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Deduced amino acid sequence of a putative sodium channel from the scyphozoan jellyfish *Cyanea capillata*

(Cnidaria/evolution/tetrodotoxin)

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ABSTRACT **Members of the phylum Cnidaria are the lowest extant organisms to possess a nervous system and are the first that are known to contain cells that produce action potentials carried exclusively by Na^+ ions. They thus occupy an important position in the evolution of Na^+ channels. A cDNA encoding a 198-kDa protein with high sequence identity to known Na^+ channels was isolated from the scyphozoan jellyfish *Cyanea capillata*. The similarity between this and other Na^+ channels is greatest in the transmembrane segments and the putative pore region and less so in the cytoplasmic loops that link the four domains of the protein. Phylogenetic analysis of the deduced protein reveals that it is closely related to known Na^+ channels, particularly those of squid and *Drosophila*, and more distantly separated from Ca^{2+} channels. Scrutiny of the *Cyanea* channel in regions corresponding to those purported to form the tetrodotoxin receptor and selectivity filter of Na^+ channels in higher animals reveals several anomalies that suggest that current models of the location of the tetrodotoxin binding site and Na^+ channel selectivity filter are incomplete.**

The Na^+ channels that are responsible for the electrical excitability of nerve and muscle in diverse species are remarkably homogeneous, both structurally and functionally, and are thought to form a superfamily of proteins together with voltage-activated Ca^{2+} and K^+ channels. Na^+ and Ca^{2+} channels consist of four domains, each composed of six transmembrane segments. Models of the evolution of these four-domain proteins from the one-domain K^+ channel envisage two rounds of gene duplication, such that domains III and IV of Na^+ and Ca^{2+} channels evolved from domains I and II, respectively, of the initial two-domain intermediate (1). Further, it has been suggested (2) that these channels initially gated the movement of Ca^{2+}, but with the advent of multicellularity and the subsequent appearance of the nervous system, channels that gated Na^+ rather than Ca^{2+} evolved from them. The selective pressure in this instance is thought to be the need to minimize the intracellular Ca^{2+} levels which would arise during repetitive spiking and which would otherwise be toxic and interfere with other Ca^{2+}-dependent processes.

Of particular interest in the context of Na^+ channel evolution is the Na^+ channel in members of the phylum Cnidaria, comprising the sea anemones, corals, and jellyfish. These are the earliest extant organisms to bear a nervous system, and action potentials in their nervous systems are produced by fast, transient Na^+ currents that are completely insensitive to tetrodotoxin (TTX) (3, 4) but, in the case of the scyphozoan jellyfish *Cyanea capillata* at least, are sensitive to a variety of agents normally thought of as Ca^{2+} channel blockers (3). Cnidarians are thought to have separated from the stem of the evolutionary tree that gave rise to protostomes and deuterostomes 800–1000 million years ago (5). The cnidarian Na^+ channel, therefore, presents a rare opportunity for determining the constraints on the structure of these channels and, perhaps, an insight into the ancestral Na^+ channel.

Here we report the structure of a cDNA isolated from the jellyfish *C. capillata*.‖ This cDNA encodes a protein with high sequence similarity to known Na^+ channels. In addition to providing useful information with which to assess the evolution of this family of proteins, the deduced amino acid sequence provides potentially important information with which to test current theories about the identities of the selectivity filter and TTX binding site of Na^+ channels.

MATERIALS AND METHODS

RNA Isolation and Library Construction. RNA was extracted from the perirhopalial tissue of *Cyanea*. The predominant nerve net in this tissue is the motor nerve net, the population of neurons used in an earlier voltage-clamp study (3). Pieces of perirhopalial tissue were rinsed in fresh seawater and then frozen in 5-g aliquots in liquid nitrogen. Total RNA was isolated by the guanidinium thiocyanate/CsCl method (6). Poly(A)$^+$ RNA was purified by oligo(dT)-cellulose chromatography on a push-column (Stratagene). An oligo(dT)/*Not* I-primed cDNA library was then constructed in the λgt22A vector (GIBCO/BRL) (7).

