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Binding Properties of ^{3}H-PbTx-3 and ^{3}H-Saxitoxin to Brain Membranes and to Skeletal Muscle Membranes of Puffer Fish *Fugu pardalis* and the Primary Structure of a Voltage-Gated Na^+ Channel α-Subunit (fMNa1) from Skeletal Muscle of *F. pardalis*[1]

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The dissociation constants for ^{3}H-saxitoxin to brain membranes and to skeletal muscle membranes of puffer fish *Fugu pardalis* have been estimated to be 190- and 460-fold, respectively, larger than those to corresponding membranes of rat, by a rapid filtration assay, while these values for ^{3}H-PbTx-3 have been estimated to be one-third and one-half of those to rat, respectively. We have obtained a cDNA, encoding an entire voltage-gated Na^+ channel α-subunit (fMNa1, 1880 residues) from skeletal muscle of *F. pardalis* by composition of the fragments obtained from cDNA library and RT-PCR products. In fMNa1 protein, the residues for ion-selective filter and voltage sensor and the charged residues in SS2 regions of domains I–IV were conserved, but the aromatic amino acid (Phe/Tyr), commonly located in the SS2 region of domain I of tetrodotoxin-sensitive Na^+ channels, was replaced by Asn. With this particular criterion, we propose that the fMNa1 protein is a tetrodotoxin-resistant Na^+ channel.

Several natural toxins have been used as useful tools in the study of excitable cells because of their potent and specific actions on voltage-gated Na^+ channels (1, 2). PbTx-3 (3), an analogue of brevetoxins, activates Na^+ channels at normal membrane resting potential by binding to the site (site 5) which is suggested to exist at the interface of domains I and IV(4). Tetrodotoxin (TTX) and saxitoxin (STX) block the Na^+ channels by binding to the common site (site 1) (5, 6). The TTX binding site was mainly investigated by site-directed mutagenesis (7, 8), structure-activities relationship of TTX analogues (9, 10), and photoaffinity labeling (11). According to the mutagenesis carried out by Terlau *et al.* (8), the TTX binding site exists in SS2 region, locating in the highly conserved pore-forming region (P-loop) between the fifth and the sixth transmembrane segments (S5–S6) in each domains I–IV. In SS2 regions, two negatively charged amino acids each in domain I (Asp, Glu) and II (two Glu), one positively charged amino acid in domain III (Lys) and one negatively charged amino acid in domain IV (Asp) were identified as the possible major determinants of TTX-sensitivity (Fig. 3).

The works of molecularly cloning of the cDNA of TTX-resistant Na^+ channels were shown in rapid progress, and sequence information of them has been accumulated (12). TTX-resistant Na^+ channels have been found in rat heart (rHNa) (13), human heart (hHNa1) (14), denervated rat skeletal muscle (rMNa2) (15), rat sensory neuron (rSNa) (16), and the TTX possessing newt *Cynops pyrrohogaster* retinal neuron (nRNa, GenBank Accession No. AF123593) (17). In these TTX-resistant Na^+ channels, the charged amino acids in SS2 regions in I-IV domains were identical with those in TTX-sensitive Na^+ channels, but the aromatic amino acid (Phe/Tyr) in SS2 of domain I in TTX-sensitive Na^+ channel was replaced by a nonaromatic amino acid, Cys in rHNa, rMNa2 and hHNa1, Ser in rSNa, and Ala in nRNa. Satin *et al.* (18) showed that substitution of Cys374 by Tyr in rHNa signifi-

Abbreviations used: nt, nucleotide; TTX, tetrodotoxin; STX, saxitoxin; Hepes, 4-(2-hydroxyl)-1-piperazineethanesulfonic acid; BSA, bovine serum albumin.

[1] The nucleotide and deduced amino acid sequences of fMNa1 reported in this paper have been deposited in GenBank (Accession No. AB030482).

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cantly decreased the IC_{50}-TTX from 950 nM to 1.3 nM (1/730). A number of similar results along this line have been successively reported as follows (19–21). Conversely, substitution of the non-aromatic amino acids of TTX-insensitive channels to the Tyr/Phe residue decreases significantly the IC_{50}-TTX. For examples, Chen *et al.* (20) reported change of Cys in rMNa2 to Tyr decreased IC_{50}-TTX from 3 μM to 8 nM (1/380), and Sivilotti *et al.* (22) also showed that substitution of Ser in rSNa by Phe decreased IC_{50}-TTX from 60 μM to 2.8 nM (1/21,000). All these data agree and suggest that the sensitivity to TTX is markedly influenced by a single amino acid displacement of aromatic residue with non-aromatic one at the same position in SS2 region. Fozzard *et al.* (23) proposed that the hydrophobic interaction of TTX with the aromatic rings of the Phe/Tyr residues might increase their TTX sensitivities.

Since puffer fish accumulate TTX at extremely high concentration in their tissue (24), it appears that they have special TTX-tolerant system. Kidokoro *et al.* (25) examined the effect of TTX on action potential of skeletal muscle fiber in various fishes, and found that muscle fiber of all puffer fish species examined were insensitive to up to 30 μM TTX, while the dissociation constants of TTX of ordinary fish were only in 10 nM order. In this article, first, the binding parameters of ^{3}H-PbTx-3 and ^{3}H-STX to brain membranes and to skeletal muscle membranes of puffer fish *Fugu pardalis* and rat have been estimated by a rapid filtration assay. Second, we have obtained a full-length cDNA sequence of a subtype of skeletal muscle Na^+ channel α-subunit from *F. pardalis* (fMNa1). We also discuss general aspect of TTX-resistance system of puffer fish.

