Volume 274, number 1,2, 207-213 FEBS 09105 November 1990

Polymerase chain reaction cloning of L-type calcium channel sequences from the heart and the brain

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Received 17 August 1990

The sequences of the highly conserved S4 regions of voltage-sensitive ion channels were used to design oligonucleotide primers for the polymerase chain reaction. Specific fragments of the cDNA encoding L-type calcium channels from the heart, brain, and skeletal muscle were amplified and cloned. The nucleotide sequences of the cardiac and brain calcium channels obtained are identical over this region, and share 78% homology with the skeletal muscle calcium channel. Comparison of the predicted amino acid sequences of our clones with those of other calcium channels reveals unexpected patterns of conservation which suggest alternative exon use.

Dihydropyridine receptor; Voltage-sensitive, Calcium ion channel; Polymerase chain reaction; Homology probing

1. INTRODUCTION

L-type calcium channels are members of a family of voltage-sensitive ion channels which share structural similarities and sequence homology [1-6]. They are found in a variety of tissues, including skeletal muscle, heart, and brain. Channels in these tissues are functionally and immunologically distinguishable [7,8]. However, they all consist of five subunits, $\alpha 1$, $\alpha 2$, β, δ, and γ. The $\alpha 1$ subunit appears to form the transmembrane channel and contains the binding site for dihydropyridines. Complementary DNA sequence for the $\alpha 1$ subunit from rabbit skeletal muscle has been determined by Tanabe et al. [5]. Ellis et al. have determined the cDNA sequence for the $\alpha 1$ and $\alpha 2$ subunits of the rabbit skeletal muscle calcium channel [9]. More recently, Mikami et al. reported the primary amino acid sequence of an $\alpha 1$ subunit from the heart [10], and Koch et al. reported partial amino acid sequence of $\alpha 1$ subunits from rat brain and aorta [11].

We used the polymerase chain reaction (PCR) to clone a specific fragment of the $\alpha 1$ subunit of the calcium channel from rabbit heart and brain tissue. Primers were synthesized using sequences from the S4 regions of the skeletal muscle calcium channel. Every third amino acid within these regions is arginine or lysine, making them highly positively charged. They are conserved not only between the repeat units of a single ion channel, but also between ion channels. Stuhmer et al. [12] have shown by site-directed mutagenesis that the S4 regions are involved in the voltage-sensing mechanism for activation of these channels.

A fragment corresponding to the region between the third and fourth S4 region of the calcium channel was amplified and cloned from cDNA of rabbit heart, brain, and skeletal muscle. The cardiac and brain clones we obtained are identical, and share 78% homology to the skeletal muscle clone. The predicted amino acid sequence of this region is similar, but not identical to that recently reported by Mikami et al. for a cardiac calcium channel clone [10].

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2. MATERIALS AND METHODS

2.1. *Oligonucleotide synthesis*

Oligonucleotides were synthesized using the phosphoramidite method with an Applied Biosystems 380B DNA synthesizer. Preparative acrylamide gel electrophoresis and Sep-Pak C-18 columns (Waters Associates) were used for desalting and purification.

2.2. *RNA isolation and characterization*

RNA was isolated from rabbit heart, brain, and skeletal muscle by homogenization in RNAzol (Cinna/Biotecx, Texas), followed by extraction with chloroform. Poly(A) RNA was selected using type 7 oligo-dT cellulose (Pharmacia).

RNA was resolved on agarose gels containing formaldehyde, and transferred to nitrocellulose. Blot hydridization was carried out in 50% formamide, 6× SSC (SSC is standard saline citrate; 1× SSC is 0.15 M NaCl, 0.015 M sodium citrate) at 42°C. Blots were washed with 0.2× SSC, 0.1% SDS at 55°C and exposed to film at −70°C in the presence of an intensifying screen.

