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Molecular cloning and characterization of a novel calcium channel from rabbit brain

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The complete amino acid sequence of a novel calcium channel (designated BII) from rabbit brain has been deduced by cloning and sequencing the cDNA. The BII calcium channel is structurally more closely related to the BI calcium channel than to the cardiac and skeletal muscle L-type calcium channels. Blot hybridization analysis of RNA from different tissues and from different regions of the brain shows that the BII calcium channel is distributed predominantly in the brain, being abundant in the cerebral cortex, hippocampus and corpus striatum.

Calcium channel; cDNA cloning; Nucleotide sequence; RNA blot hybridization; Rabbit brain

1. INTRODUCTION

Voltage-dependent calcium channels are essential for the regulation of a variety of cellular functions, including membrane excitability, muscle contraction, synaptic transmission and other forms of secretion. At least four types of calcium channel (designated T-, L-, N- and P-type calcium channels) have been distinguished by their electrophysiological and pharmacological properties [1–3]. Recently, attempts have been made to understand the molecular basis of the functional heterogeneity of the calcium channel. cDNA cloning studies have revealed the existence of multiple calcium channel gene products. Expression of the cDNAs for the dihydropyridine (DHP) receptor from skeletal muscle, heart, smooth muscle and brain yields DHP-sensitive L-type calcium channels [4–10]. On the other hand, the brain calcium channel BI exhibits calcium channel activity that is insensitive both to nifedipine and to ω-conotoxin (ω-CgTx), resembling P-type calcium channels in neurons [11].

Here we report the complete nucleotide sequence and deduced amino-acid sequence of a novel calcium channel from rabbit brain (designated BII). The tissue distribution of BII calcium channel mRNA has also been studied by blot hybridization analysis.

Abbreviations: DHP, dihydropyridine; ω-CgTx, ω-conotoxin.

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2. MATERIALS AND METHODS

An oligo(dT)-primed, size-selected (>~2 kilobase pair (kb)) cDNA library was constructed [4] in phage λgt10 using poly(A)+ RNA prepared [7] from adult rabbit brain. It was screened with a BI calcium channel cDNA probe, that is, the *Eco*RI(3,727)/*Eco*RI(5,050) fragment from clone λCB3 [11]; restriction endonuclease sites are identified by numbers (in parentheses) indicating the 5'-terminal nucleotide generated by cleavage. Thus clone λCBA240 (carrying nucleotides 3,689–6,293×) was isolated; numbers in parentheses indicate the nucleotide residues of the cDNA carried by the clone. Restriction fragments from λCBA240 were used as probes to clone adjacent cDNAs and similar cloning procedures were repeated. The synthetic primer complementary to nucleotide residues 4,242–4,258 was elongated and cloned into λgt10 by the procedures described previously [4] (size selection >~1 kb). λCBP201 (132–4,253×) was isolated from the synthetic primer-derived cDNAs. λCB204 (−598 to 1,648) and λCB215 (−598 to 1,083) was isolated from randomly primed, size-selected (>~1 kb) cDNA library, and λCB236 (6,186–8,010), λCB244 (6,023–6,864) and λCB264 (5,446–8,010 with a deletion of 6,300–7,115) from another randomly primed cDNA library. Appropriate restriction fragments from the isolated clones were subcloned into pBluescript KS(−), M13mp18 or M13mp19. Nested deletions were made [4] and DNA sequencing was carried out on both strands by the dideoxy chain-termination method [12]. RNA blot hybridization analysis was performed as in [13].

3. RESULTS AND DISCUSSION

Fig. 1 shows the primary structure of the rabbit BII calcium channel deduced from the nucleotide sequence of the cloned cDNA (for cloning procedures see section 2); the open reading frame corresponding to the amino acid sequences of the skeletal muscle, cardiac and BI calcium channels [4,11,14] was adopted and the translation initiation site was assigned to the first ATG triplet