Library Screening. Polymerase chain reaction (PCR) was performed on this library, using degenerate primers corresponding to highly conserved regions at the 3′ ends of transmembrane segment S6 in domains III and IV of other Na^+ channels; specifically, from V1256 to N1262 and from N1552 to L1559 of the rat brain I Na^+ channel. Reaction mixtures (100 μl) contained 10 mM Tris (pH 8.8), 1.5 mM Mg^{2+}, 50 mM KCl, 0.1% Triton X-100, 200 μM dNTPs, 300 pmol of each of the primers, 5 μl of the cDNA, and 2.5 units of *Taq* DNA polymerase (Promega). This yielded a 918-bp product with high sequence homology to known Na^+ channels, which was used to probe $\approx 3 \times 10^6$ plaques from the λgt22A library. Ten positive plaques, all with seemingly identical 2- to 2.5-kb inserts, were isolated and subcloned by PCR into the PCR1000 vector (Invitrogen). One insert (pMH4C) was sequenced completely, and the sequence was used to identify oligonucleotide primers for use in isolating additional fragments. A 1-kb fragment 5′ to pMH4C (MAH1) was isolated by PCR of an ethanol-precipitated (i.e., concentrated) sample of the library, using an exact antisense primer and a vector primer. Additional PCR failed to provide any further fragments, so a minilibrary was constructed in the pSPORT vector (GIBCO/BRL) by using an exact primer

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Abbreviation: TTX, tetrodotoxin.

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‖The sequence reported in this paper has been deposited in the GenBank database (accession no. L15445).

from the 5′ end of MAH1 to prime first-strand synthesis. This yielded an additional 1.5 kb (p2A). The original λ clone, pMH4C, had a potential poly(A) tail, but this was not preceded by any stop codons and, when the sequence was aligned with the sequences of other Na^+ channels, was seen to occur too close to the last transmembrane segment to be correct. Subsequent inverse PCR (8) on genomic DNA (prepared from planula larvae) yielded a 438-bp fragment (pMbo) 3′ to this spurious poly(A) tail.

Two additional fragments (p3C1 and p38gt) that form the 5′ and 3′ ends of the cDNA, respectively, were isolated as follows. The first, p3C1 (1.2 kb), was obtained from a second 5′ minilibrary, which was constructed in λgt22A by using an exact antisense primer from the 5′ end of p2A for first-strand synthesis. This library was screened with a labeled 150-bp PCR product generated from p2A by using two primers 5′ to that used for first-strand synthesis. Out of 1000 plaques screened, 3 were positive and all bore the same 1.2-kb insert. The second fragment, p38gt (900 bp), was obtained by PCR of the original library by using an exact, sense primer to a region at the 3′ end of pMbo and a reverse, vector primer, followed by a second round of PCR with a second, exact, nested primer. This fragment has a termination codon in frame with the more 5′ fragments, and a 43-base poly(A) tail preceded by an AATAAA consensus sequence, suggesting that this time, it is the real termination of the message. All six fragments were fully sequenced by the dideoxy chain-termination method (9). Each fragment was sequenced from both ends, and sequencing anomalies were clarified by sequencing from genomic DNA.

Phylogenetic Analysis. Amino acid sequences were first aligned by the multiple alignment program CLUSTAL V (10). Default values of 10 were selected for open and floating gap penalties, and the PAM 250 weight matrix (11) was used. The alignment obtained was used to conduct phylogenetic analyses with the aid of the PAUP (version 3.0s) program (12), which searches for trees of maximum parsimony. All trees shown were constructed by using the "branch and bound" option, which is guaranteed to find all minimum-length trees. Gaps were treated as single substitutions. PAUP was also used to perform parsimony "bootstrapping," to place confidence limits on groupings in the trees.

RESULTS AND DISCUSSION

Primary Structure of the Putative Na^+ Channel from *Cyanea*. A 918-bp PCR product with high sequence similarity to known Na^+ channels was amplified from a *Cyanea* cDNA library by using degenerate primers. This was used to isolate a total of six overlapping cDNAs which, when aligned, form a single, 6050-bp sequence that includes a 43-bp poly(A) tail. This nucleotide sequence contains a 5217-bp open reading frame that encodes a 1739-aa protein with a calculated molecular mass of 198 kDa. The first ATG methionine codon appears at base 98. We believe that this is the initiation codon for the *Cyanea* Na^+ channel, for three reasons. (*i*) It is the first potential initiation codon we find, and the sequence preceding it contains a number of nonsense codons. (*ii*) It is in frame with the 5217-base open reading frame of the *Cyanea* channel cDNA, and its position aligns well with other known Na^+ channel initiation sites. (*iii*) The sequence surrounding this ATG corresponds to the consensus sequence for initiation of translation (13).