2.3. *cDNA synthesis*

First strand cDNA was synthesized using murine Maloney virus reverse transcriptase (Bethesda Research Laboratories). The cDNA was extracted with phenol/chloroform and precipitated with ethanol. Quantitation of yield and size was done by including [^{32}P]dCTP in the reaction and calculating the incorporation of ^{32}P, and by autoradiography of a denaturing alkaline agarose gel.

2.4. *Polymerase chain reaction*

20 pmol of each primer and 100 ng of first-strand cDNA were used in each 100 μl PCR reaction. Reaction components were obtained from Cetus Corporation. Reaction temperatures were varied using a

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Eel Na IIIS4                    Asn Leu Arg Thr Ile Arg Ala Leu Arg Pro Leu Arg Ala Leu Ser Arg
                                AAT CTC AGA ACT ATC AGG GCT CTT CGC CCT TTA CGT GCC CTT TCC AGA

Rat Nal IIIS4          Ala Ile Lys Ser Leu Arg Thr Leu Arg Ala Leu Arg Pro Leu Arg Ala Leu Ser Arg Phe
                       GCC ATC AAG TCC CTA AGG ACA CTA AGA GCT CTG AGA CCC CTA AGA GCC TTA TCA CGA TTT

Rabbit Ca IIIS4        Val Val Lys Ile Leu Arg Val Leu Arg Val Leu Arg Pro Leu Arg Ala Ile Asn Arg Ala
                       GTG GTA AAG ATC CTG AGA GTG CTA AGG GTG CTC CGG CCC CTG CGA GCC ATC AAC AGA GCC
                                                                  PRIMER 1

Eel Na IVS4            Arg Leu Ala Arg Ile Ala Arg Val Leu Arg Leu Ile Arg Ala Ala Lys Gly Ile Arg
                       CGC TTG GCA AGG ATT GCT CGG GTG TTG CGT CTC ATC AGA GCA GCA AAG GGC ATA AGG

Rat Nal IVS4           Arg Leu Ala Arg Ile Gly Arg Ile Leu Arg Leu Ile Lys Gly Ala Lys Gly Ile Arg
                       CGC CTG GCC AGG ATT GGA CGA ATC CTA CGC CTG ATC AAA GGC GCC AAG GGG ATC CGC

Rabbit Ca IVS4         Ile Ser Ser Ala Phe Phe Arg Leu Phe Arg Val Met Arg Leu Ile Lys Leu Leu Ser Arg Ala
                       ATC TCC AGT GCC TTC TTC CGC CTG TTC CGG GTC ATG AGG CTG ATC AAG CTG CTG AGT CGG GCC
                                                                  PRIMER 2
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Fig. 1. Comparison of homology between third and fourth S4 regions of the eel and rat sodium channels and rabbit skeletal muscle calcium channel. The locations of primers 1 and 2 are underlined.

thermal cycler (Perkin Elmer). A typical cycle consisted of denaturation at 94°C for 1 min, annealing at 58°C for 2 min, and polymerization at 72°C for 3 min. The cycle was repeated 40 times. Reaction products were resolved on NuSieve agarose gels (Seakem).

PCR reaction products were extracted with phenol/chloroform, and precipitated with ethanol. They were digested with *Hin*dIII, and ligated to pUC19 vector digested with *Hin*dIII. Ligation mixtures were transformed into competent DH5α cells, and positive clones were identified by hybridization to ^{32}P-labelled size-fractionated DNA from the PCR reaction.

2.5. *DNA sequencing*

DNA was subcloned into M13 mp18 or mp19, and single-stranded phage DNA was isolated. DNA sequencing was done by the chain termination method using Sequenase enzyme (US Biochemicals) in the presence of [^{35}S]dATP. Sequencing reactions were resolved on 8% and 6% acrylamide gels containing 8 M urea; the gels were dried and exposed to film at room temperature. Sequencing of both strands was done for confirmation.

3. RESULTS

3.1. *Design of oligonucleotide primers and PCR cloning*

Sodium channels and calcium channels contain 4 internal repeat units, designated I–IV. Each of these units can be divided into 6 membrane-spanning segments, S1 through S6. The S4 regions are highly positively charged, and form the presumed voltage-sensor of these channels. The potassium channel is similar, but contains only one set of the 6 segments.

