 320, 188–192.
[10] Kayano, T., Noda, M., Flockerzi, V., Takahashi, H. and Numa, S. (1988) FEBS Lett. 228, 187–194.
[11] 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.
[12] 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., Sig-

worth, F.J., Goodman, R.H., Agnew, W.S. and Mandel, G. (1989) Neuron 3, 33–49.
[13] 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.
[14] Noda, M., Suzuki, H., Numa, S. and Stühmer, W. (1989) FEBS Lett. 259, 213–216.
[15] Pusch, M., Noda, M., Stühmer, W., Numa, S. and Conti, F. (1991) Eur. Biophys. J. 20, 127–133.
[16] Guy, H.R. and Seetharamulu, P. (1986) Proc. Natl. Acad. Sci. USA 83, 508–512.
[17] Guy, H.R. and Conti, F. (1990) Trends Neurosci. 13, 201–206.
[18] MacKinnon, R. and Yellen, G. (1990) Science 250, 276–279.
[19] Yellen, G., Jurman, M.E., Abramson, T. and MacKinnon, R. (1991) Science 251, 939–942.
[20] Yool, A.J. and Schwarz, T.L. (1991) Nature 349, 700–704.
[21] Hartmann, H.A., Kirsch, G.E., Drewe, J.A., Taglialatela, M., Joho, R.H. and Brown, A.M. (1991) Science 251, 942–944.
[22] Stühmer, W., Conti, F., Suzuki, H., Wang, X., Noda, M., Yahagi, N., Kubo, H. and Numa, S. (1989) Nature 339, 597–603.
[23] Melton, D.A., Krieg, P.A., Rebagliati, M.R., Maniatis, T., Zinn, K. and Green, M.R. (1984) Nucleic Acids Res. 12, 7035–7056.
[24] Sayers, J.R. and Eckstein, F. (1989) in: Protein Function – A Practical Approach (Creighton, T.E. ed.) pp. 279–295, IRL Press, Oxford.
[25] Konarska, M.M., Padgett, R.A. and Sharp, P.A. (1984) Cell 38, 731–736.
[26] Methfessel, C., Witzemann, V., Takahashi, T., Mishina, M., Numa, S. and Sakmann, B. (1986) Pflügers Arch. Gesamte Physiol. Menschen Tiere 407, 577–588.
[27] Sigworth, F.J. (1980) J. Physiol. 307, 97–129.
[28] Heinemann, S.H. and Conti, F. (1991) Methods Enzymol., (in press).
[29] Cribbs, L.L., Satin, J., Fozzard, H.A. and Rogart, R.B. (1990) FEBS Lett. 275, 195–200.
[30] Imoto, K., Busch, C., Sakmann, B., Mishina, M., Konno, T., Nakai, J., Bujo, H., Mori, Y., Fukuda, K. and Numa, S. (1988) Nature 335, 645–648.
[31] Imoto, K., Konno, T., Nakai, J., Wang, F., Mishina, M. and Numa, S. (1991) FEBS Lett. 289, 193–200.