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PRIMARY STRUCTURE OF SQUID SODIUM CHANNEL DEDUCED FROM THE COMPLEMENTARY DNA SEQUENCE

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SUMMARY: The complete amino acid sequence of a sodium channel from squid *Loligo bleekeri* has been deduced by cloning and sequence analysis of the complementary DNA. The deduced sequence revealed an organization virtually identical to the vertebrate sodium channel proteins; four homologous domains containing all six membrane-spanning structures are repeated in tandem with connecting linkers of various sizes. A unique feature of the squid Na channel is the 1,522 residue sequence, approximately three fourths of those of the rat sodium channels I, II and III.

The sodium channel is a voltage-gated ionic channel essential for the generation of action potentials (1, 2). The complete amino acid sequences of the neural sodium channels deduced by cloning and sequence analysis of the cDNAs have all been limited to vertebrates; that is, those from the electric organ of the eel *Electrophorous electricus* (3) and from three distinct sodium channels (designated rat sodium channels I, II and III) of rat brain (4, 5). For invertebrate sodium channels, a partial amino acid sequence deduced from *Drosophila* genomic clones isolated with a vertebrate sodium channel complementary DNA probe has so far been deduced (6). Squid is another important invertebrate for neurophysiology (1, 7). The squid giant axon has long been used for experiments to elucidate molecular mechanisms both of action potential generation and transmission (1, 8), axon-Schwann cell interactions (9) and of axonal transport (10). The present paper reports the isolation of the protein-coding sequence of the squid cDNA and the complete amino acid sequence of this novel sodium channel deduced from the cDNA sequence. Comparisons of the amino acid sequence of the squid sodium channel are made with those of the vertebrate and *Drosophila* sodium channels.

MATERIALS AND METHODS

PCR: We adopted the PCR (polymerase chain reaction) method (11) to isolate part of squid sodium channel gene from genomic DNA of the optic lobe of squid *Loligo bleekeri*. The live squids were captured in the Sagami Gulf of Japan and transported to the Electrotechnical Laboratory (12) where they were sacrificed for the present experiments. Reaction solution for the PCR contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin, 200 mM NTP, 200 mM DNA primers and 25 units/ml Taq DNA polymerase (Cetus Co.) (13). For the DNA primers, we synthesized several 20 - 24 mer mixed oligonucleotides based on the

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amino acid sequences commonly conserved among rat I, II & III and eel Na channels, as shown in Fig.1a. The DNA primers were synthesized with the Cyclone plus DNA synthesizer (Milligen Co.). The PCR was performed in the 100 ml reaction solution with the DNA primers under the conditions of 94°C for 1 min, 54°C for 2 min and 72°C for 3 min in succession, and subjected to 50 cycles of the amplification. DNAs thus amplified were analyzed with 1.5% agarose gel electrophoresis. Major bands excised from the gel were ligated into the M13 mp19 vector, respectively, to be amplified and sequenced with an automatic sequencer (ABI 370A).We also adopted the inverse PCR method (14). For this, 1 mg squid genomic DNA was digested with 1 unit of restriction endonuclease Alu I at 37°C for 30 min, to be ligated with T4 DNA ligase. Circular DNA was used for this template to carry out the inverse PCR with the primer shown in Fig.1b under the same conditions for the PCR as described above. Amplified DNA was ligated into Puc 119 and M13 mp19 vectors.

cDNA cloning: Total RNA was extracted from the squid optic lobe by the guanidium thiocyanate method (15). Poly $(A)^+$ RNA was isolated by repeating oligo (dT)-cellulose chromatography twice (Pharmacia). cDNA was synthesized from 5 mg mRNA of adult squid optic lobe with reverse transcriptase (Amersham, RPN.1256Y). The cDNAs were ligated into λZAP II and transfected into XL-1 Blue cells. The cells were then inoculated into 35 culture dishes (Greiner 94/16) and transferred to the nylon membrane (Amersham, Hybond-N). The filters were treated with alkaline, neutralized and irradiated with UV to crosslink the DNA to the filter. Hybridization with the DNA probe was performed in a hybridization buffer (6X SSC, 5X Dehaldt's solution, 0.5% SDS, 20 mg/ml Sermon sperm DNA) at 65°C over night. After hybridization, filters were washed in 0.2X SSC at 65°C for 20 min.