MATERIALS AND METHODS

Binding assay. 42-^{3}H-PbTx-3 (0.66 TBq/mmol) and 11-^{3}H-STX (0.74–1.44 TBq/mmol) were products of Amarsham (Buckinghamshire, UK). The brain and skeletal muscle were immediately excised from puffer fish *Fugu pardalis* (captured in the Sendai Bay, Japan) and rat (Wistar strain) after sacrificing. Brain membranes (P3) and skeletal muscle membranes (F-III) were prepared essentially by the procedures described by Hartshorne and Catterall (26) and Krall and Korenman (27), respectively. Protein concentration was measured according to the method of Lowry *et al.* (28). Brain membranes (50 μg of protein/ml) and skeletal muscle membranes (150 μg of protein/ml of *F. pardalis*, 100 μg of protein/ml of rat) were individually incubated with increasing concentration of ^{3}H-STX (0.1–37.5 nM) and ^{3}H-PbTx-3 (0.1–10 nM) in 1 ml of the incubation medium containing 50 mM Hepes/Tris, 130 mM choline chloride, 5.5 mM glucose, 0.8 mM $MgSO_4$, and 5.4 mM KCl (pH 7.4) (for ^{3}H-PbTx-3, 1 mg/ml BSA and 0.01% Polyoxyethylene-10-tridecylether were added to the above medium) (3, 26). After 15 min of incubation at 0°C, the mixture was detected into the inlets of vacuum chamber equipped with GC/C glass fiber filter (Whatman International, Ltd., Maidstone, UK) (4, 29). To remove unbound ^{3}H-PbTx-3 and ^{3}H-STX, the filters were washed thrice with 1 ml of the washing medium containing 5 mM Hepes/Tris, 163 mM choline chloride, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$ (pH 7.4) (for ^{3}H-PbTx-3, 1 mg/ml BSA was added), and bound radioactivity was determined with 8 ml of EX-H scintillation cocktail (Dojindo Labs, Kumamoto, Japan). To measure the nonspecific binding, excess unlabeled PbTx-3 or TTX (1000 times molar concentration of the labeled toxin) was added to the incubation medium. The assay was performed thrice at each probe concentration, and repeated thrice for each animal and for membrane fraction.

RT-PCR. A sense primer (TNAGRGAYCCNTGGAAYTGG) and an antisense primer (RTTYTGYTCTTCRTANGCCAT) were designed from the conserved amino acid sequence located between IS3 and IS6 of Na^+ channels (between nt 676–1427 of fMNa1) according to the previous reports (13–17, 34, 36–39). PCRs were carried out with the conditions as follows: each primer (60 pmol) were used in a PCR (80 μl) with one twentieth of first strand cDNA synthesized from 1 μg of total RNA (see the next paragraph) as a template. Pfu DNA Polymerase (1.6 unit, Stratagene, La Jolla, CA) was added to the reaction mixture containing, 1× Pfu DNA Polymerase buffer ($MgCl_2$ free), 0.2 mM each of dNTPs and 3 mM $MgCl_2$. The thermal cycling parameters were 30 cycles of 95°C, 30 s; 55°C, 1 min; 72°C, 2 min, followed by at 72°C for 7 min. PCR products were subcloned in pGEM-T vector (Promega, Madison, WI) and subjected to further analysis.

Construction of cDNA libraries and screening of the cDNA clones. Adult *Fugu pardalis* captured domestically (in the Sendai Bay, Japan) were used to prepare total RNA from their skeletal muscle by the guanidinium thiocyanate-cesium chloride method. Poly(A)$^+$ RNA was isolated by Oligotex-dT30 super (Japan Roche, Tokyo). First strand cDNA was synthesized from 5 μg mRNA using oligo-dT or random hexameric primers with SuperScript RNase H^- reverse transcriptase (GIBCO BRL, Rockville, MD). The first strand cDNA was further processed to prepare ds (double strand) cDNA and average size of 2.3 kb, with *Eco*RI–*Not*I–*Bam*HI adapter (Takara, Tokyo), were prepared. The cDNA library was constructed using lambda gt10 (Stratagene, La Jolla, CA). One library from oligo-dT primed cDNA and two libraries from random primed cDNA, with a complexity of 4×10^5 pfu (plaque-forming units) each, were obtained. Hybond-N^+ nylon membrane filter (Amarsham Pharmacia Biotech, Tokyo) were lifted from plates with 5×10^4 pfu per plate. Candidate phage clones, harboring Na^+ channel cDNA, were screened from the libraries by hybridization with appropriate DNA fragment labeled with [α-^{32}P]dCTP using random priming procedure, as a probe. Hybridization was carried out using the method of Church and Gilbert (30).

Northern blot analysis. Fifteen micrograms of total RNA from skeletal muscle, brain, gill, heart, kidney, ovary, skin, and testis of *F. pardalis*, prepared by using the QuickPrep Total RNA Extraction Kit (Amarsham Pharmacia Biotech, Tokyo), was subjected to electrophoresis through 1.2% agarose-formaldehyde gels and transferred to Hybond-N nylon membrane (Amarsham Pharmacia Biotech, Tokyo). DNA probe specific for fMNa1 (184 bp fragment of nt 951-1134 obtained by PCR) labeled with [α-^{32}P]dCTP was generated and then hybridized at 65°C with the blot. A cDNA fragment (near 5′-end, 564 bp, GenBank Accession No. AB029314) of *F. pardalis* skeletal muscle 18S rRNA was cloned by RT-PCR by the authors and used as the probe for rehybridization to detect 18S rRNA as loading control. The final wash was with 0.1× SSC–0.5% SDS at 65°C for 15 min.

Southern blot analysis. Southern blot analysis was performed on DNA extracted from *F. pardalis* testis. The DNA was digested with *Hin*dIII, *Eco*RI, or *Bam*HI restriction enzymes, separated on a 1% agarose gel, and transferred to a Hybond N^+ nylon membrane (Amarsham Pharmacia Biotech, Tokyo). The same fMNa1 specific DNA probe used for northern blot analysis (nt 951–1134) was hybridized at 65°C with the blot. The final wash was with 0.1× SSC–0.5% SDS at 62°C for 30 min.

