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Molecular Cloning and Characterization of a Putative Neural Calcium Channel α_1-Subunit from Squid Optic Lobe

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The complete amino acid sequence of a putative calcium channel α_1-subunit, SQCC1, from the optic lobe of the squid *Loligo bleekeri* has been deduced by cloning and sequence analysis of the complementary DNA. The open reading frame encodes 2206 amino acids, which corresponds to a molecular weight of 251,451. The deduced amino acid sequence shares general structural features with the other voltage-dependent calcium channels; it consists of four repeated units of homology. Each motif has five hydrophobic segments and one positively charged segment. The transcriptional products were detected in all nervous systems examined; optic lobe, cerebral ganglia and giant stellate ganglia. However, it was not detected in the mantle muscle, heart and stomach, indicating SQCC1 is a calcium channel α_1-subunit specific for squid nervous system. SQCC1 is more closely related in its amino acid sequence patterns to dihydropyridine-insensitive calcium channels rather than dihydropyridine-sensitive ones.

Voltage-dependent calcium channels play essential roles in the regulation of a variety of cellular functions, including membrane excitability, enzymatic activity, axonal outgrowth, muscle contraction, synaptic transmission and secretion (1,2). On the basis of their electrophysiological and pharmacological properties, at least five types of voltage-dependent calcium channels (designated N, T, L, Q and P-type) have been distinguished (3, 4) and can be grouped into two major classes by their dihydropyridine (DHP) sensitivity. One is the DHP sensitive L-type calcium channel subfamily and the other is the DHP insensitive non-L-type subfamily.

In the squid nervous system, Ll'nas *et al.* reported that a calcium channel of squid optic lobe showed P-type like physiological properties (5). Further, Charlton and Augustine noted that the pharmacological and functional properties of presynaptic calcium channels of the squid giant synapse did not correspond to those of N, T or L-type calcium channels (6). Although these reports suggest the presence of P-type or non N, T and L-type calcium channels in the squid nervous system, the molecular features of squid calcium channels are still unknown.

The present paper reports the complete sequence of a squid putative neural calcium channel α_1-subunit cDNA (SQCC1) cloned from a squid optic lobe cDNA library and the tissue distribution of SQCC1 and its novelty and commonness compared to the calcium channels previously reported.

MATERIALS AND METHODS

Animals. Live squid, *Loligo bleekeri,* were captured in the Sagami Gulf of Japan and transported to the Electrotechnical Laboratory (7) or the Marine Biological Station of the University of Tokyo at Misaki, Japan, where they were sacrificed for the present experiments.

cDNA library construction and cloning. Total RNA was extracted from the squid optic lobe by the guanidium thiocyanate method. Poly (A)+ RNA was isolated by repeating oligo (dT)-cellulose chromatography twice (Pharmacia Biotech). The random primed cDNAs were ligated into λZAP II (Stratagene) and transfected into XL-1 Blue cells to construct the squid optic lobe cDNA library. The cells were then inoculated into plastic culture dishes and transferred onto nylon membrane filters, Hybond-N (Amersham). The filters were treated with alkaline, neutralized and irradiated with UV to cross-link the DNA to the filter. Hybridization with the DNA probe was performed in a hybridization buffer (6× SSC, 5× Denhaldt's solution, 0.5 % SDS, 20 μg/ml Salmon sperm DNA) at 65 °C over night. After hybridization, filters were washed in 0.2× SSC at 65 °C for 20 min, and analyzed by X-ray film autoradiography or the bio-image analyzer, BAS2000 (Fuji Photo Film).

PCR. Double-strand cDNAs for PCR were synthesized from purified mRNA. The reaction solution for the PCR contained 10 mM Tris-