Fig. 1 shows the homology between the third and fourth S4 regions from the eel and rat sodium channels and the rabbit skeletal muscle channel. We chose the third and fourth S4 segment of the rabbit skeletal muscle calcium channel to generate the PCR primers underlined. Primer 1, from the IIIS4 segment, spans 16 amino acid codons including a proline which is conserved between he sodium and calcium channels. Primer 2, from the IVS4 segment, is the reverse complement of a region which spans 17 amino acid codons including 3 phenylalanine residues found only in the calcium channel. A hexanucleotide containing the *Hin*dIII site was added to the 5′ end of both primers.

Poly(A) mRNA from rabbit heart, brain, and skeletal muscle was used as template for reverse transcription. The single stranded cDNA was used as template with primers 1 and 2 in a PCR reaction. Fig. 2 shows an agarose gel of the reaction products as a function of annealing temperature. Very little specific product is seen with temperatures below 50°C. At

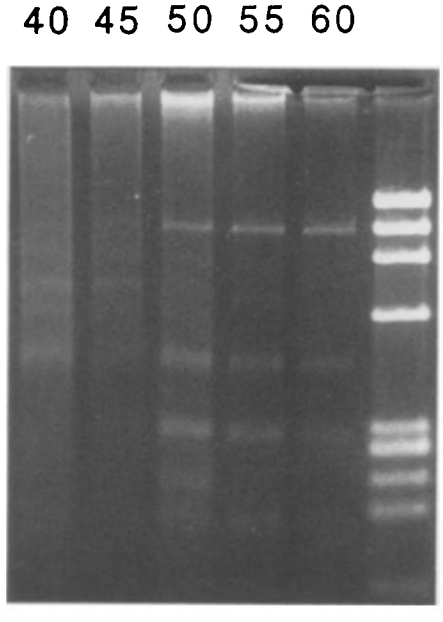

Fig. 2. Polymerase chain reaction products. Reaction products were resolved on a 1.5% NuSieve agarose gel and stained with ethidium bromide. The effect of varying the annealing temperature is shown. Markers are PhiX *Hae*III digest fragments.

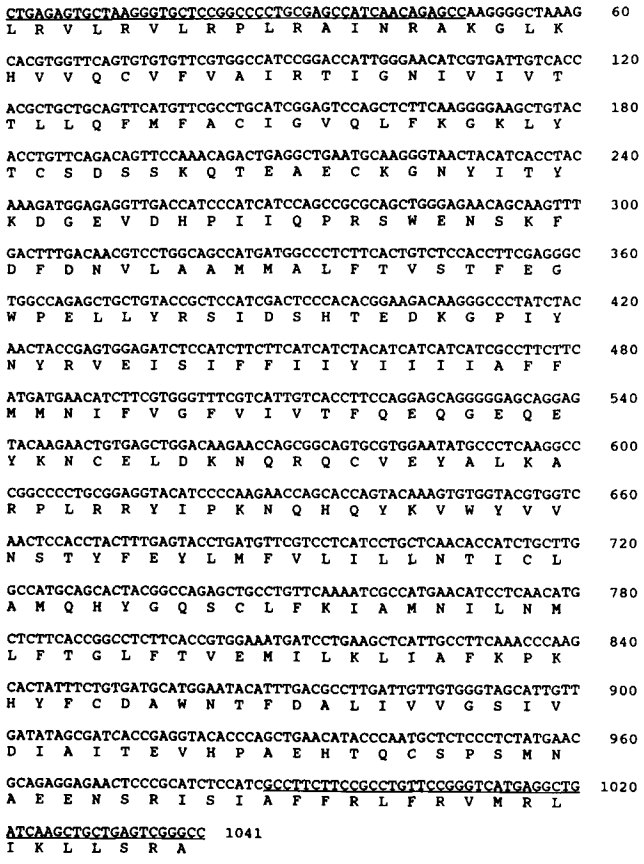

Fig. 3. Nucleotide sequence of pCC-1 and pBC-1, cardiac and brain calcium channel clones. The predicted amino acid sequences are given below the nucleotide sequence in one-letter code. The PCR primers are underlined.

temperatures up to 60°C, a major reaction product of about 1000 bp in size is seen, which corresponds to the distance between the adjacent S4 regions. The PCR products were digested with *Hin*dIII and cloned into pUC 19.