Sequencing of the cDNA and analysis of deduced amino-acid sequences. The deletion mutants of cDNA fragments prepared by exonuclease III were sequenced in both directions using a Perkin–Elmer ABI 373A sequencer and an ABI Prism Dye-Primer Cycle

Sequencing Kit (PE Applied, Chiba, Japan). GENETYX-MAC ver. 8.0 (Software Development Co., Ltd., Tokyo) and Wisconsin Package Version 9.1, Genetics Computer Group (GCG) (Madison, WI, via Human Genome Center, Institute of Medical Science, University of Tokyo) were used for analysis of DNA and amino-acid sequences. A phylogenetic analysis of Na^+ channel amino acid sequences was carried out using the Neighbor-Joining method (31) by CLUSTALW analyzing System of DDBJ. GenBank and EMBL were used for database searches via DDBJ.

RESULTS

Binding Properties of ^{3}H-PbTx-3 and ^{3}H-STX to Brain Membranes and to Skeletal Muscle Membranes of Puffer Fish (Fugu pardalis) and Rat

Saturation binding of ^{3}H-PbTx-3 and ^{3}H-STX to brain membranes and to skeletal muscle membranes of *F. pardalis* (A1, B1, C1, D1) and the linear transformation of the data (Scatchard plots), together with those of rat (A2, B2, C2, D2), were shown in Fig. 1. The average ratios of nonspecific binding to total binding on all assay were less than 20%, but that for ^{3}H-STX to *F. pardalis* skeletal muscle membranes was relatively higher (43%) (Fig. 1-C1). This high ratio was caused by low total binding. The level of its nonspecific binding itself was less than that for ^{3}H-PbTx-3 to the same membrane at the same concentration of labeled toxin (Fig. 1-D1). The values of dissociation constants (K_d) and the total concentration of the binding sites (B_{max}) were determined from the linear regression lines of Scatchard plots and summarized in Table 1. The values of K_d for ^{3}H-PbTx-3 to brain membranes and to skeletal muscle membranes of *F. pardalis* were estimated to be one-third and half, respectively, of those to the corresponding membranes of rat. In contrast, the values of K_d for ^{3}H-STX to brain membranes and to skeletal muscle membranes of *F. pardalis* were estimated to be 190- and 460-fold, respectively, larger than those to the corresponding membranes of rat. These data suggest that Na^+ channels in brain and skeletal muscle in *F. pardalis* are resistant to STX, but sensitive to PbTx-3. The B_{max} value of ^{3}H-STX to the skeletal muscle membranes was presumed to be overestimated for the reason of low specific binding, because this value was considerably larger than that of ^{3}H-PbTx-3 to the same membranes. Except this, the difference of the values of B_{max} for both labeled toxins to both membranes between *F. pardalis* and rat were less than twice.

Cloning of cDNA of Na^+ Channel from Fugu pardalis Skeletal Muscle (fMNa1)

With degenerate primers designed from the amino acid sequences between IS3 and IS6 region of published Na^+ channels, RT-PCR (see Materials and Methods) was performed using total RNA prepared from skeletal muscle of *F. pardalis* as a template. The PCR products (752 bp) were subcloned and the sequences were determined. The sequences of the PCR products suggested the presence of at least two subtypes of Na^+ channels, and they were designated as fMNa1 and fMNa2 (the sequence between IS3 and IS6 of fMNa2 was deposited in GenBank Accession No. AB032022). The cloned PCR product of fMNa1 (nt 676–1427) was then used as a probe to screen two random primed cDNA libraries, and two positive cDNA clones B2 (nt 1–1466), spanning to the putative 5′ end of the transcript, and 2R11 (nt 750–4259), flanking to B2 clone with 717 bp of overlapping region, were obtained. Complementary DNA fragments digested with *Bam*HI or *Not*I from these phage clones were subcloned into pBluescriptKSII$^-$ vectors for sequencing. In succession, the region between nt 3902 and nt 4255 (354 bp) of the clone 2R11, amplified by PCR, was used as a probe, and obtained the positive clone FM9601 harboring the 3′ region (nt 4205–6722) of fMNa1 cDNA from an oligo-dT primed cDNA library (4×10^5). RT-PCR was carried out to obtain the fragment extending from nt 3902 to nt 4384 with a sense primer (CTACGACAACGTGGCAAAAG) and an antisense primer (TTGAAGAGGAAGTCCTCC). DNA sequence of several PCR clones which covered the boundary region between the clones 2R11 and FM9601, suggested the continuity of the sequence from the clone 2R11 to the clone FM9601.

In comparison with the amino acid sequences of reported Na^+ channels (13–17, 34, 36–39), the clone 2R11 appeared to have two frame shifts which terminated the translation in the interdomain loops between domain I and II, and between domain II and III. To interpret this possible cessation of the translation in the cDNA sequence of fMNa1, RT-PCR was performed. The region of the cDNA between nt 1376 and nt 3302 (between IS6 and IIIS1 region) was amplified from total RNA as the template, with a sense primer (CCTCATCAACCTTATCCT) and antisense primer (AATAGTGAAGCAGGTCTT). A major fragment with approximately 2 kb was cloned into pGEM-T vector. Comparison of the sequences obtained from above RT-PCR products with that from the clone 2R11 showed that a 142-bp-long fragment (nt 1671–1812) between IS6 and IIS1 region was deleted in the clone 2R11 and some of the RT-PCR clones, and this deletion caused a frame shift. In addition to the deletion, an insertion of a 243-bp-long fragment[4] between nt 3210 and nt 3211, between IIS6 and IIIS1 region, was found in the clone

[4] Nucleotide sequence of an insertion of a 243-bp-long fragment between nt 3210 and nt 3211 of fMNa1: GTATGAACCACCTACACGTTGTTCTCGTTGTCACAAGCAAGATTGTCATTTACAGAAACCAGAAATTTAAGGTGAGGAAAGTGGTGCCGATGGCCAGCTGTGGAGACGGACAGAGGAGAAGAGCAAACACATACATCATACATGTAGTTATGCACTAGGATGAGCTTGACTACACATTAAAGGAGCTAAAACTGGGGGTCCACCATGTTGGTGAGGTCTCACCCACAGTTTCCTGTCGCTCAG.