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HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.01 % gelatin, 200 μM NTP, 1 μM DNA primers and 25 units/ml Taq DNA polymerase (Cetus). For the DNA primers, we synthesized eleven 14-20 mer mixed oligonucleotides, based on the amino acid sequences commonly conserved among BI-2, BII-2, BIII, D, Sk and C calcium channels. Fig. 2 shows the position of a successful primer pair (P5, P7) by double under line. Primer P5 lies on II S5-6 and has the sequence 5′[C/T]T[A/G/C/T]AC[A/G/C/T]GG[A/G/C/T]GA[A/G]GA[C/T]TGGA3′. Primer P7 lies on IIS6 and has the sequence 5′GC[A/G/T]AT [A/G/C/T]GC[A/G/C/T]A[A/G][A/G]AA[A/G/C/T]AC[A/G]TT3′. A thermal cycler, model PC-700 (ASTEC), was used with the DNA primers which were synthesized with the Cyclone plus DNA synthesizer (Milligen). The PCR with the DNA primers was performed in a 100 μl of the reaction solution under the conditions of 94 °C for 1 min, 40 °C for 2 min, 72 °C for 3 min in succession, and subjected to 50 cycles of the amplification. DNAs amplified were analyzed with agarose gel electrophoresis. Major bands were excised from the gel, ligated into the M 13 mp19 vector, respectively, to be amplified and sequenced.

Sequencing. After screening, positive colonies were plaque purified, excised into pBluescript II SK(−) phagemid. Inserts of each clone were digested with Kilo-Sequence Deletion Kit (Takara) and deletion mutants were sequenced. Both directions of the cDNA were sequenced by the dideoxy chain termination method with Sequenase ver. 2.0 (United Sates Biochemical) or Sequencing High (TOYOBO) with [α-^{35}S]dCTP (Amersham) and partially sequenced with dye-labeled oligo primer (ABI 370A auto-sequencer).

Northern hybridization analysis. Three different probes, SQN, SQ3 and SQC, were obtained from SQCC1 by restriction endonuclease digesting at respective site (Fig. 1B). 30 μg/lane of the total RNA was electrophoresed in 0.9 % formaldehyde denatured agarose gel and transferred to nylon membrane filters, Hybond-N (Amarsham) with 20× SSC for 48 hr. The filter was irradiated with UV to cross-link the total RNA to the filter. Hybridization with the DNA probe was performed in a hybridization buffer (5× SSPE, 5× Denhardt's solution, 0.5% SDS, 20 μg/ml salmon sperm DNA, 10 ng/ml DNA probe) at 42 °C overnight. Using high stringency, filters were washed first in 2× SSC, 0.2 % SDS at 65 °C for 20 min twice, and then in 0.2× SSC, 0.1 % SDS at 65 °C for 25 min twice. For the re-hybridization with G3PDH, filters were washed in 5 mM Tris-HCl (pH.8.0), 2 mM Na2EDTA, 0.1× Denhardt's solution at 65 °C for 2hr. In the low stringency, re-hybridization was performed at 38 °C overnight. After re-hybridization, filter was washed in 2× SSC, 0.2 % SDS at 55 °C for 30 min. Specific DNA probes were labeled with [α-^{32}P] dCTP (Amarsham) by the Megaprime DNA labeling kit (Amarsham). The patterns of the northern hybridization were analyzed by the bio-image analyzer, BAS2000 (Fuji Photo Film).

RESULTS AND DISCUSSION

The product from primer pair P5/P7 had a deduced amino acid sequence very similar to that of vertebrate calcium channel α_1-subunit between IIS5-6 and IIS6. Using the PCR product as a probe, the squid optic lobe cDNA library in λZAP II was screened under high stringency condition. Out of 300,000 clones, we found only one positive clone, SQCR1 of about 5.4 kbp length (Fig. 1A). The 650 bp DNA fragment, which was located at the 5′ end of SQCR1, was amplified by PCR and used as a new probe (SQ650). This located one more clone, SQCR2 of about 2.4 kbp length (Fig. 1A). The full-length cDNA sequence of a squid putative calcium channel α_1-subunit, SQCC1 was constructed by the DNA sequences of SQCR1 and SQCR2 (Fig. 1A). The restriction endnuclease maps used for the cDNA fragment, SQCR1 and SQCR2, are shown in Fig. 1A. Fig. 2 shows the nucleotide sequence of the cDNA, together with the amino acid sequence for SQCC1, determined using the clones of SQCR1 and SQCR2.