Sequencing: Insert of each clone was digested with exonuclease III and deletion mutants were sequenced. Both strands of the cDNA have been sequenced by the dideoxy chain termination method with [α-^{32}P]dCTP (Amersham) and further with dye-labeled oligo primer (ABI 370A).

RESULTS AND DISCUSSION

The conserved sequence of sodium channel genes was amplified with the PCR only for the IV S5-6 primers in the domain IV (Fig.1a). The synthesized DNA (IV S6A) of about 150 bp (Fig.1b), encoding upstream region of IV S6 and a part of IV S6 segment of the squid sodium channel, was obtained. Amplified DNA was sequenced, after it was ligated into Puc 119 vector. Following the inverse PCR was applied to the genomic DNA of squid with the primers as shown in Fig.1b, DNAs were isolated and sequenced, thereby the probe *IV* 16 of 960 bp was acquired. With this probe, the random primed squid cDNA library in λZAP II was screened. Out of 250,000 clones, we found six positive clones, one of which was SQSR22 of 3.3 kbp. The 800 bp of SQSR22, which was located at the 5' end, was used as the probe *IV* 7 to obtain the cDNA clone covering further upstream region. This resulted in finding two more clones. One of them was SQSR2-7 of 3.8 kbp (Fig.2). The restriction endonuclease maps used for the cDNA segments, SQSR2-7 and SQSR22, are shown in Fig.2.

Fig.3 shows the nucleotide sequence of the cDNA, together with the amino acid sequence, for squid sodium channel I, determined with the clones of SQSR2-7 and SQSR22. An intron was also observed in IV S3 based on the genomic DNA analysis (Fig.3). The translational initiation site was assigned to the methionine codon composed of nucleotide residues 583-585 because this is the first ATG triplet that appears downstream of a nonsense codon, TAT (nucleotides 547-549), and is contained in the nucleotide sequence ATTATGG of the residues 580-586, which has a favored sequence of A (or G) XXATGG (X representing any nucleotide) around the initiation codon ATG in the eukaryote. Thus, the open reading frame consists of 4,566 nucleotides, indicating that squid sodium channel I is composed of 1,522 amino acids (Fig.3) and has the molecular weight of 174,105 daltons estimated from the amino acid sequence. The

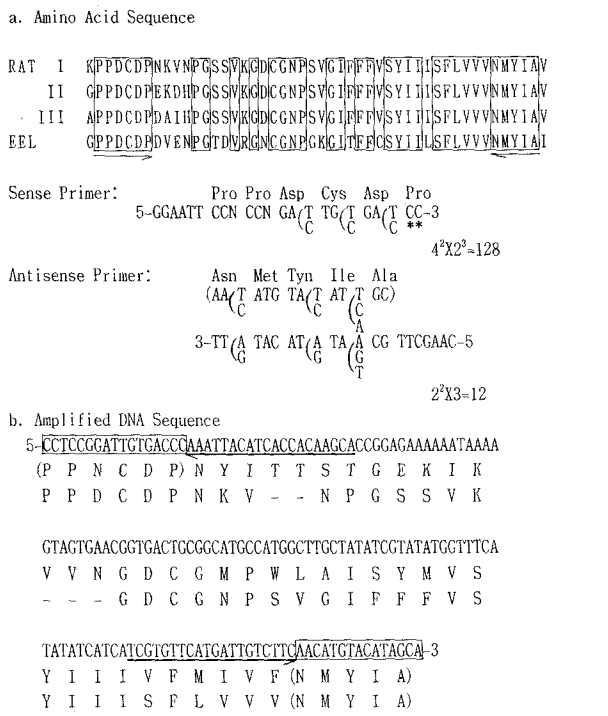

Fig.1. The experimental procedure of PCR used for squid cDNA cloning. *a*: The sense and antisense primers for the RCR extracted from the amino acid sequences of rat I, II & III and eel sodium channels. The sense primer of the amino acid sequence PPDCDP was used, corresponding to the one 1298-1302 between IV S5 and IV S6 (Fig.3). The inclusion of the next two nucleotides from the amino acid^{1303} is indicated by (**). The antisense primer of the amino acid NMYIA was used, corresponding to the sequence 1344-1348 in IV S6 (Fig.3). For the both primers, the degenerated combinations of codons were all synthesized for the PCR. *b*: The sequence of DNA (SQSO3) cloned after the PCR. The nucleotide and amino acid sequences are represented at *upper* and *middle* columns, respectively, with the comparison to the amino acid sequence of rat sodium channel I. The positions of inverse PCR primers are shown with the arrows.