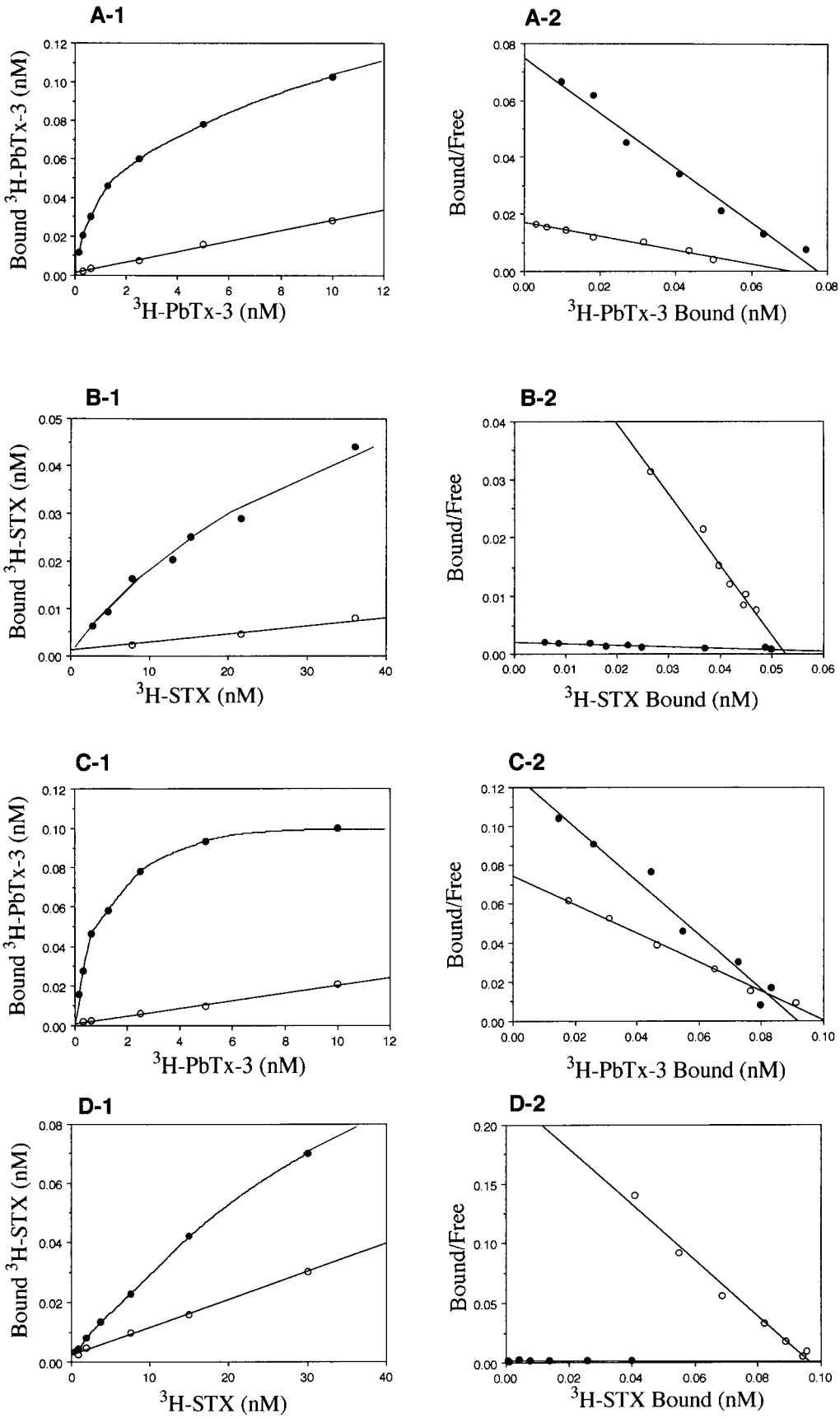
A-1
Bound ^{3}H-PbTx-3 (nM)
^{3}H-PbTx-3 (nM)
A-2
Bound/Free
^{3}H-PbTx-3 Bound (nM)
B-1
Bound ^{3}H-STX (nM)
^{3}H-STX (nM)
B-2
Bound/Free
^{3}H-STX Bound (nM)
C-1
Bound ^{3}H-PbTx-3 (nM)
^{3}H-PbTx-3 (nM)
C-2
Bound/Free
^{3}H-PbTx-3 Bound (nM)
D-1
Bound ^{3}H-STX (nM)
^{3}H-STX (nM)
D-2
Bound/Free
^{3}H-STX Bound (nM)

TABLE 1

Binding Properties of ^{3}H-PbTx-3 and ^{3}H-STX to Brain Membranes and to Skeletal Muscle Membranes of Puffer Fish *Fugu pardalis* and Rat

	Brain				Skeletal muscle			
	^{3}H-PbTx-3		^{3}H-STX		^{3}H-PbTx-3		^{3}H-STX	
Animal	K_d (nM)	B_{max} (pmol/mg)	K_d (nM)	B_{max} (pmol/mg)	K_d (nM)	B_{max} (pmol/mg)	K_d (nM)	B_{max} (pmol/mg)
Puffer fish (*F. pardalis*)	1.07 ± 0.06	1.45 ± 0.16	36.0 ± 8.06	1.49 ± 0.29	0.72 ± 0.07	0.63 ± 0.10	198 ± 24	2.7 ± 1.5
Rat	3.12 ± 0.60	1.38 ± 0.04	0.19 ± 0.05	1.05 ± 0.04	1.45 ± 0.13	1.17 ± 0.13	0.43 ± 0.04	0.82 ± 0.08

Note. The dissociation constants (K_d) and the total concentration of the binding sites (B_{max}) for ^{3}H-PbTx-3 and ^{3}H-STX to brain membranes and to skeletal muscle membranes were estimated with standard deviations ($n = 3$) from the linear regression lines of Scatchard plots shown in Fig. 1.