The translational initiation site was assigned to the methionine codon, composed of nucleotide residues 330-332, because the first ATG triplet that appears downstream of a nonsense codon, TAA (nucleotides 279-281), is contained in the specific nucleotide sequence AGGATGA of the residues 327-333. The specific sequence has a favored sequence of a general represen-

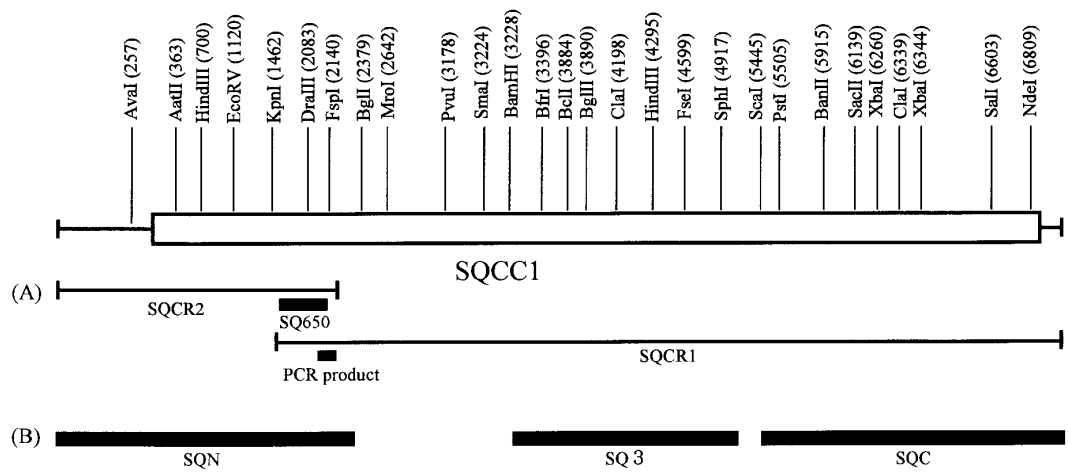

FIG. 1. The restriction endonuclease map of SQCC1. The map stands for the cleavege sites at the 5′ terminal nucleotide by respective restriction endonucleases. Protein coding regions are indicated by open boxes. (A)The probes, PCR product and SQ650, used for screening of a cDNA library are indicated by closed boxes. The obtained clones, SQCR1 and SQCR2, are indicated open boxes. (B)The probes, SQN, SQ3 and SQC, used for northern hybridizations are indicated by closed boxes.