3.2. *Nucleotide sequence*

The nucleotide sequence of one cardiac tissue clone (pCC-1), two independently isolated brain tissue clones (pBC-1 and pBC-2), and one skeletal muscle clone (pSC-1) were determined. The sequences of the two brain clones and the heart clone were identical, and differed from the skeletal muscle clone which was obtained using the same PCR primers.

Fig. 3 shows the nucleotide sequence of pCC-1 and pBC-1 with the predicted amino acid sequence. Fig. 4 compares the nucleotide sequences of pCC-1 with pSC-1. The cardiac and brain sequence reported here share 78% homology with the skeletal sequence over this region. Alignment of the cardiac/brain sequence to the skeletal muscle sequence requires several gaps at the 3′ end of this region. The sequence of pSC-1 agrees with that reported by Tanabe et al. [5] for the skeletal muscle calcium channel.

3.3. *Northern blot*

RNA from the heart, brain, a skeletal muscle, and liver were subjected to blot hybridization analysis using the pCC-1 insert as probe, as shown in Fig. 6. The heart and brain contain two major hybridizing RNA species, of size 7.5 and 8.7 kb whereas skeletal muscle contains a single hybridizing species of 6.0 kb.

4. DISCUSSION

4.1. Cloning approach

The structure of several ion channels has been determined at the amino acid sequence level by cloning of the cDNAs which encode these channels. The major subunits of the sodium channels of the electric eel [1] and rat brain [2], the potassium channels of *Drosophila* [3] and mouse brain [4], and the calcium channel of rabbit skeletal muscle [5] share significant sequence homology, suggesting that these and other voltage-sensitive ion channels arose by divergence from a common ancestral sequence.

Of the 3 kinds of calcium channels described [14], L-type channels are responsible for the slow inward current of calcium. Tanabe et al. [15] showed that an expression plasmid containing the α1 cDNA restored both the excitation-contraction coupling and slow calcium current defect in mice with muscular dysgenesis, suggesting a dual role for this protein.

Reconstitution experiments in planar lipid bilayers have shown that cardiac calcium channels have significantly different functional properties from skeletal calcium channels [7]. Furthermore, antibodies raised against the α1 subunit are tissue-specific, whereas those against α2 subunit show wide crossreactivity [8].

Despite the functional differences between L-type channels in different tissues, the α1 subunits of the calcium channels from the heart, brain, and skeletal muscle were likely to share significant homology in the highly conserved S4 regions which are involved in voltage-sensing. We synthesized primers spanning 16 and 17 amino acid codons of these regions of the rabbit skeletal muscle calcium channel. These primers were used in PCR reactions with single stranded cDNA from cardiac, brain, and skeletal muscle as the template. Several independent clones were obtained from these tissues, representing the specific region between the third and fourth S4 region of the α1 subunit.

4.2. *Polymerase chain reaction cloning*

The polymerase chain reaction is a powerful method for the enrichment of specific target sequences [16]. It has been used for the cross-species isolation of homologous genes [17,18], and for isolation of members of gene families [19]. Here, we have used it to clone a previously uncharacterized gene for an ion