2R11 and some of the RT-PCR clones. This insertion also generated another frame shift which caused an in-frame termination codon. According to the sequencing analysis, the clones from RT-PCR products were classified into four groups (Fig. 2); Group (1), clones containing only deletion at the same region as found in 2R11 (IS6–IIS1); Group (2), clones containing only insertion at the same region as found in 2R11 (IIS6–IIIS1); Group (3) containing both of the deletion and the insertion; and Group (4), clones containing neither of the deletion nor the insertion, whose sequence was proved to be all in-frame and without termination codon. Clones in Group (1) and Group (3) predicted a truncated "one-domain" protein, clones in Group (2) predicted a truncated "two-domain" protein, and clones in Group (4) predicted a protein of entire "four-domain." These deletion and insertion are supposed to be risen from alternative splicing, since the two nucleotide sequence upstream from the deletion and the 3′-end sequences of the deleted fragment were both AG, and the two nucleotide sequences of the 5′-end and 3′-end of the inserted fragment[4] were GT and AG, respectively. The sequence of the clone in Group (4) was used for the following analysis in this section.

Structure–Function Considerations for the Na^+ Channel protein, fMNa1

Using the sequence information obtained from the clones from cDNA library (B2, 2R11, and FM9601) and the products of RT-PCR (Group (4)), we integrated the whole cDNA sequence (6722 bp) to reveal the putative full coding sequence of Na^+ channel fMNa1 from *F. pardalis* skeletal muscle. fMNa1 is composed of 1880 amino acids, and has the molecular weight of 212 kilodaltons, estimated from the amino acid sequence. Nucleotide position 141 is assumed as the translation start site because it is the first Met of the open reading frame, encoding the longest amino acid sequence, that can align well with those of other known Na^+ channels. In addition, the nucleotide sequence AAGATGG from nt 138–144 matches well with the Kozak sequence (A/G)XXATGG (X representing any nucleotide), which is frequently observed motif around initiation codon of the transcript in the eukaryotic cell (32). A termination codon, TAA at nt 5781–5783 in the clone FM9601 results in a 939-bp length of 3′-untranslated region. However, in this 3′-untranslated region in the clone FM9601, no consensus polyadenylation signal was observed around the 3′-terminal end next to 13 bp length of terminal poly(A) tail. Lacking of the polyadenylation signal is supposed to be caused by deletion of the signal during splicing process, and further analysis of genomic sequence is necessary to prove this hypothesis. The hydropathy profile of the deduced protein sequence of fMNa1 is similar to those of reported Na^+ channels, and the structure of fMNa1 has features generally observed in other Na^+ channels.

FIG. 1. Binding of ^{3}H-PbTx-3 and ^{3}H-STX to brain synaptic membrane and to skeletal muscle plasma membrane of puffer fish *F. pardalis* and rat. (A-1 and B-1) *F. pardalis* brain membranes (50 μg of protein/ml) were incubated with increasing concentrations of ^{3}H-PbTx-3 (0.2–10 nM) and ^{3}H-STX (3–37.5 nM), respectively. (C-1 and D-1) *F. pardalis* skeletal muscle membranes (150 μg of protein/ml) were incubated with increasing concentrations of ^{3}H-PbTx-3 (0.2–10 nM) and ^{3}H-STX (0.1–30 nM), respectively. After incubation for 15 min at 0°C, bound toxins were measured using the rapid filtration assay as described under Materials and Methods (3, 29). Total binding (●) and nonspecific binding measured in presence of excess unlabeled PbTx-3 or TTX (1000 times molar concentration of the labeled toxin) (○) are plotted versus the concentration of labeled toxin. Data shown were representative of three replicates. Similarly, rat brain membranes (50 μg of protein/ml) and rat skeletal muscle membranes (100 μg of protein/ml) were individually incubated with increasing concentrations of ^{3}H-PbTx-3 and ^{3}H-STX (data not shown), and bound toxins were measured by the same method. A-2, B-2, C-2, and D-2. Linear transformation of the data of *F. pardalis* (●) in A-1, B-1, C-1, and D-1, and those of rat (○), resulted in calculated K_d values and B_{max} values summarized in Table 1.

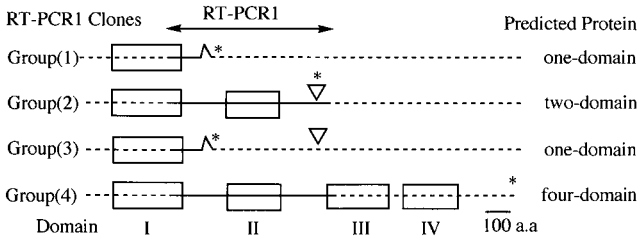

FIG. 2. Classification of the clones from RT-PCR products (nt 1376–3302 of fMNa1), and the configuration of the predicted proteins from those clones. The RT-PCR (nt 1376–3302 of fMNa1) clones were classified into four Groups (1–4) according to their sequences. The sign of ∧ on the lines for Group (1) and Group (3), and the sign of ▽ on the lines for Group (2) and Group (3) indicate the deleted portion and the inserted portion, respectively. The broken lines indicate putative transcripts, and the asterisks indicate the position of termination codons.