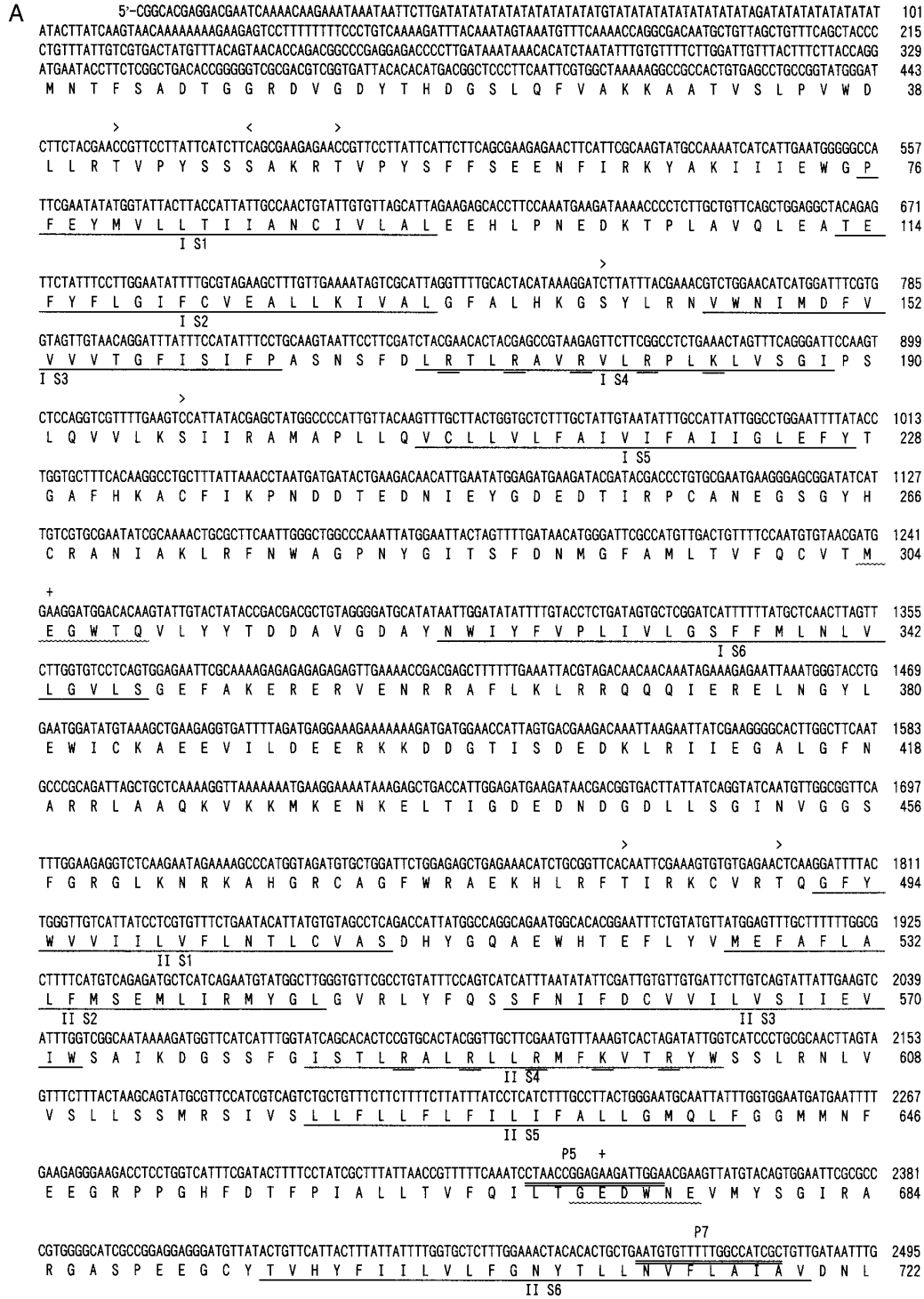

FIG. 2. Nucleotide sequence of the cDNA encoding a squid putative calcium channel (upper) and its deduced amino acid sequence (lower). The sequence was determined with the clones of SQCR1 and SQCR2 (Fig. 1). The nucleotide sequence is numbered, starting from the first residue of SQCR2. The positively charged amino acids (R and K) in S4 is indicated by a double underline. The potential cyclic adenosine monophosphate (cAMP)-dependent phosphorylation sites (>) and protein kinase C phosphorylation sites (<) are shown. The proposed calcium-binding domain (the EF hand) is indicated as labeled broken line and the putative ion-selective pore-forming regions (〰) and conserved glutamate (+) are shown. The nucleotide sequence data reported in this paper will appear in the DDBJ, EMBL and GenBank nucleotide sequence databases with the Accession No. D86600.