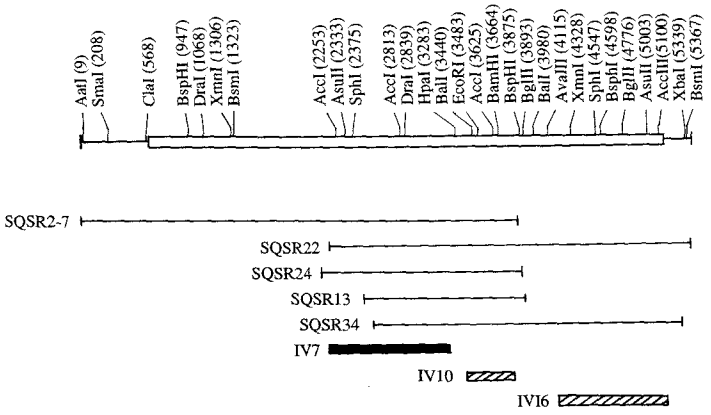

Fig.2. Restriction endonuclease map of squid cDNAs of SQSR2-7 and SQSR22. The map stands for the cleavage sites at the 5' terminal nucleotide by respective restriction endonucleases. Protein-coding regions are indicated by open boxes; the probe IV 7 to screen upperstream is indicated by a closed box while probes, IV 10 and *IV* I6, are both represented by hatched boxes.