The interdomain linker between domains III and IV in fMNa1, including the three hydrophobic amino acids (Ile-Phe-Met) is highly conserved as those in almost all known Na^+ channels (33). Voltage-sensing segments are presumed to locate in S4 of domain I to IV, which has positively charged Arg or Lys residues repeating every three amino acids (1, 34). In the protein of fMNa1, these important regions for functions of Na^+ channel are all well conserved. These data suggest that the protein of fMNa1 has fundamental functions as common Na^+ channels.

The sequences of SS1 and SS2 regions of four domains (I-IV) of the protein of fMNa1 were compared with those of various Na^+ channels (Fig. 3). In Fig. 3, upper four are Na^+ channels whose TTX-sensitivities have not been determined, center five are TTX-resistant Na^+ channels, and four lower are TTX-sensitive ones. In fMNa1 protein, the charged amino acids (indicated by asterisks) that have been previously identified as the major determinants for TTX-sensitivity by *in vitro* mutagenesis (8) and the ion filter together with Ala1565 (35), are all conserved. In con-

		Domain I	Domain II	Domain III	Domain IV
	TTX	↓			
	Sensitivity	$*_{383}$ $*_{386}$	* *	*	$*_{1569}$
fMNa1	ND	RLMTQDNWES	RILCGEWIET	IATFKGWMDI	ITTSAGWDTL
fMNa2	ND	RLMTQDFWEN			
fBNa1	ND	RLMTQDFWEN			
fBNa2	ND	RLMTQDFWEN	RVLCGEWIET	VATFKGWMDI	ITTSGGWDGL
rHNa	R	RLMTQDCWER	RILCGEWIET	VATFKGWMDI	ITTSAGWDGL
hHNa1	R	RLMTQDCWER	RILCGEWIET	VATFKGWMDI	ITTSAGWDGL
rMNa2	R	RLMTQDCWER	RILCGEWIET	VATFKGWMDI	ITTSTGWDGL
rSNa	R	RLMTQDSWER	RILCGEWIET	VATFKGWMDI	ITTSAGWDGL
nRNa	R	RLMTQDAWEN	RVLCGEWIET	IATFKGWMDI	VTTSAGWDGL
rMNa1	S	RLMTQDYWEN	RILCGEWIET	VATFKGWMDI	ITTSAGWDGL
hoMNa	S	RLMTQDYWEN	RVLCGEWIET	VATFKGWMDI	ITTSAGWDGL
rBNa2	S	RLMTQDFWEN	RVLCGEWIET	VATFKGWMDI	ITTSAGWDGL
rBNa3	S	RLMTQDYWEN	RVLCGEWIET	VATFKGWMDI	ITTSAGWDGL
		SS1 SS2	SS1 SS2	SS1 SS2	SS1 SS2

FIG. 3. Alignment of amino acid sequences for SS1 and SS2 regions of four domains I-IV in various Na^+ channels. fMNa1 and fMNa2, *Fugu pardalis* skeletal muscle channels I and II; fBNa1 and fBNa2, *F. rubripes* brain channels I and II (42); rHNa, rat heart channel (13), hHNa1, human heart channel I (14); rMNa2, rat denervated skeletal muscle channel (15); rSNa, rat sensory neuron channel (16); nRNa, newt retina channels (17); rMNa1, rat innervated skeletal muscle channel I (39); hoMNa2, horse skeletal muscle channel (GenBank Accession #U25990), rBNa2 and rBNa3, rat brain channel II (36) and III (37). SS1 and SS2 regions are underlined. Sensitivity of each channel to TTX is indicated as ND (not determined), R (resistant) or S (sensitive), in the column of TTX sensitivity. The charged amino acids identified as major determinants of TTX-sensitivity (8) are indicated by asterisks. An arrow indicates the aromatic (or nonaromatic) amino acid in SS2 region in domain I. The amino acid positions are representatively numbered as that in fMNa1.

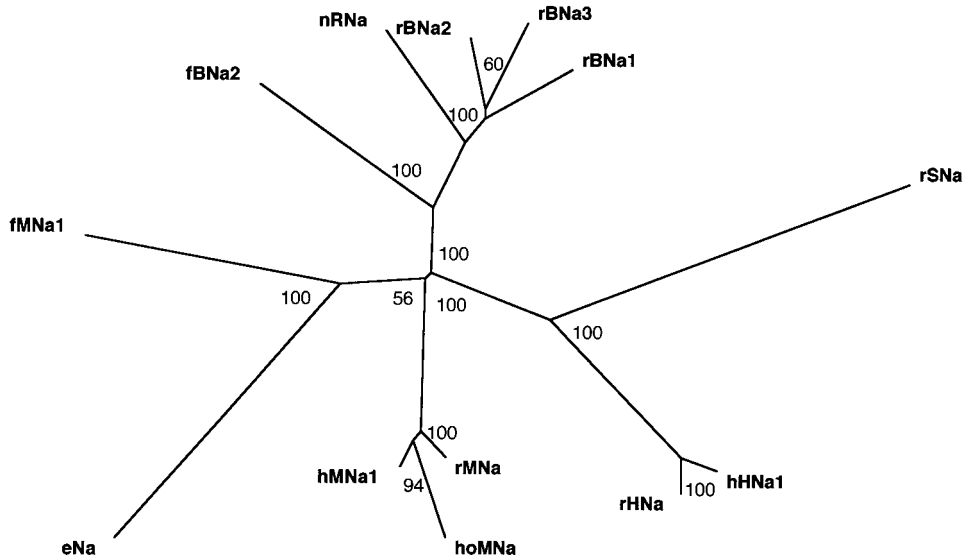

FIG. 4. Phylogenetic tree of Na^+ channel amino acid sequences. The tree was generated by the Neighbor-Joining method (31) based on the maximum likelihood distances by CLUSTALW analyzing System of DDBJ. Numbers shown are percentages of 100 bootstrap replicates in which the same internal branch was observed. Lines are drawn proportional to inferred phylogenetic distances. rBNa1, rat brain channel I (36); eNa, electric eel channel (34); see legend to Fig. 3 for other channels.