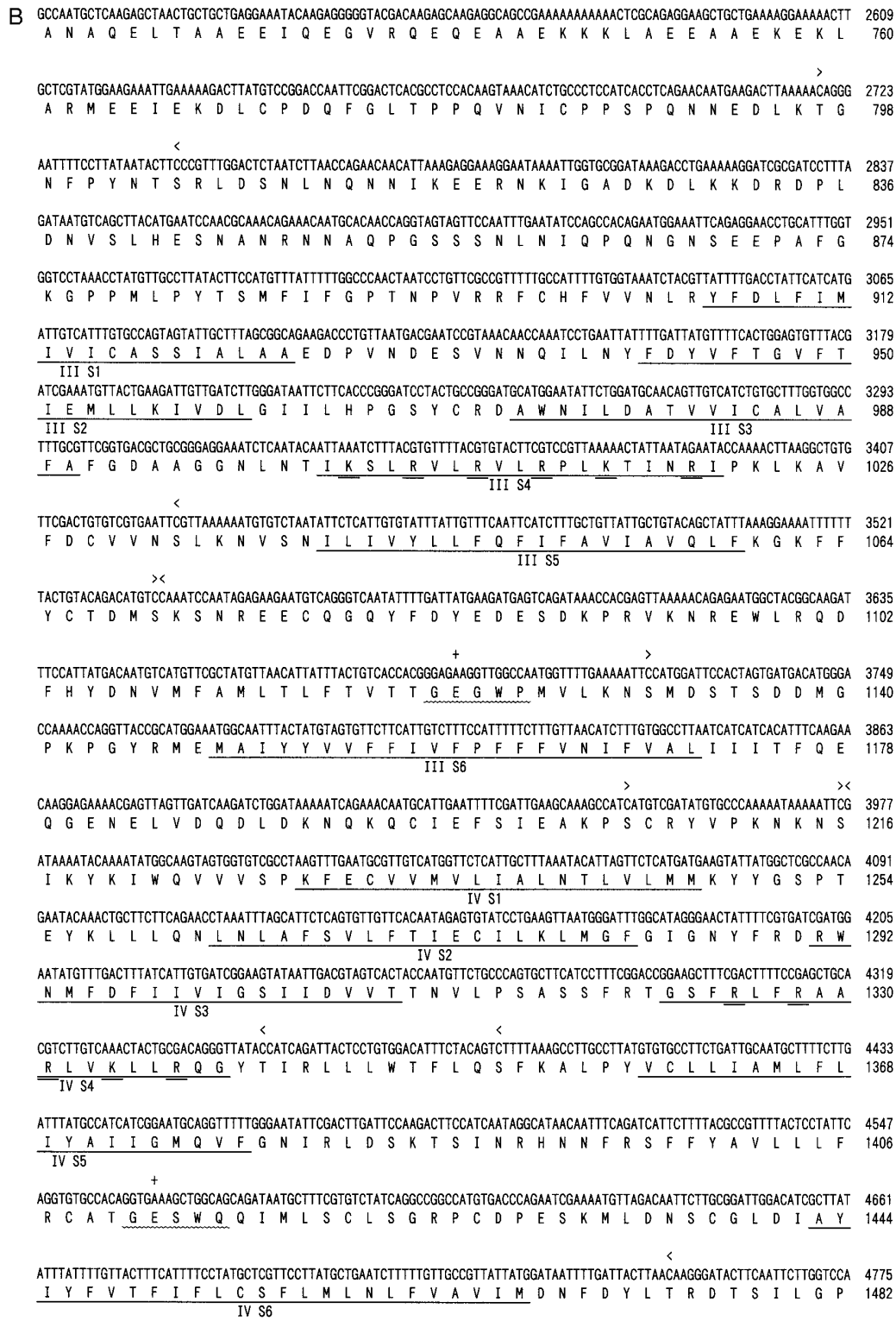

FIG. 2—*Continued*

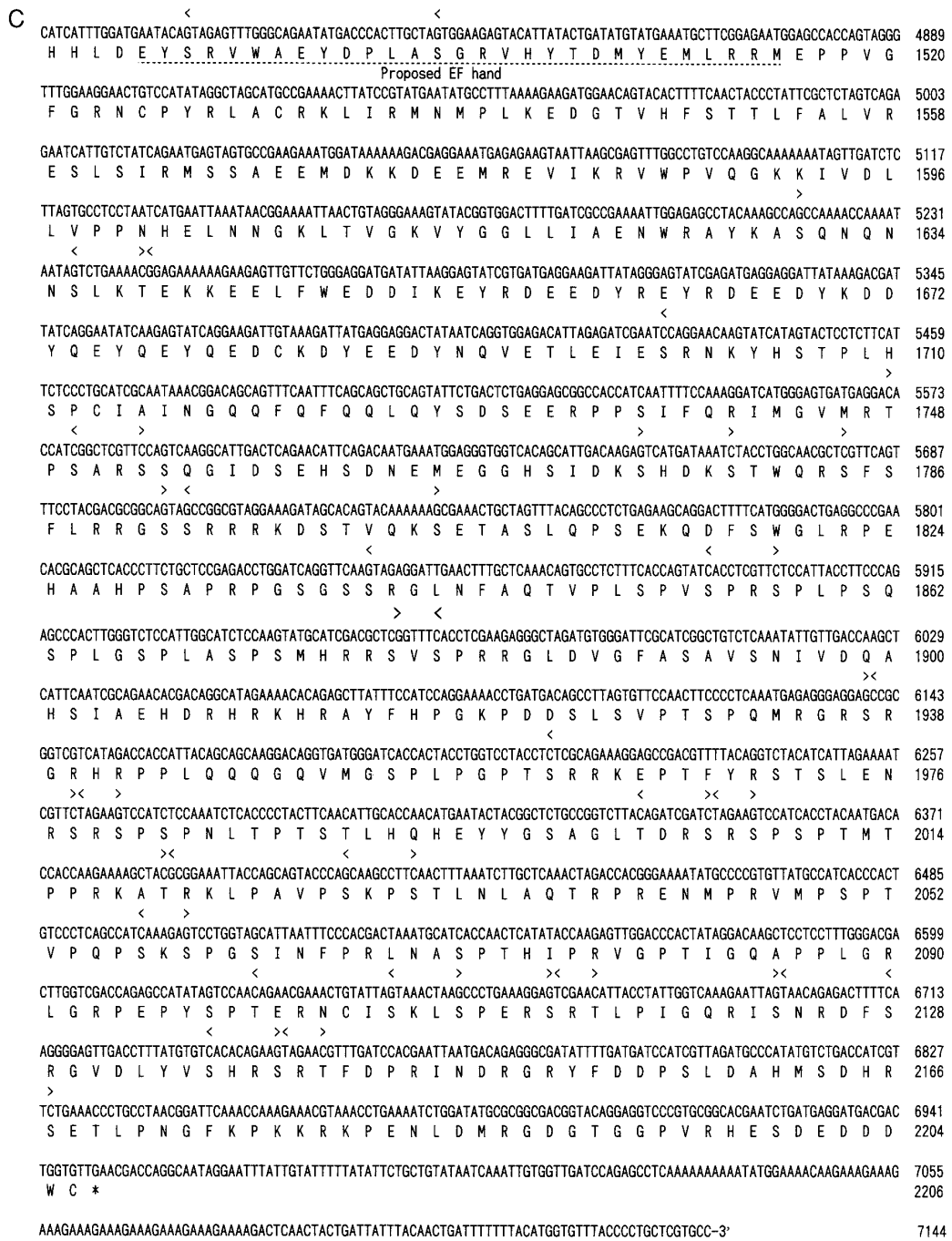

FIG. 2—*Continued*

tation (A or G)XXATG(A or G) (X being any nucleotide) around the initiation codon ATG in the eukaryote (8). SQCC1 is found to be AT rich; the content of AT and CG are 59.3 % and 40.7 %, respectively.

SQCC1 encodes a 2206 amino acid residue, which corresponds to a molecular weight of 251,451. The amino acid sequence of SQCC1 shares some general structural features with the α_1-subunit of the voltage-dependent calcium channels and sodium channels, thus apparently having a similar topology; [1] it consists of four repeated units of homology, [2] each repeat having five hydrophobic regions (S1, S2, S3, S5 and S6) and one positively charged segment (S4) (Fig. 2) (9). As for the segment 4 (S4) , which probably represents a voltage-sensor region (10, 11, 12) or core pore formation region (13) (Fig. 2), SQCC1 shows the same general pattern as the majority

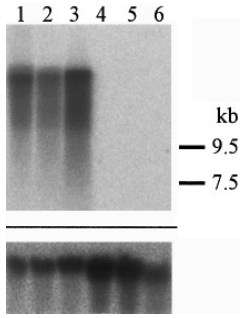

FIG. 3. Tissue distribution of SQCC1 mRNA in various tissues by northern hybridization analysis (30μg/lane total RNA) (1) optic lobe; (2) cerebral ganglia; (3) stellate ganglia; (4) mantle muscle; (5) heart; (6) stomach. The bottom shows the result of reprobing with human G3PDH.

of other calcium channels, with five positively charged side chains in the S4 helices in domains I, II and IV, and six in domain III (Fig. 2). As expected for a calcium channel α_1-subunit, conserved glutamate residues, believed to form a calcium binding site (selectivity filter) (14), were found in the putative pore-forming regions in all four domains (Glu-305, Glu-672, Glu-1122, Glu-1412) (Fig. 2). The potential cyclic adenosine monophosphate (cAMP)-dependent phosphorylation sites and protein kinase C phosphorylation sites (15) found in the cytoplasmic side of SQCC1 are indicated by the tips of the arrows, $>$ and $<$, respectively, in Fig. 2. Another feature commonly found in both sodium and calcium channel α_1-subunits is a protein motif known as the EF hand, which consists of two α-helices flanking a calcium-binding loop (16). In the Tufty-Kretsinger test (17) the SQCC1 sequence has 11 matches (out of 16 possibilities) for residues important for calcium binding. As indicated by the broken-line beginning 19 amino acids downstream from the IV S6 region in Fig. 2, an EF hand is present in the squid sequence.