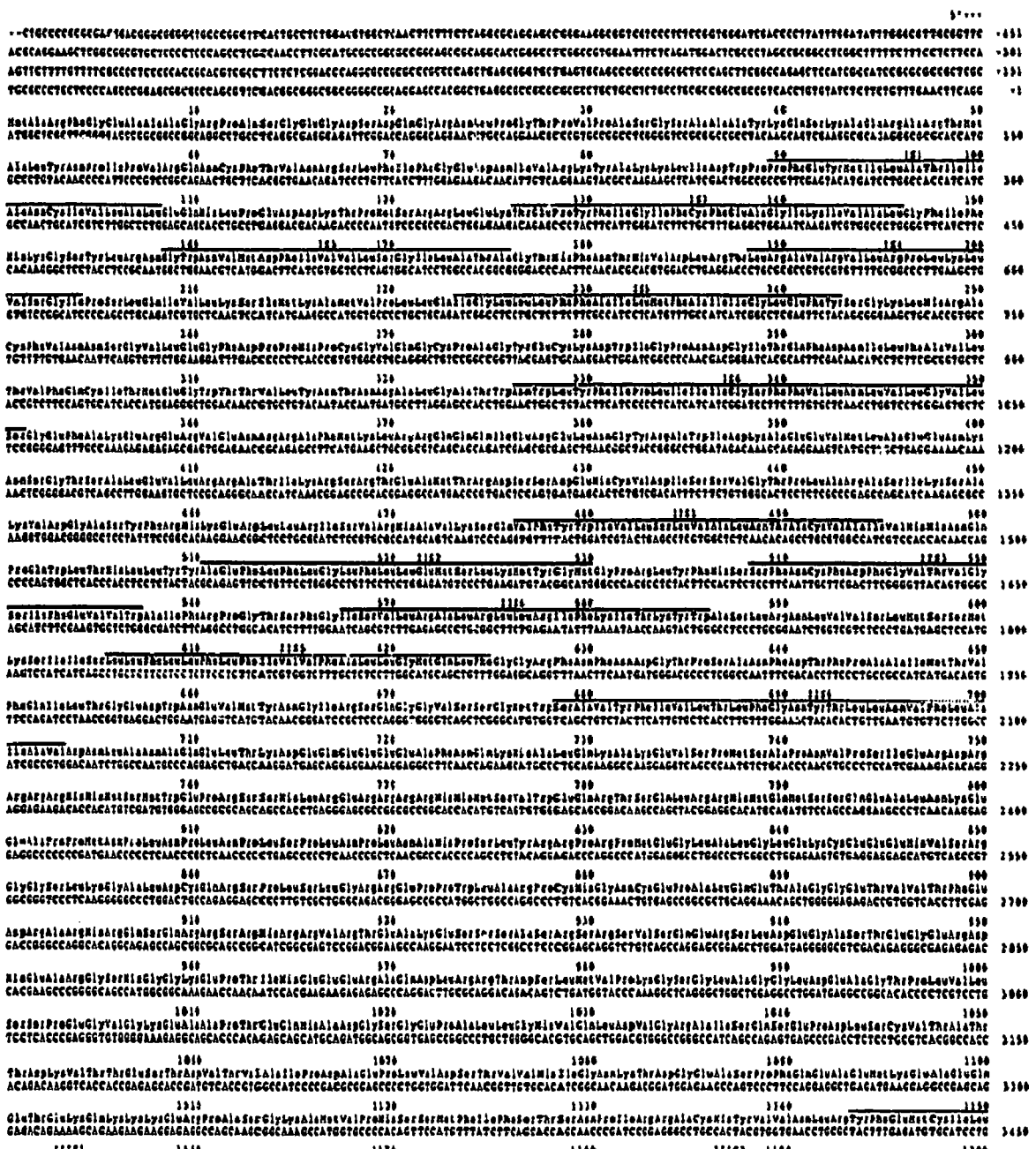
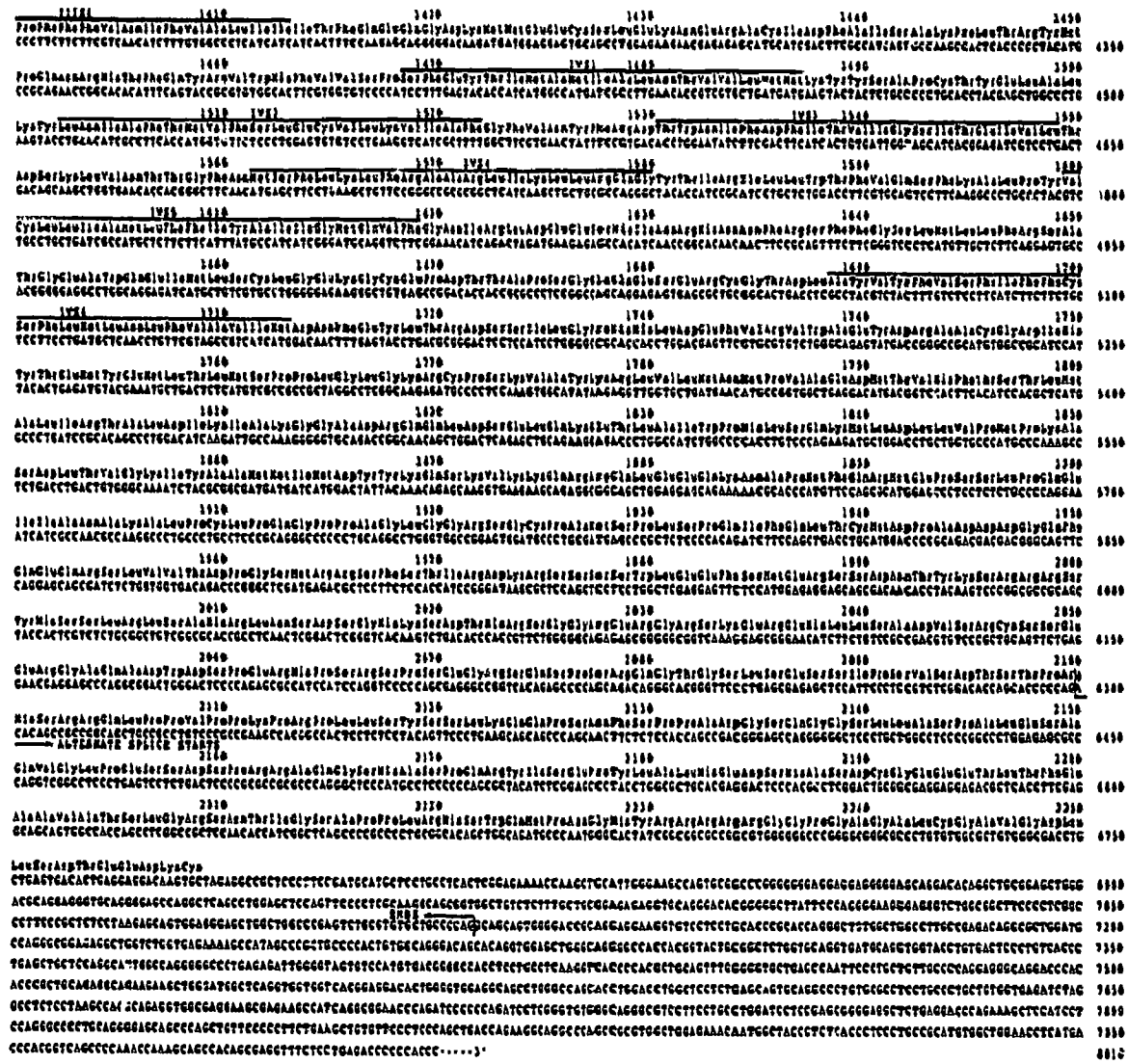