trast, the amino acid replacements are observed at positions 383 and 386 in domain I and 1569 in domain IV in fMNa1 protein. At position 383, the aromatic amino acid Phe/Tyr in TTX-sensitive Na^+ channels is replaced by Asn in fMNa1 protein (indicated by arrow). The amino acid replacements at this position with non-aromatic amino acids (Ser, Cys, and Ala) have been reported in several TTX-resistant Na^+ channels, as rHNa (13), hHNa (14), rMNa2 (15), rSNa (16), and nRNa (17). With taking this type of amino acid replacement into account, the fMNa1 protein might be resistant to TTX with the similar mechanism of known TTX resistant Na^+ channels. The Ser386 in the protein of fMNa1 may not be important for TTX-sensitivity, since the substitution of Asn to Arg at this position of rBNa2 changed IC_{50}-TTX only twice (from 18 to 37 nM) (8), and mutation of Arg in rHNa at this position to Asn did not alter the sensitivity to TTX (18). It is of note that Thr1569 is the first amino acid that is observed in this position, since Gly has been identified in all other Na^+ channels. Further analysis by site-directed mutagenesis is needed to determine the effect of this residue in fMNa1 protein on the TTX sensitivity.

The amino acid sequence comparison by calculating identity among the Na^+ channel representatives shown in Fig. 4 (60–70%) did not tell whether fMNa1 is closer to TTX-sensitive or TTX-resistant channels. A phylogenetic analysis of the residue sequences of Na^+ channels carried out based on the maximum likelihood of distances shows that Na^+ channels fall into four major groups (Fig. 4). fMNa1 and eNa form a distinct class with 100% reproducibility of bootstrap replicates, suggesting that fMNa1 protein and eNa protein (34) have evolved from a common ancestor. TTX-sensitive rBNa1, rBNa2 (36), rBNa3 (37), and TTX-resistant nRNa (17) and fBNa2 (42) forms a class of nervous Na^+ channels. In contrast, three TTX-resistant Na^+ channels, rSNa (16), hHNa1 (14) and rHNa (13), form a distinct class with 100% reproducibility. Other one class is formed by skeletal muscle Na^+ channels, hMNa1 (38), hoMNa, and rMNa1 (39).

Tissue Specific Expression of fMNa1

As shown in Fig. 5, a transcript with a size of approximately 7.5 kb was detected only in the skeletal muscle RNA on northern blots with a probe derived from fMNa1 cDNA (nt 951–1134). The Probe has only 48% homology to the corresponding region of fMNa2, suggesting no possibility to detect fMNa2 transcript in this blot analysis. Therefore, it is considered that fMNa1 is transcribed preferentially in adult skeletal muscle of *F. pardalis.* The profile of mRNA expression of fMNa1 in tissues of *F. pardalis* is apparently different from that of TTX-resistant rat skeletal muscle Na^+ channel (rMNa2) (15), whose mRNA was reported to be expressed at high level both in skeletal and cardiac muscles in the early development stage and after denervation, while only in cardiac muscle in the normally innervated adult.

DISCUSSION

Puffer fish is believed to have special TTX-tolerant system. The average concentration of TTX in skeletal muscle of *Fugu pardalis* collected in north Japan was

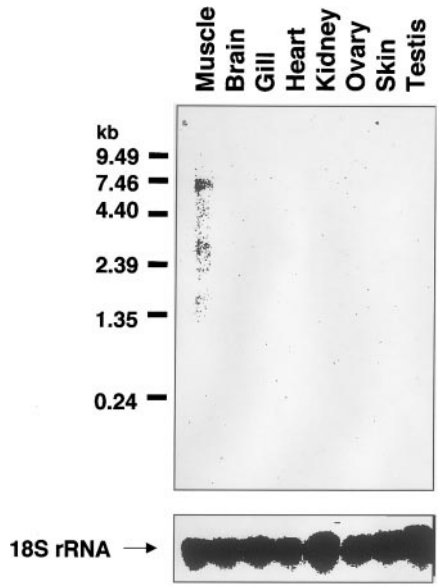

FIG. 5. Northern blot analysis of fMNa1 in various tissues. Total RNA (15 μg) was subjected to denaturing 1% agarose gel electrophoresis, electrotransfer, hybridization with fMNa1 subtype specific probe (nt 950–1134), and then rehybridization with *F. pardalis* skeletal muscle 18S rRNA probe (564 bp fragment obtained by RT-PCR and sequenced by authors, GenBank Accession No. AB029314) for quality and quantity control for total RNA. Lane 1, skeletal muscle; Lane 2, brain; Lane 3, gill; Lane 4, heart; Lane 5, kidney; Lane 6, ovary; Lane 7, skin; Lane 8, testis. Numbers at the left of the panel indicate the location of size standards (GIBCO BRL, Rockville, MD, in kilobases) run on the same gels.