Northern hybridizations of squid optic lobe, cerebral ganglia, stellate ganglia, mantle muscle and stomach total RNA were performed with SQN, SQ3, SQC probes. All probes gave similar results. Transcription of SQCC1 gene was detected not only in the optic lobe but also in both the cerebral ganglia and stellate ganglia. On the other hand, no signals were detected in mantle muscle and stomach (Fig. 3) or in heart. To test for mRNA recovery and gel loading differences, filters were re-hybridized with human glyceraldehyde 3-phospate dehydrogenase (G3PDH) DNA (Clontec), which is ubiquitously expressed throughout the organism (18), as a control probe in the low stringency. All tissues examined expressed G3PDH uniformly. This result suggests that the non-reaction of squid muscle, heart and stomach total RNA is not due to degradation caused by the RNase (Fig. 3). As a result, it is concluded that SQCC1 gene is expressed only in the nervous tissues and not in the muscle, heart and stomach. This is consistent with the observation for vertebrate calcium channels that different subtypes are located in either nervous or non-nervous tissue (19).

According to the sequence comparison of SQCC1 with the overall amino acid sequence of sodium channels (a squid putative sodium channel SQSC1 (20) and rat type II (21)) and other calcium channel α_1-subunit (BI-2: rabbit brain, BII-2: rabbit brain, BIII: rabbit brain, rbA-I: rat brain, rbB-I: rat brain, doe-4: marine ray electric lobe, brain, D: human neuronal cell, C: rabbit heart, Sk: rabbit skeletal muscle, rbC-I: rat brain, CpSkm: carp skeletal muscle, Mdlα_1: house fly muscle, Dmca1D: fruit fly head), the amino acids identities between the SQCC1/SQSC1, SQCC1/rat type II pairs were only 22 %, 20 %, respectively. SQCC1 is more closely related to those of DHP-insensitive non L-type calcium channels (42 % to 45 %) than to those of DHP-sensitive L-type calcium channels (36 % to 39 %) and fly calcium channels (32 % and 37 %) (Fig. 4A). Percentage identity of two conserved regions of the squid channel (position 53-737, including domain I, II and 878-1633, domain III, IV) is higher to those of DHP-insensitive non L-type channels (61 % to 63 %) compared to DHP-sensitive L-type (46 % to 51 %) and fly calcium channels (49 %)(Fig. 4A). To establish possible evolutionary relationships among these voltage-dependent calcium channels, a phylogenetic tree was constructed by the UPGMA method, as depicted in Figure 4B. The calculation was based on the overall amino acid sequences. According to the phylogenetic tree, SQCC1 is grouped within the DHP-insensitive non L-type calcium channel α_1-subunit subfamily.

Amongst the invertebrates, putative calcium channels have been cloned from housefly (*Musca domestica*) muscle and fruitfly (*Drosophila melanogaster*) head preparations, calcium channel α_1-subunits Mdlα_1 (22) and Dmca1D (23), respectively. The spatial distributions of these clones within the tissues have been examined in detail and demonstrating that Mdlα_1 is predominantly expressed in the larvae body wall musculature and is grouped with the DHP-sensitive calcium channel subfamily. Dmca1D is expressed in the nervous system of *Drosophila* embryo and in the adult body, head and legs. Dmca1D is thought to be non-specifically expressed among whole parts of fly organs in adult *Drosophila* tissues. According to the phylogenetic tree, Dmca1D and Mdlα_1 are grouped with the DHP-sensitive L-type subfamily, separate from subfamily consisting of D, Sk, C type calcium channels (Fig. 4B).