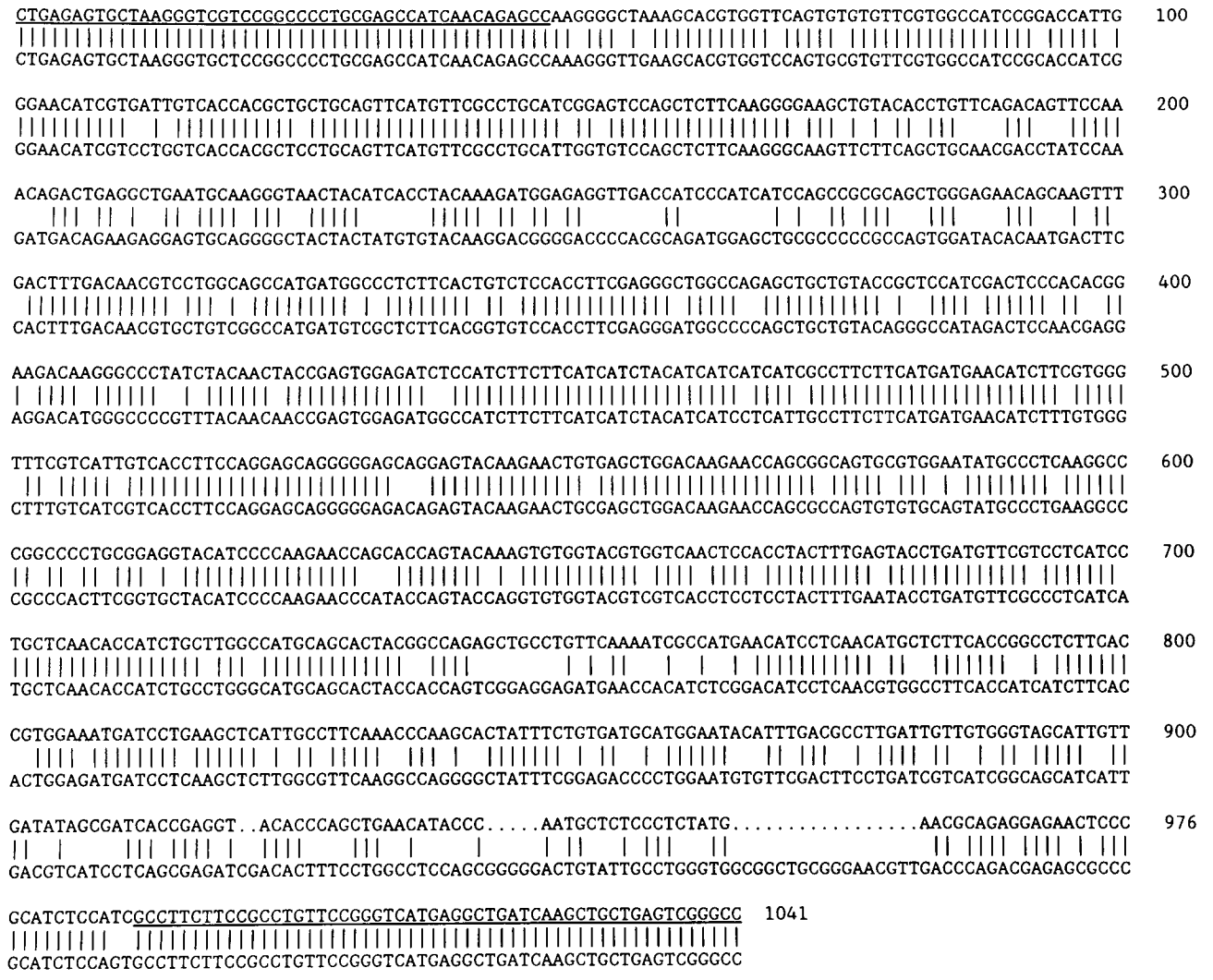

Fig. 4. Nucleotide sequences of pCC-1 and pSC-1, cardiac and skeletal muscle calcium channel clones. The top line is pCC-1, and the lower line is pSC-1. Identical nucleotides are indicated by vertical bars.

channel, making use of the high degree of conservation in the S4 regions.