The deduced amino acid sequence of this protein consists of four homologous domains connected by long hydrophilic linkers of various lengths. A hydrophobicity profile of the protein (not shown) shows that each domain contains six transmembrane segments. Database searches using the FASTA (14) or BLAZE (IntelliGenetics) algorithms for the entire protein, or segments thereof, invariably yield high similarity scores for alignments with Na^+ channels and less so for alignment with Ca^{2+} channels. This, together with the secondary structure revealed by its amino acid sequence and the hydrophobicity profile, suggests that this protein is a Na^+ channel. Based on the known topology of other Na^+ channels, the *Cyanea* Na^+ channel protein bears 34 potential phosphorylation sites and 5 N-linked glycosylation sites (residues 295, 322, 1129, 1136, and 1490).

Alignment with Other Na^+ Channels. The high degree of sequence similarity between the putative jellyfish channel (CYNA1) and other Na^+ channels is evident in Fig. 1, where CYNA1 has been aligned with the eel Na^+ channel (15), the mammalian TTX-resistant cardiac Na^+ channel (16), and the rat brain II Na^+ channel (17). The degree of similarity is greatest in the transmembrane segments and less so in the large cytoplasmic loops that connect individual domains. This is particularly true for the loop between domains I and II, where the jellyfish channel resembles that of the squid (18), the *Drosophila* locus *para* (19) (not shown), and, to a lesser degree, eel (Fig. 1) in being considerably shorter than the equivalent regions of mammalian channels. The S4 region of each of the four domains has a series of positively charged amino acids at every third position, as is the case in other voltage-activated channels (20–22). Although conservation is poor in the cytoplasmic loops, the IFM motif of the III/IV loop that has been shown to be essential for inactivation (23) is functionally conserved (IFL at 1235–1237) in the jellyfish channel. The presence of this inactivation motif is consistent with the properties of the native Na^+ channel in *Cyanea* (3).

If one expands the number of Na^+ channels under comparison (Table 1), one finds that the degree of identity at the amino acid level between the *Cyanea* putative Na^+ channel and others, within the transmembrane segments of the various domains, ranges from as little as 21% (cardiac domain IV segment S3) to as much as 79% (rat brain domain III segment S4). The overall level of identity between the transmembrane segments of CYNA1 and the other five Na^+ channels ranges from 51% (squid and eel) to 55% (rat brain II). Transmembrane segments S4–S6 are consistently more identical than S1–S3 (Table 1).

The loops that connect the S5 and S6 segments in each of the four domains are believed to dip down into the membrane, forming the pore of the channel. Much of the pharmacology and selectivity of voltage-activated cation channels is thought to reside in these loops (24–28). Scrutiny of the *Cyanea* channel in the S5–S6 region (Fig. 2) reveals several interesting features. The TTX binding site of the rat brain II Na^+ channel has been reported to include D384 and E387 (domain I), E942 and E945 (domain II), K1422 and M1425 (domain III), and A1714 and D1717 (domain IV) (24, 25). Furthermore, a Y410C mutation of the skeletal muscle Na^+ channel confers TTX resistance and other cardiac channel properties on the channel (26), whereas the reverse mutation of the TTX-resistant cardiac channel (C374Y) results in an increase in TTX sensitivity (27). All of the relevant residues in domain I of the TTX-sensitive mammalian channels are conserved in CYNA1 (Figs. 1 and 2). Alignment of CYNA1 with rat brain II reveals differences in domains II–IV (Fig. 2). Specifically, in domain II, the negatively charged E942 of rat brain II is replaced by positively charged K694; E945 of rat brain II is conserved in CYNA1 (E697). The altered charge at the first position in this domain could influence TTX binding, since the TTX binding site is thought to bear a negative charge (29). However, in domain III, the charges are reversed; the positively charged K1422 of rat brain II is replaced by negative E1168 in CYNA1. Thus, in domains II and III, the total number of negative charges is the same in CYNA1 as in rat brain II. Finally, in domain IV, the only difference is at position 1717, where the negatively charged D1717 is replaced by a neutral amino acid (N1467). When this