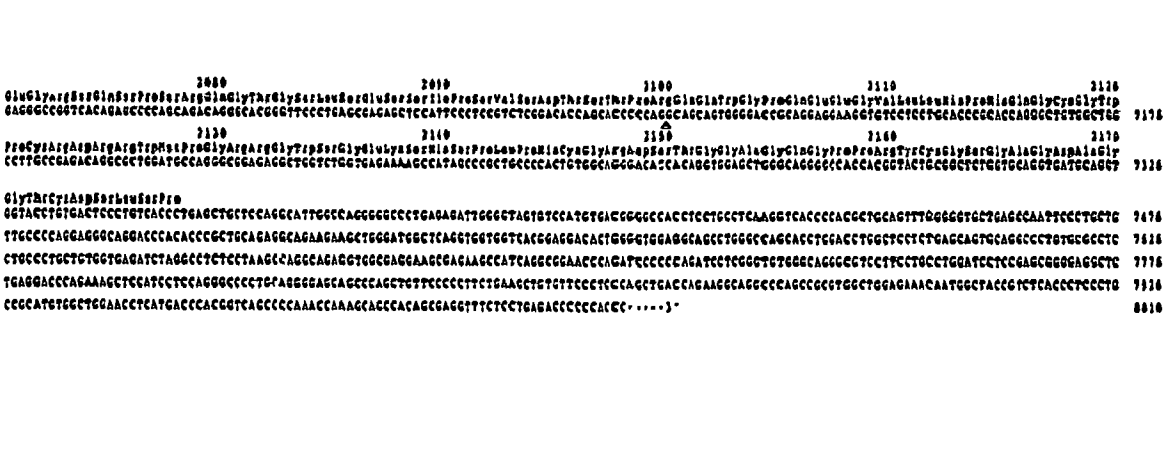