The third and fourth S4 segment were chosen for several reasons. First, as the first strand of cDNA was synthesized using oligo-dT as primer, it was likely that sequences closer to the 3′ end of the cDNA would be represented. Secondly, examination of the sequences in Fig. 1 shows that there is a proline residue which is conserved between the two sodium channels and the skeletal calcium channel only in the third S4 region, suggesting an important structural role for this amino acid. Third, Lotan et al. [13] have shown that an oligonucleotide from the IVS4 region inhibits the expression of calcium channels in *Xenopus* oocytes microinjected with cardiac RNA, suggesting that there is sufficient sequence homology in this region for the complementary oligonucleotide to hybridize to cardiac calcium channel mRNA and prevent its translation.

This technique will be generally applicable to other tissues and other ion channels. Preparation of RNA and synthesis of cDNA to provide template for the PCR reaction is achieved from very little tissue, making this method preferable to the generation of representative cDNA libraries, followed by screening for the clone of interest.

4.3. *Nucleotide sequence*

The nucleotide sequences of one cardiac and two brain clones we obtained are identical, demonstrating that the heart and brain contain channels which are identical in sequence in this specific region between the third and fourth S4 region. Whether these channels are identical outside this region is not known. Of interest, photoaffinity-labelling experiments suggest that the $\alpha 1$ subunits in hippocampal and cardiac membranes are the same size, 195 kDa, and are larger than the subunit labelled from skeletal muscle membranes [20].

Mikami et al. [10] reported the amino acid sequence of a rabbit cardiac calcium channel. The amino acid sequence of our cardiac and brain clones differ from

A

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LRVLRVLRPLRAINRAKGLKHVVQCVFVAIRTIGNIVIVTTLLQFMFACIGVQLFKGKLY   60
LRVLRVLRPLRAINRAKGLKHVVQCVFVAIRTIGNIVIVTTLLQFMFACIGVQLFKGKLY

TCSDSSKQTEAECKGNYITYKDGEVDHPIIQPRSWENSKFDFDNVLAAMMALFTVSTFEG  120
TCSDSSKQTEAECKGNYITYKDGEVDHPIIQPRSWENSKFDFDNVLAAMMALFTVSTFEG

WPELLYRSIDSHTEDKGPIYNYRVEISIFFIIYIIIIAFFMMNIFVGFVIVTFQEQGEQE  180
WPELLYRSIDSHTEDKGPIYNYRVEISIFFIIYIIIIAFFMMNIFVGFVIVTFQEQGEQE

YKNCELDKNQRQCVEYALKARPLRRYIPKNQHQYKVWYVVNSTYFEYLMFVLILLNTICL  240
YKNCELDKNQRQCVEYALKARPLRRYIPKNQHQYKVWYVVNSTYFEYLMFVLILLNTICL

AMQHYGQSCLFKIAMNILNMLFTGLFTVEMILKLIAFKPKHYFCDAWNTFDALIVVGSIV  300
AMQHYGQSCLFKIAMNILNMLFTGLFTVEMILKLIAFKPKGYFSDPWNVFDFLIVIGSII

DIAITEVHPAEHTQCSPSMNAEENSRISIAFFRLFRVMRLIKLLSRA  347
DVILSETNPAEHTQCSPSMNAEENSRISITFFRLFRVMRLVKLLSRG
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B

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Skeletal muscle     GYFGDPWNVFDFLIVIGSIIDVILSEIDTFLASSGGLYCL
Heart (Mikami)      GYFSDPWNVFDFLIVIGSIIDVILSETNPAEHTQCSPSMN

Heart (Mikami)      GYFSDPWNVFDFLIVIGSIIDVILSETNPAEHTQCSPSMNAEENSRISI
Heart/brain (pCC-1) HYFCDAWNTFDALIVVGSIVDIAITEVHPAEHTQCSPSMNAEENSRISI