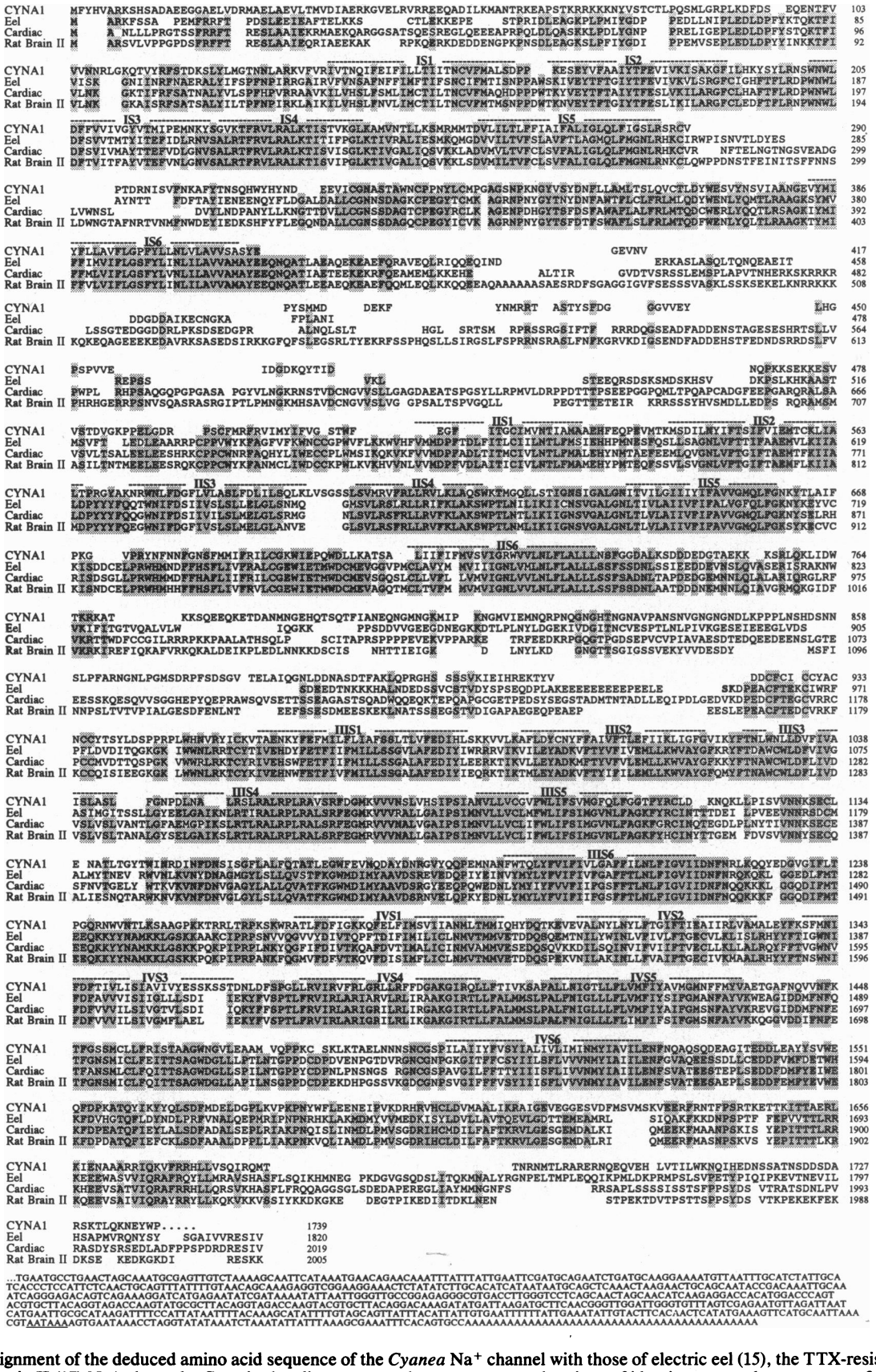

FIG. 1. Alignment of the deduced amino acid sequence of the *Cyanea* Na^+ channel with those of electric eel (15), the TTX-resistant cardiac (16), and rat brain II (17) Na^+ channels. Gaps in the alignment are shown as gaps, and regions of identity among three or more of the channels are shaded. Putative transmembrane segments (S1–S6) are indicated in domains I–IV.

latter mutation was introduced into the rat brain II Na^+ channel, however, the TTX sensitivity decreased only relatively slightly (25). All known Na^+ currents in cnidarians are completely insensitive to TTX (3, 4). Therefore, if subsequent expression experiments confirm that CYNA1 encodes a functional Na^+ channel, one must assume either that the