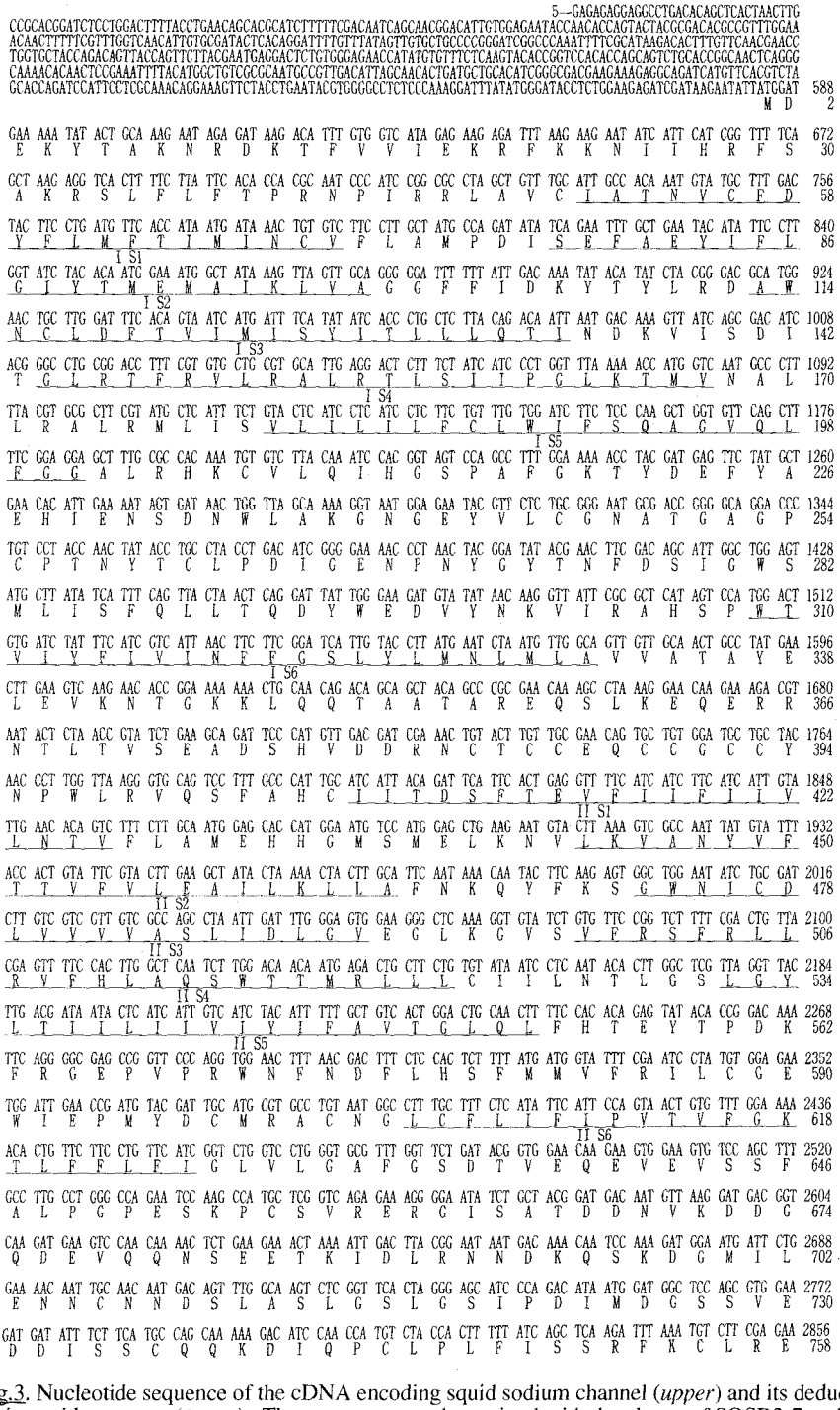

Fig.3. Nucleotide sequence of the cDNA encoding squid sodium channel (*upper*) and its deduced amino acid sequence (*lower*). The sequence was determined with the clones of SQSR2-7 and SQSR22 (Fig.2). Both strands of the cDNA were sequenced with either Sequenase ver 2.0 (USB) or klenow large fragment (Takara Co.). The nucleotide sequence is numbered, starting from the first residue of SQSR2-7. The position where an intron is inserted is indicated by an arrow (↓). This was determined by the genomic DNA analysis on the repeat IV.