Heart/brain (pCC-1) HYFCDAWNTFDALIVVGSIVDIAITEVHPAEHTQCSPSMN
Rat brain (Koch)    HYFTDAWNTFDALIVVGSVVDIAITEVNPSDSENIPLPTA
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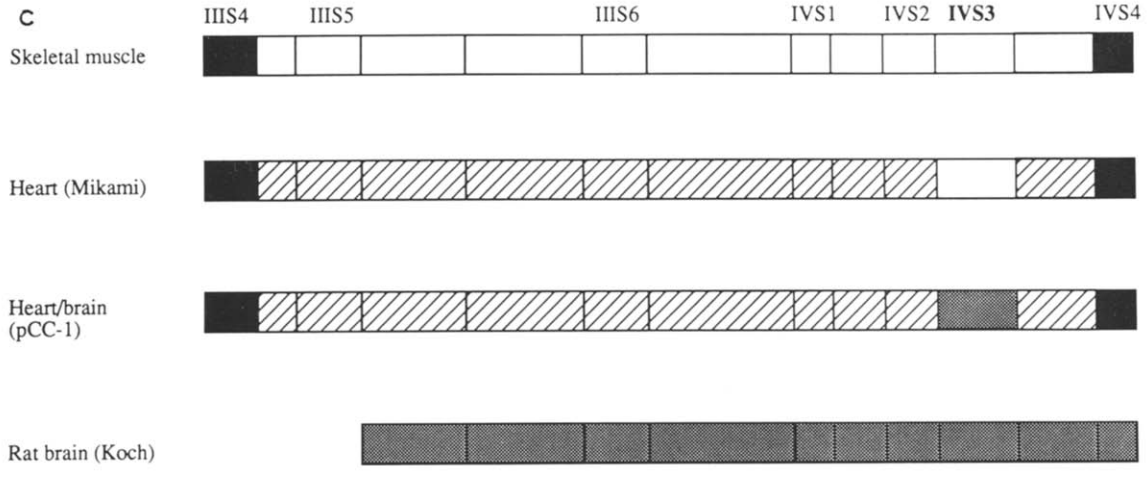

Fig. 5. Comparison of predicted amino acid sequences of part of pCC-1 with other reported amino acid sequences of calcium channels. (A) Amino acid sequence of pCC-1 is shown in the top line, with the PCR primers underlined. Cardiac calcium channel sequence by Mikami is shown in the lower line. Identical amino acids are indicated by vertical bars. The IVS3 segment where the sequences differ is shown in bold type. (B) The sequences of the calcium channels of rabbit skeletal muscle, heart (Mikami), heart/brain (pCC-1), and rat brain (Koch) are shown. The IVS3 segment is shown in bold type. (C) Schematic diagram of the homologies between pCC-1 and other calcium channels. The amino acid sequence of pCC-1 is identical to that of Mikami's cardiac clone except in the IVS3 region. This region in Mimaki's clone is nearly identical to the skeletal muscle channel. This region in pCC-1 is very homologous to the rat brain sequence.