Table 1. Percent identity between CYNA1 and other Na^+ channels

Domain	Species	Transmembrane segment S1	S2	S3	S4	S5	S6
I	Squid	50	42	35	64	60	48
	Drosophila	36	50	48	65	48	48
	Eel	38	60	43	65	60	56
	Cardiac	42	60	39	70	60	59
	Rat brain II	42	56	39	70	69	59
II	Squid	30	40	45	76	67	40
	Drosophila	52	44	37	76	71	64
	Eel	35	44	42	50	62	60
	Cardiac	39	52	42	58	71	64
	Rat brain II	39	40	46	58	71	68
III	Squid	55	31	47	74	77	58
	Drosophila	48	35	47	58	73	65
	Eel	48	27	37	74	73	77
	Cardiac	52	35	53	68	73	62
	Rat brain II	45	31	53	79	73	69
IV	Squid	38	32	24	48	59	59
	Drosophila	38	36	30	64	70	52
	Eel	34	28	29	56	67	52
	Cardiac	38	36	21	64	74	48
	Rat brain II	45	36	25	64	63	56
Mean		42.2	40.8	39.1	65.1	67.1	58.2
SEM		1.5	2.2	2.0	1.9	1.6	1.9

Values are percent identities derived from CLUSTAL V alignments of the *Cyanea* channel with squid (18), *Drosophila* (19), eel (15), TTX-resistant cardiac (16), and rat brain II (17) Na^+ channels.

several residues discussed above are not sufficient for TTX binding or that it is not the overall charge profile of the pore *per se* that determines its TTX sensitivity, but also the three-dimensional orientation of those charges. It will, therefore, be interesting to see whether TTX sensitivity can be conferred on CYNA1 by simultaneous K694E and E1168K mutations.

Some of the same mutations of the rat brain II Na^+ channel alter its ionic selectivity to resemble that of a Ca^{2+} channel.

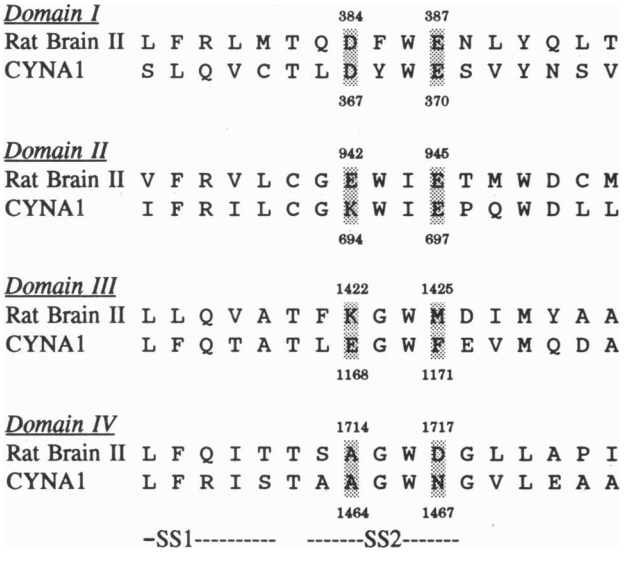

FIG. 2. Alignment of the *Cyanea* sequence with that of rat brain II in the pore region of the channels. Specific amino acids that are thought to form the TTX binding site of the rat brain II channel (24, 25) are shaded, together with their counterparts in the jellyfish channel. Note that the charges associated with E942 and K1422 are reversed in the jellyfish channel. SS1 and SS2 are as defined in ref. 25.

Specifically, mutation of either K1422 or A1714, or both, to glutamic acid confers Ca^{2+} selectivity to the channel (28). However, CYNA1 already bears glutamic acid at the position corresponding to K1422 in the rat brain II sequence (Fig. 2), implying once again that the determinants of ionic selectivity may be more complicated than current models suggest.

Comparison of the sequence of the *Cyanea* channel with those of several other Na^+ channels reveals a remarkably conserved region at the cytoplasmic end of transmembrane segment S6 in each of the four domains (Fig. 3). This high level of identity does not extend to the equivalent regions of Ca^{2+} channels. While such high levels of identity are common among different mammalian Na^+ channel subtypes, particularly within the various transmembrane segments, the degree of identity typically decreases when some of the more distant Na^+ channels (e.g., eel, *Drosophila* para) are included in the alignment. Such a high degree of identity in these regions between Na^+ channels from such evolutionarily distant species suggests that there may be severe constraints on the degree to which this part of the Na^+ channel can mutate without losing its functionality. To date, however, no clear function has been attributed to this region.