Fig. 1. Nucleotide sequence of the cDNA encoding the BII calcium channel and the deduced amino acid sequence. (A) The nucleotide sequence of BII-1 was determined using clone λCB204 (carrying nucleotides −598 to 1,648), λCBP201 (132–4,253), λCBA40 (3,689–6,293) and λCB236 (6,186–8,010). Nucleotide residues are numbered in the 5′ to 3′ direction from the first residue of the ATG initiation triplet and the preceding residues are indicated by negative numbers. The 3′-terminal nucleotide residue, 8,010, is not followed by a poly(dA) tract. Numbers of the nucleotide residues at the right-hand end of the individual lines are given. Amino acid residues are numbered from the initiating methionine. The putative transmembrane segments S1–S6 in each of the repeats I–IV are overlined; the termini of each segment have been tentatively assigned. The tentative boundaries of the inserted sequence of 816 nucleotide residues are shown. (B) The 3′-terminal region of the cDNA encoding BII-2. The nucleotide sequence of BII-2 was determined using clones λCB204, λCBP201, λCBA40 and λCB264 (5,446–8,010 with a deletion of 6,300–7,115). The arrowhead indicates the position where the 816 nucleotide sequence is inserted in BII-1.





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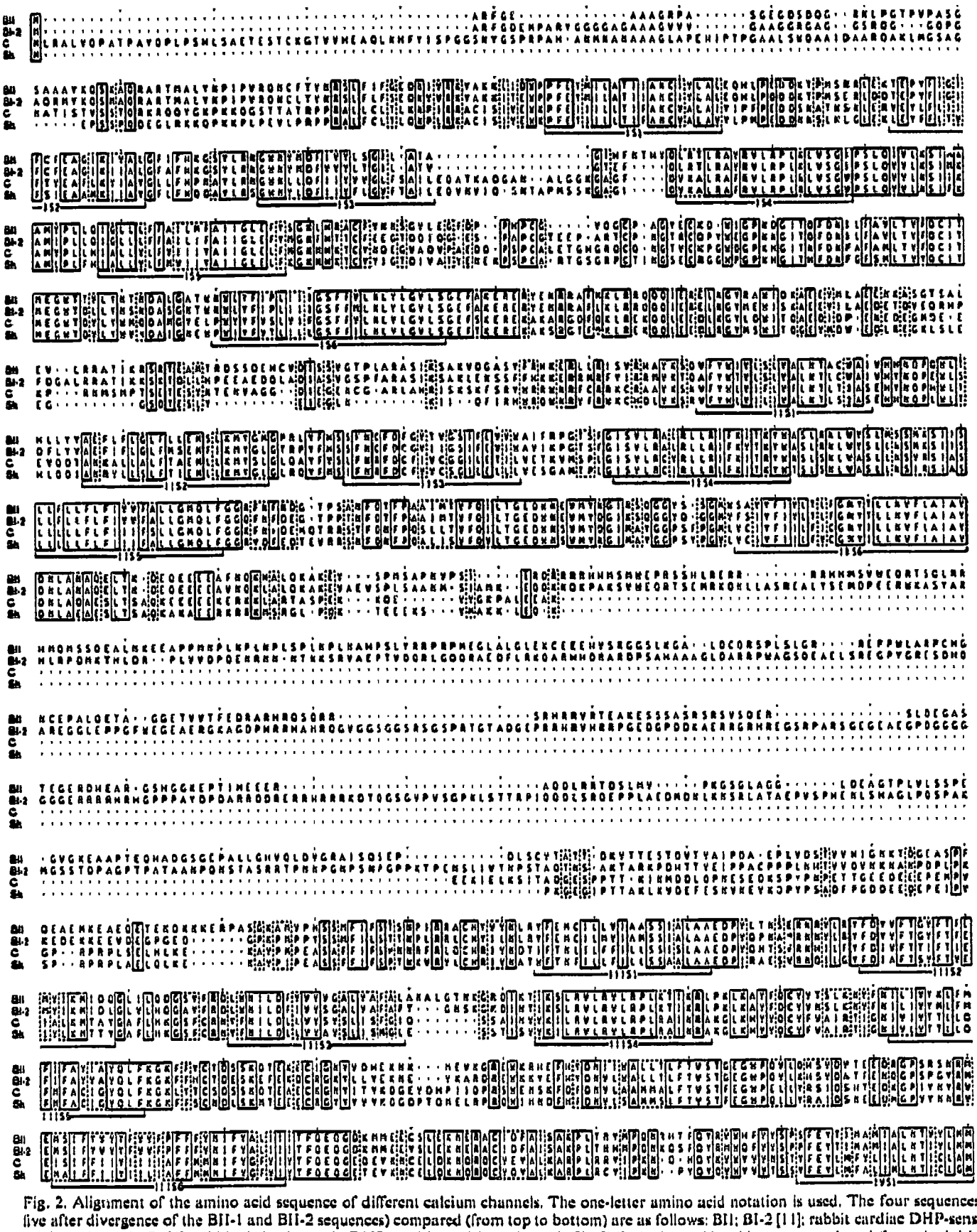