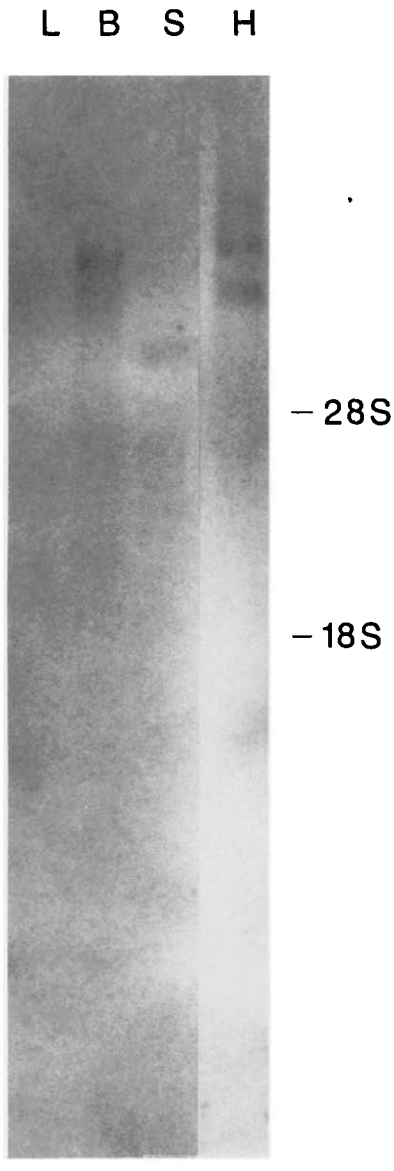

Fig. 6. RNA blot hybridization. RNAs from liver (L), brain (B), skeletal muscle (S), and heart (H) were resolved by electrophoresis on agarose gels containing formaldehyde, and transferred to nitrocellulose. The blot was probed with ^{32}P-labelled pCC-1 clone insert, washed in 0.2× SSC at 55°C, and exposed to film at −70°C with an intensifying screen.

Mikami's reported amino acid sequence only near the C-terminus of this region, as shown in bold type in Fig. 5A. Following the numbering convention of the sodium channels and the existing calcium channels, this corresponds to the IVS3 segment. Interestingly, the IVS3 segment of Mikami's cardiac clone is nearly identical to the IVS3 region of the rabbit skeletal muscle channel (Fig. 5B).

Koch et al. have reported partial amino acid sequence of L-type calcium channels from rat brain and aorta [11]. They obtained a clone which encodes 250 amino acids from the aorta, and one which encodes 608 amino acids from the brain. Comparison of their rat brain sequence with our pCC-1 clone reveals that while the two sequences share 72% amino acid homology overall, the homology is 90% in the IVS3 segment, the very region where our clones differ from Mikami's cardiac clone. This sequence homology exists despite species differences between rabbit and rat. Fig. 5B shows the homologies between these sequences. Fig. 5C shows a schematic diagram of these homologies.

Our clone and Mikami's cardiac clone resume their homology immediately following the IVS3 segment, through the end of the IVS4 segment. The identity of the two sequences over most of the 1 kb region examined, coupled with the differences in the IVS3 segment, raises the question of how genetic diversity of channels in different tissues is achieved. Possibilities include alternative exon use to generate different forms of L-type calcium channels, or the existence of two or more genes whose sequence is identical in certain regions and divergent in other regions. The striking similarity of our pCC-1 clone to the rat brain channel only in the IVS3 segment, where our sequence differs from Mikami's cardiac clone, suggests that there may be a functional reason for conservation of these sequences. Changes in this membrane-spanning region could lead to differences in the functional properties of these channels. Together, these sequences suggest that there may be additional mechanisms, such as alternative exon use, to combine different functional domains to generate diversity of calcium channels.

4.4. *RNA blot hybridization*

RNA blot hybridization demonstrates the existence of two RNA species in the brain and the heart which hybridize to the pCC-1 clone. The sizes of these RNAs are estimated to be 7.5 and 8.7 kb using ribosomal RNAs as markers. Skeletal muscle contains a single hybridizing RNA which is 6.0 kb in size. These data are consistent with photoaffinity studies which suggest a larger size for the α1 subunit in the brain and heart than in skeletal muscle. The nature of the differences between the two RNA species seen in heart and brain are not yet known. They may represent use of alternative polyadenylation sites or use of different exons from a single gene. Alternatively, these transcripts may arise from separate genes.

4.5. *Conclusion*

There appear to be several forms of the α1 subunit of L-type calcium channels. The skeletal muscle channel and the cardiac channel share significant homology, but appear to be the product of separate genes. We have shown here that there are sequences common to calcium channels in the heart and the brain. Furthermore, comparison of our clones to those of others reveals unexpected patterns of homology, which suggests that there may be interesting mechanisms of combining different domains to generate diversity of calcium channels. The

availability of these clones will hopefully facilitate the detailed study of the determinants of the functional properties of L-type calcium channels from different tissues.

Acknowledgements: We thank Philip L. Huang for key discussions and critical reading of the manuscript. Paul L. Huang is a Merck Fellow of the American College of Cardiology. This work is supported in part by National Institutes of Health, Grant T32HL07208-14.

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