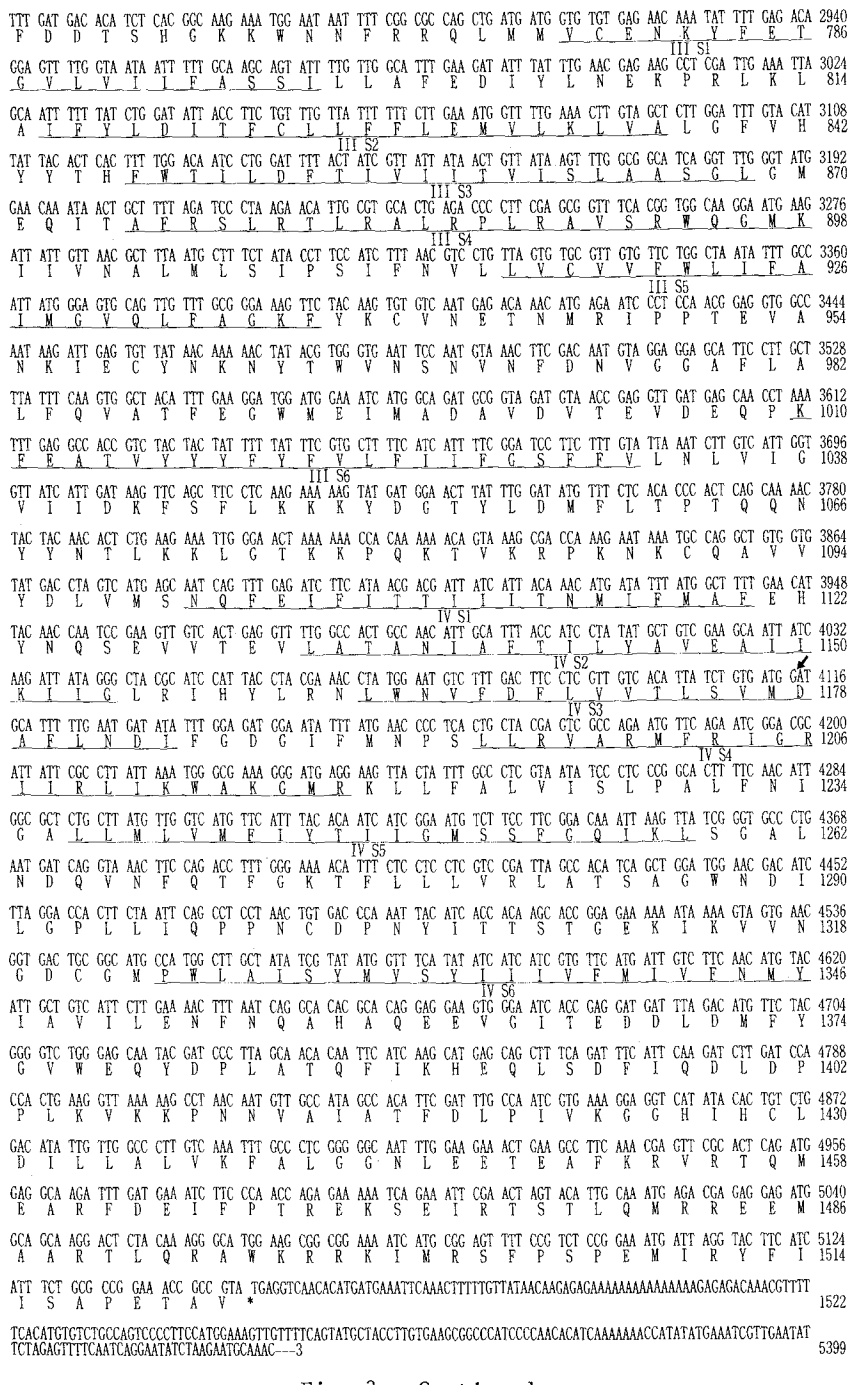

Fig. 3 - Continued

1,522 amino acid sequence is far shorter than those of vertebrate sodium channels; 2,009, 2,005, 1,951 and 1,820 for rat I, II, III and eel (3, 4, 5). This is one of the features of the squid sodium channel, which is mainly because the sequences exposed to the cytoplasmic side are shorter (Fig.4). The squid cDNA is AT rich since the content of AT and GC were 58 and 42%, respectively (Fig.3.).

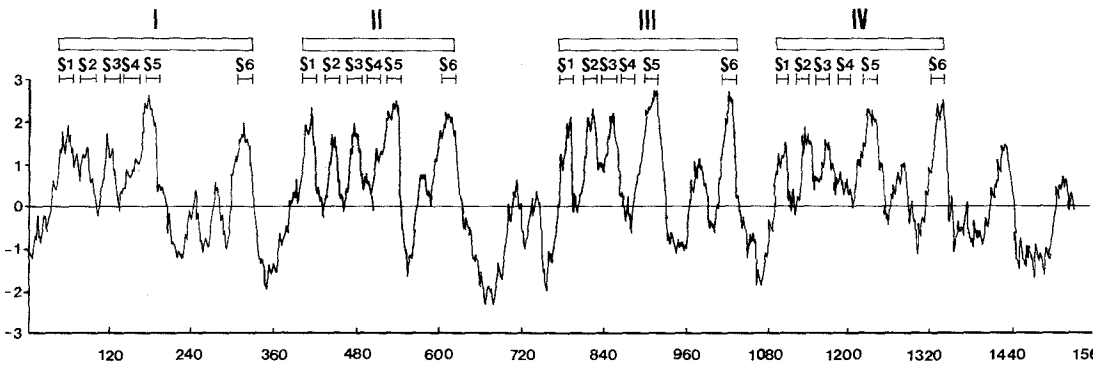

Fig.4. Hydropathy profile of squid sodium channel. The profile was obtained with use of the Kyte and Doolittle program (Kyte, J. and Doolittle, R.F. (1982) J. Mol. Biol. 157, 105) where we run it with a window of 19 residues. Locations of homologous repeats I, II, III and IV are indicated by open boxes. Altogether 24 hydrophobic peaks are found and indicated by their corresponding segments S1-S6.

The alignment of the amino acid sequences of squid sodium channel can be composed with rat sodium channels (4, 5) and the *Electrophorus* sodium channel (3). The degree of the amino acid sequence homology is 48, 47, 46 and 42% for rat I/squid, rat II/squid, rat III/squid and eel/squid, respectively; the degree is rather low as compared with that of 87 and 61% for rat I/rat II and rat I/eel, respectively. For evaluation of amino acid sequence homology, a gap was counted as one substitution. The degree of the nucleotide sequence homology between rat (or eel) and squid sodium channels is even lower. This could be one of the reasons why the cloning of squid sodium channel cDNA has long been unsuccessful with the rat cDNAs. In fact we had tried to screen cDNA library of squid optic lobe with the rat sodium channel cDNA which was kindly offered by Professor M. Noda, but we failed at both high and low stringencies.