Fig. 2. Alignment of the amino acid sequence of different calcium channels. The one-letter amino acid notation is used. The four sequences (or five after divergence of the BII-1 and BII-2 sequences) compared (from top to bottom) are as follows: BII; BI-2 [11]; rabbit cardiac DHP-sensitive calcium channel (C) [4]; rabbit skeletal muscle DHP-sensitive calcium channel (Sk) [14]. Amino acid residues are numbered from the initiating methionine, and numbers of the amino acid residues on each line are given on the right-hand side. The arrowhead indicates where the BII-1 and BII-2 proteins diverge in sequence; residues 2,040–2,110 of BII-1, which immediately precede the position indicated by the arrowhead, are not displayed. Sets of four (or five) identical residues at one position are enclosed with solid lines, and sets of four (or five) identical or conservative residues [20] at one position with broken lines. The putative transmembrane segments, S1–S6, in each of the repeats, I–IV, are shown (see legend to Fig. 1).

Fig. 2 continued

that appears downstream of a nonsense codon, TGA (nucleotide residues −78 to −76; for residue numbers see Fig. 1) in-frame. An inserted sequence of 816 nucleotide residues which contains in-frame translational termination codon is found in two clones (λCB236 and λCB244), whereas it is missing in another clone (λCB264). This insertion–deletion, which probably results from alternative RNA splicing, gives rise to distinct mRNAs encoding two isoforms of the BII calcium channel (BII-1 and BII-2). These isoforms differ from each other in the carboxy-terminal sequence beginning with amino acid residue 2,101. BII-1 and BII-2 are composed of 2,259 and 2,178 amino acid residues, respectively, with relative molecular masses of 254,249 and 245,595.

The BII calcium channel shares general structural features with voltage-dependent calcium channels [4–6,11,14–19] and sodium channels [20], thus apparently having the same transmembrane topology as proposed for voltage-gated sodium channels [20,21]. The BII calcium channel contains four repated units of homology (amino acid residues 76–354, 464–706, 1,130–1,414 and 1,453–1,716). Each repeat has five hydrophobic agents (S1, S2, S3, S5 and S6) and one positively charged segment (S4) [4–6,11,14–19], which probably represents a voltage-sensing region [22]. The glutamic acid residues in the SS1–SS2 regions [21], which may be critical for ion selectivity of calcium channels [23], and the conserved charged residues in segments S2 and S4 [4–6,11,14–19] are also retained. The BII calcium channel has three potential *N*-glycosylation sites [24] assigned to the extracellular side (Asn residues 254, 1,556 and 1,561), one of which (Asn residue 1,561) is conserved in the BI calcium channel [11]. All the potential cycle AMP-dependent phosphorylation sites [25] found in the BII calcium channel (residues 414, 420, 919, 978, 1,773, 1,969, 1,977, 1,978 and 2,000 in BII-1 and, in addition, residue 2,135 in BII-2) are assigned to the cytoplasmic side.