estimated to be 13.7 μM (40). Kidokoro *et al.* examined the effect of TTX on action potentials of muscle fiber in some species of puffer fish, and showed 30 μM TTX had no effect on their action potential (25), although *F. pardalis* itself was not examined. In the present study (Table 1), STX-resistance of *F. pardalis* skeletal muscle Na^+ channel was proved by the markedly larger (460-fold) K_d for ^{3}H-STX to its skeletal muscle membranes than that to rat's. Moreover, presence of at least two subtypes of Na^+ channel (fMNa1, fMNa2) was shown in skeletal muscle of *F. pardalis*, and a full-length cDNA encoding fMNa1 was obtained. In fMNa1 protein, the amino acid of Phe/Tyr, commonly locating in the SS2 region of domain I of TTX-sensitive Na^+ channels, was replaced by Asn (Fig. 3). All TTX-resistant Na^+ channels of mammals have the similar amino acid displacements at this position with non-aromatic amino acid, and showed resistance to 10 μM order of TTX (13–17). According to all these circumstantial evidences, we propose that fMNa1 protein is TTX-resistant. If fMNa2 protein is co-expressed with fMNa1 protein at the similar level in *F. pardalis* skeletal muscle, fMNa2 protein might be also TTX-resistant, although the similar amino acid replacement that found in fMNa1 was not shown in its partial sequence of fMNa2 (Fig. 3). To make more straightforward evidence to prove TTX-resistance of fMNa1 protein, expression in mammalian cell lines and in *Xenopus oocytes* is now being undertaken. The significance of the amino acid substitution of 1569Thr with Gly found in domain IV in fMNa1 protein is worth to be examined by using the other type of Na^+ channels as a reference.

The TTX-resistance of nervous system of puffer fish has not been examined by electrophysiological method, to our knowledge. Although the evidence that TTX directly effects on the central nervous system is scanty, Tambyah *et al.* suggested that TTX might cross the human blood-brain barrier to brainstem functions according to the symptoms of the severely TTX poisoned patient (41). In the present study, the value of K_d for ^{3}H-STX to *F. pardalis* brain membranes was nearly 190-fold larger than that to rat's, but nearly one-sixth of that to skeletal muscle membranes of *F. pardalis* itself. The value of K_d for ^{3}H-PbTx-3 to *F. pardalis* brain membranes was one-third of that to rat's (Table 1). This result suggests that brain of *F. pardalis* is also STX–resistant, but less resistant to STX than its skeletal muscle. Nakazawa *et al.* (42) reported partial cDNA sequences of the puffer fish *F. rubripes* brain Na^+ channel (fBNa1 and fBNa2). The longer sequence (fBNa2) covers most of the entire sequence but lacking the 5′ sequence, encoding the *N*-terminal half of domain I. The shorter sequence (fBNa1) covers the 5′ sequence, but two sequences of the overlapping region are different. The nucleotide sequences of the P regions in the four domains did not show significant deviation from other TTX-sensitive sodium channels (Fig. 3), leaving the question about the mechanism of TTX-resistance of puffer fish brain unsolved.

The cDNA sequences of the clone 2R11 and the clones of RT-PCR to amplify between IS6 and IIIS1 of fMNa1 predicted one-, two- and four-domain Na^+ channel proteins, which were probably generated by alternative splicing (Fig. 2). Occurrence of alternative splicing of voltage-gated Na^+ channel transcripts was reported by Schaller *et al.* (42) in rat brain, skeletal and cardiac muscles between IS6 and IIS1 region, close to the position where the putative alternative splicing occurred in the clone 2R11 in this work. The difference between these alternative splicings is that all splicing variants found in rat tissues do not introduce premature stop codons, while those of fMNa1 introduce them, resulting in the formation of truncated Na^+ channel proteins. Mutually exclusive exon splicing which generated developmentally regulated isoforms was found in rat (rBNa3) (44) and human (SCN3A) (45) brain Na^+ channels at the region of S3 and most of S4 in domain I. Moreover, in the human brain Na^+ channel SCN8A gene, Meisler *et al.* identified two alternatively spliced exons, 18N and 18A, that encoded S3 and S4 in domain III (46). Transcripts with exon 18N have an in-frame stop codon that predicts the synthesis of a truncated two-domain protein. They also indicated that *Fugu*

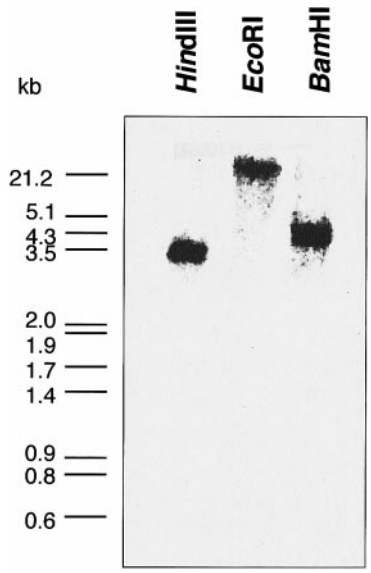

FIG. 6. Southern blot analysis of *Fugu pardalis* DNA. Genomic DNA (10 μg/lane) was digested with the indicated enzymes and hybridized to a fMNa1 subtype specific probe (nt 950–1134). Numbers at the left of the panel indicate the location of size standards (λ DNA-*Hind*III–*Eco*RI double digest, in kilobases) run on the same gels.

rubripes had the similar structure in its exons 18N and 18A to that of human. As shown in Fig. 6, Southern blot hybridization of genomic DNA from *F. pardalis* with the probe specific for fMNa1 (nt 951–1134) gave a single band in each restriction enzyme digestion. The result suggests that deletion and insertion shown in the clone 2R11 could not be originated from multigenes or pseudogene. The four-domain Na^+ channel protein translated from fMNa1 gene is presumably functional and plays a main role as a general Na^+ channel. One- and two-domain proteins, however, are unlikely to function as Na^+ channels, since Numa *et al.* (47) showed that expression of the construct produced a protein containing domain I and II of the rat brain Na^+ channel Scn2a without producing Na^+ currents when tested in the *Xenopus oocyte* system.

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