The squid sodium channel is assumed to have the same transmembrane topology as proposed for other sodium channels (4, 5) in which the four repeated domains (I, II, III and IV) of homology, each containing the six membrane-spanning segments (S1, S2, S3, S4, S5 and S6), are oriented in a pseudosymmetric fashion across the membrane, and in which the amino- and carboxy-terminal residue on the cytoplasmic side of the membrane. These were predicted by analysis for local hydropathicity on the deduced squid amino-acid sequence (Fig.4). Segments S1, S2, S3, S5 and S6 are apolar, while segment S4 and a linker between S5 and S6 are slightly apolar and partly even hydrophilic (Fig.4). These are quite similar to those of vertebrate sodium channels (3 - 5). For these characteristics of the segments, Guy *et al.* (16) proposed a model with four homologous domains but each containing eight homologous transmembrane segments, S1 through S8, where three segments (S1, S2 and S3) are relatively apolar and two segments (S5 and S8) are quite apolar. S8 is the nomenclature that Noda *et al.* (4) called S6. The close resemblance of sodium channel topology between vertebrate (rat and eel) and invertebrate (squid and fly) is consistent with the idea that sodium channel evolution took place before the separation of vertebrate and invertebrate species (2, 6).

Based on the sodium channel model by Noda *et al.* (3, 4) where sodium channel has four homologous domains, each having six homologous transmembrane segments, S1 - S6, we will compare amino-acid sequence of squid sodium channel with those of vertebrate sodium channels

in more detail. For S4 segments which have widely been recognized to serve as voltage sensors (3, 5, 17, 18), squid S4s contain 4, 3, 5 and 8 basic residues (arginine R or lysine K) at every third position in the domains I, II, III and IV (Fig.3), respectively, while those of rat or eel contain 4, 5, 5 and 8 basic residues correspondingly (3 - 5). The difference is on II-S4 where two lysine residues of vertebrate are substituted for histidine^{510} and glutamine^{513} residues of squid (Fig.3). As for S2, it is noted that glutamic acid (negatively charged) and lysine (positively charged), which are located in the 15th and 19th positions for each domain, are all conserved for vertebrate and squid sodium channels in their identical positions. In addition, segment S2 in repeats I and III contains a conserved glutamic acid or aspartic acid residue both for vertebrate and squid, which locates 10 residues upstream from the above-mentioned glutamic acid. Note that the aspartic acid residue in III-S2 is replaced by an asparagine residue in *Dorosophila*. However, lysine (a positively charged residue) conserved in III-S2 for every vertebrate, which locates one residue downstream from the just above-mentioned aspartic acid residue, is substituted for isoleucine^{821} in squid. Segment S3 in every repeat contains a conserved aspartic acid residue (the 6th position in each domain) for both vertebrate and squid at an equivalent position. In II-S3, a negatively charged residue, 10 residues downstream from the above-mentioned aspartic acid, is conserved for both vertebrate (all glutamic acid) and squid (aspartic acid). In IV-S3, a negative charged residue, 15 residues downstream from the aspartic acid in the 6th position, is conserved both for vertebrate and squid. But it is noted that, in I-S3, a conserved glutamic acid residue for vertebrate, located at the position 11 residue downstream from the aspartic acid (the 6th position in I-S3), is substituted for a leucine residue in squid.

A cytoplasmic linker of squid between domains III and IV is quite similar, in its amino-acid sequence, to those of vertebrate (17, 19, 20), particularly for their positively charged positions; three successively aligned lysine$^{1048\text{-}1050}$ residues, two successively aligned lysine^{1072,73} & ^{1077,78} residues, a lysine^{1081} and arginine^{1085} residue are all conserved for squid and vertebrate, except that lysine residues^{1065,66}, conserved lysine residues for vertebrate, are replaced by non-charged ones, glutamine and aspargine in squid. This exception is, however, consistent with the site directed mutagenesis experiment (20) showing that inactivation is not altered in rat III sodium channels expressed in *Xenopus* oocytes where lysine residues mentioned above are substituted by non-charged ones. A serine residue, which can be phosphorylated in a PKC (protein kinase C) dependent manner to cause both inactivation slow and reduce peak sodium current in rat II sodium channel (21), is replaced by threonine^{1076} at the equivalent position for squid. This suggests that potentiality of phosphorylation at the position is conserved. Two potentially glycosylated sites of aspargine^{231} and aspargine^{248} residues are conserved between I-S5 and I-S6 for squid and vertebrate (4, 5, 22). The aspargine^{248} residue could be glycosylated while the aspargine^{231} residue could not be glycosylated because of the presence of an aspartic acid^{233} residue in its next neighbour (23). In squid one more potential site of glycosylation between I-S5 and I-S6 is on an aspargine^{258} residue.