Cnidaria are thought to have separated from the main phylogenetic line that gave rise to the remainder of the animal kingdom some 800–1000 million years ago (5). That the level of sequence identity between CYNA1 and other Na^+ channels is so high, in the transmembrane segments at least, despite this enormous evolutionary interval, suggests that there are severe constraints on the degree to which the Na^+ channel can mutate and still remain functional.

Phylogenetic Analysis. The program PAUP (12) was used to find the most parsimonious tree for the relationship between the different domains of CYNA1 and between CYNA1 and a variety of Na^+ channel and Ca^{2+} channel amino acid sequences (Fig. 4). Comparison between the different domains

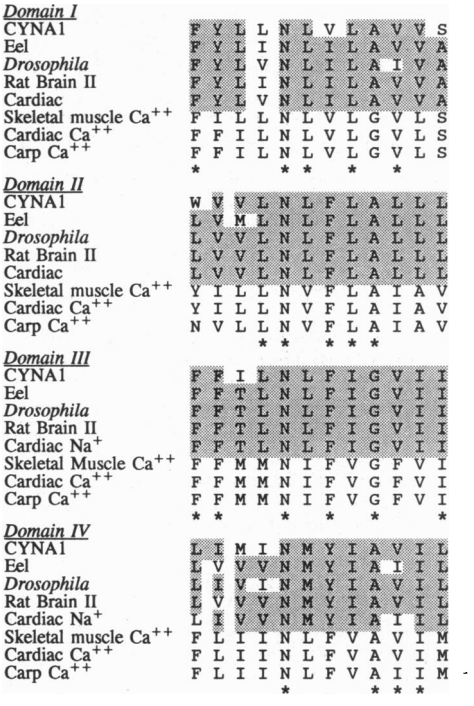

FIG. 3. Alignment of the *Cyanea* Na^+ channel with those of the eel (15), the *Drosophila* locus *para* (19), rat brain II (17), and cardiac muscle (16) over the last 12 aa in each of the four domains. The equivalent regions of three Ca^{2+} channels—skeletal muscle (30), cardiac (31), and carp (32)—are aligned below. Areas of identity within the Na^+ channels are shaded; residues shared between Na^+ channels and Ca^{2+} channels are marked with asterisks.

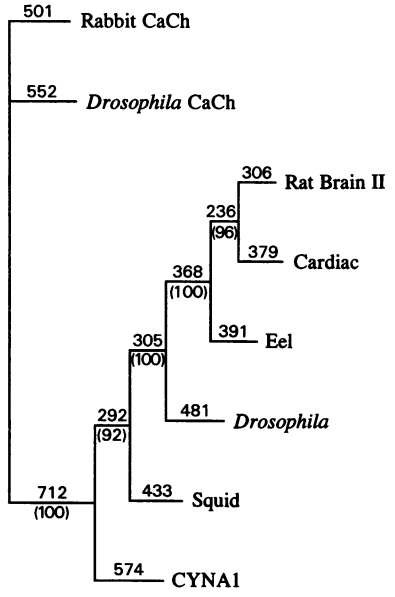

FIG. 4. The single most parsimonious phylogenetic tree created by use of PAUP (12), showing the relationship between six Na^+ channels [rat brain II (17), TTX-resistant cardiac (16), eel (15), *Drosophila* para (19), squid (18), and CYNA1] and two Ca^{2+} channels (CaCh) [rabbit skeletal muscle (30) and *Drosophila* (unpublished)]. Bootstrap values (in parentheses) and branch lengths are indicated.

of CYNA1 shows that domains II and IV are more closely related to one another than they are to domains I and III (data not shown). This confirms the model of gene duplication recently proposed (1) and indicates that the two domains created by the initial round of gene duplication of the single domain precursor must have undergone considerable divergence prior to the second round of gene duplication that ultimately created domains III and IV.

A phylogenetic comparison between CYNA1 and a variety of Na^+ and Ca^{2+} channels (Fig. 4) reveals that CYNA1 fits into a grouping with other known Na^+ channels. In contrast, two Ca^{2+} channel sequences, one vertebrate and one invertebrate, fall outside of the Na^+ channel grouping and are well separated from CYNA1. The *Cyanea* channel is, as one might expect, more distantly related to rat brain II Na^+ channel than the other Na^+ channel sequences. Its closest relation is the squid Na^+ channel (18).

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