SQCC1 is expressed in adult squid nervous system, of optic lobe, cerebral ganglia and stellate ganglia, but not in other squid tissues examined, i.e., mantle muscle, heart and stomach. SQCC1 has a higher amino acid identity to the DHP-insensitive non L-type calcium channels and grouped to this calcium channel

A

	SQCC1	BI-2	BII-2	BIII	rbAI	rbBI	doe-4	D	Sk	C	CpSkm	rbCI	Dmca1D	Mdlα1
SQCC1		61	61	63	63	63	62	49	49	51	46	50	49	49
BI-2	43		74	79	95	79	76	49	48	49	46	49	48	48
BII-2	42	56		76	76	76	74	49	49	50	46	49	49	49
BIII	44	61	56		81	97	82	51	50	51	47	51	50	50
rbAI	43	83	58	61		81	78	50	49	51	47	50	50	50
rbBI	45	60	56	91	60		82	51	50	51	47	51	50	50
doe-4	45	60	56	68	59	68		49	49	50	47	50	49	49
D	38	35	35	37	35	36	36		74	83	67	82	67	66
Sk	38	34	37	36	36	37	37	57		75	74	74	64	64
C	39	35	36	36	36	37	37	66	58		68	98	66	66
CpSkm	36	33	35	35	35	35	36	52	66	53		69	59	59
rbCI	39	36	36	37	36	37	37	67	59	93	54		66	66
Dmca1D	32	27	30	30	30	31	31	43	41	44	34	43		83
Mdlα1	37	33	36	35	36	35	35	49	53	49	50	49	51	

B

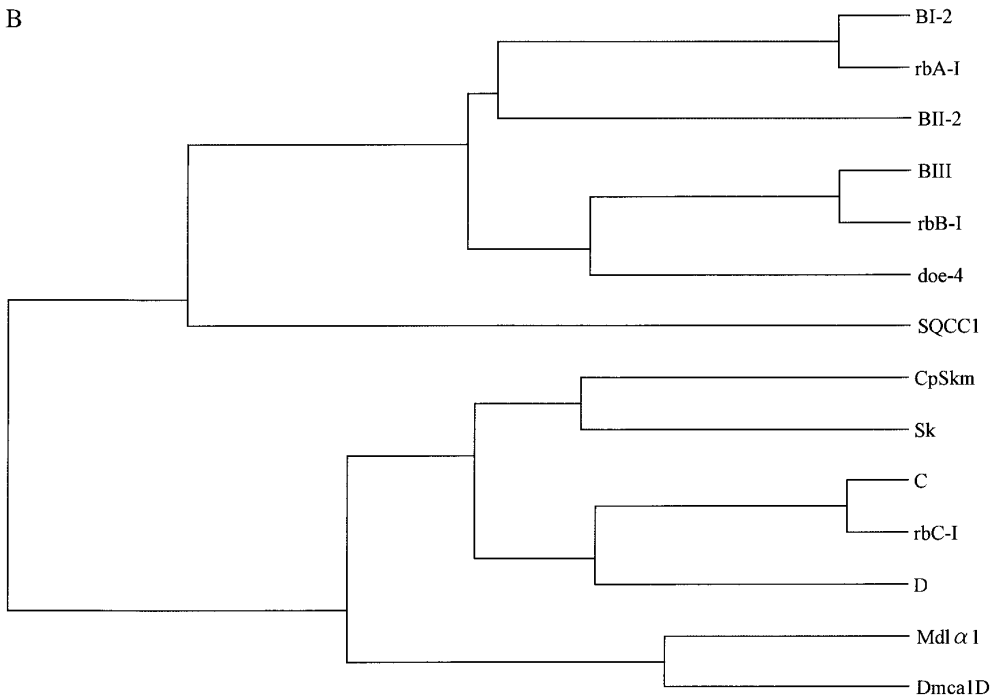

FIG. 4. Identity matrix and phylogenetic tree for members of the calcium channel family. (A) Percentages of amino acid identity of calcium channel pairs as determined the GENETYX program. Gaps are counted as one substitution regardless of their length. Lower left corner of the diagonal: overall amino acid identity. Upper right corner: percentage identity within the totality of two conserved regions (position 53-737 and 878-1633 (SQCC1 counting)) (B) Phylogenetic tree obtained with GENETYX program using the UPGMA method. The length of the horizontal lines connecting one sequence to another is proportional to the estimated genetic distance between the sequences.

subfamily in the phylogenetic tree (Fig. 4B). These results suggest that, although the electrophysiological and pharmacological properties of SQCC1 are still unknown, SQCC1 is a first molecular-cloned, putative DHP-insensitive calcium channel α_1-subunit, specifically expressed in the invertebrate nervous system. Functional expressions of SQCC1 are now in progress.

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