In conclusion, the structural features characteristic of squid sodium channel are shared by vertebrate sodium channels. We should here point out that the deduced amino acid sequence of squid is different from that of *Drosophila* at some critical points; in the squid sodium channel the II-S4 segment contains 3 positively charged residues at every third position, while the *Drosophila*

sodium channel contains 5, the same as in vertebrate, in its equivalent segment. In a III-IV linker, the phosphorylated site (serine and threonine^{1076} residues for vertebrate and squid, respectively) is replaced by an arginine residue in *Drosophila*, suggesting that it is non-phosphorylated.

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REFERENCES

1 Hodgkin, A.L. and Huxley, A.F. (1952) J. Physiol. (London) 117, 500-544.
2 Hille, B. (1984) *Ionic Channels of Excitable Membranes*, Sinauer, Sunderland, M.A.
3 Noda, M., Shimizu, S., Tanabe, T., Takai, T., Kayano, T., Ikeda, T., Takahashi, H., Nakayama, H., Kanaoka, Y., Minamino, N., Kanagawa, K., Matsuo, H., Raftery, M.A., Hirose, T., Inayama, S., Hayashida, H., Miyata, T. and Numa, S. (1984) Nature 312, 121-127.
4 Noda, M., Ikeda, T., Kayano, T., Suzuki, H., Takeshima, H., Kurasaki, M., Takahashi, H. and Numa, S. (1986) Nature 320, 188-192.
5 Kayano, T., Noda, M., Flockerzi, V., Takahashi, H. and Numa, S. (1988) FEBS Lett. 228, 187-194.
6 Salkoff, L., Butler, A., Wei, A., Scavarda, N., Giffen, K., Ifune, C., Goodman, R. and Mandel, G. (1987) Science 237, 744-749.
7 Armstrong, C.M. and Bezanilla, F. (1977) J. Gen. Physiol. 70, 567-590.
8 Tsukita, S., Tsukita, S., Kobayashi, T. and Matsumoto, G. (1986) J. Cell Biol. 102, 1710-1725.
9 Lieberman, E.M., Abbott, N.J. and Hassan, S. (1989) Glia 2, 94-102.
10 Vale, R.D., Schnapp, B.J., Reese, T.S. and Sheetz, M.P. (1985) Cell 40, 449-454.
11 Saiki, R.K., Sharf, S., Faloona, F., Mullis, K.B., Horn, G., Ehrlich, H.A. and Arnheim, N. (1985) Science 230, 1350-1354.
12 Ichikawa, M., Urayama, M. and Matsumoto, G. (1991) J. Membrane Biol. 120, 211-222.
13 Saiki, R.K., Gelfand, D.H., Stoffel, S., Scharff, S.J., Higuchi, R., Horn, G.T., Mullis, K. and Erlich, H.A. (1988) Science 239, 487-491.
14 Triglia, T., Peterson, M., Kemp, D. (1988) Nucleic Acid Res. 16, 81-86.
15 Chargwin J.M., Przybyla A.E., MacDonald R.J. and William J.R. (1979) Biochemistry 18, 5294-5299.
16 Guy, H.R. and Seetharamulu, P. (1986) Proc. Natl. Acad. Sci. USA 83, 508-512.
17 Stühmer, W., Conti, F., Suzuki, H., Wang, X., Noda, M., Yahagi, N., Kubo, H. and Numa, S. (1989) Nature 339, 597-603.
18 Catterall, W.A. (1986) TINS 9, 7-10.
19 Vassilev, P.M., Scheuer, T. and Catterall, W. (1988) Science 241, 1658-1661.
20 Moorman, J.R., Kirsch, G.E., Brown, A.M. and Joho, R.H. (1990) Science 250, 688-691.
21 West, J.W., Numann, R., Murphy, B.J., Scheuer, T. and Catterall, W.A. (1991) Science 254, 866-868.
22 Trimmer, J.S. and Agnew, W.S. (1989) Annu. Rev. Physiol. 51, 401-418.
23 Hubbard, S.C. and Ivatt, R.J. (1981) Annu. Rev. Biochem. 50, 555-583.