Fig. 2 shows an alignment of the amino acid sequence of the BII channel isoforms with those of the BI-2, cardiac and skeletal muscle calcium channels. The amino acid sequence of the BII calcium channel is more closely related to that of the BI-2 (59% amino acid

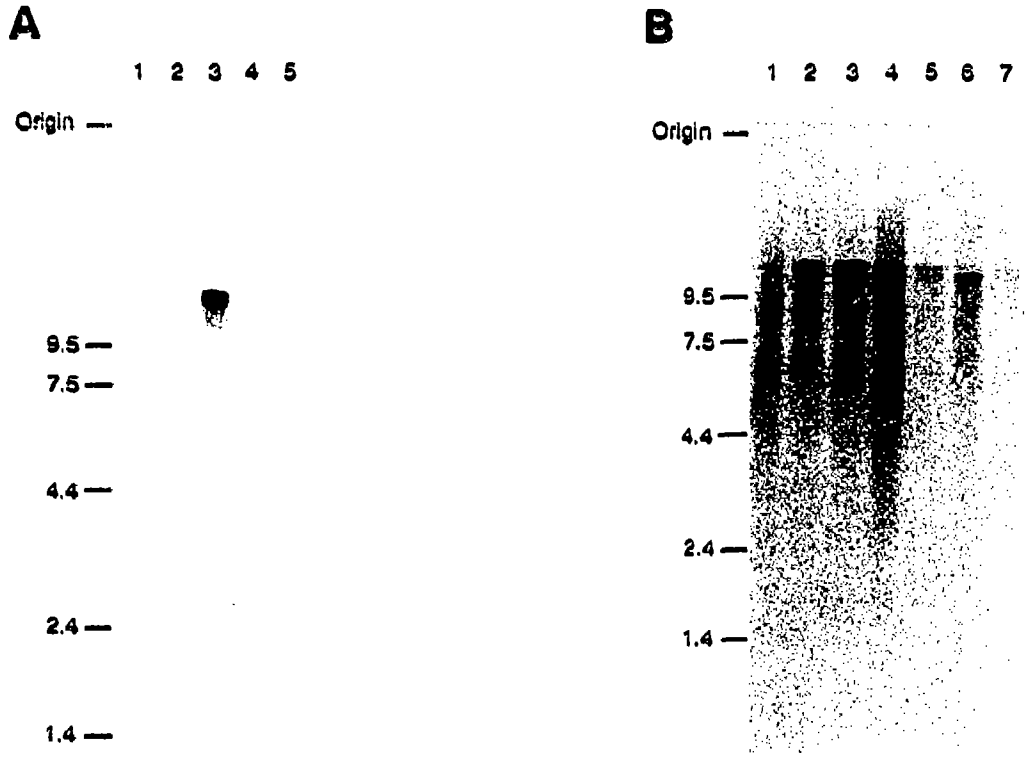

Fig. 3. Autoradiograms of blot hybridization analysis with a BII calcium channel cDNA probe of RNA from different rabbit tissues (A) and different regions of rabbit brain (B). (A) Analysis of poly(A)$^+$ RNA (10 μg each) prepared [7] from skeletal muscle (lane 1), heart (lane 2), brain (lane 3), stomach (lane 4) and kidney (lane 5) from adult rabbits. The probe was the 0.60-kb *Sal*I(1,298)/*Bam*HI(1,988) fragment derived from λCBP201 and labeled by the random primer method [28]. Similar results were obtained in the experiment in which the 2.6-kb *Eco*RI(3,689)/*Eco*RI(6,293) fragment from λCBA240 was used as a probe (data not shown). (B) Analysis of total RNA (15 μg each) prepared [7] from olfactory bulb (lane 1), cerebral cortex (lane 2), hippocampus (lane 3), corpus striatum (lane 4), midbrain (lane 5), cerebellum (lane 6) and medulla-pons (lane 7) from adult rabbits. The probe was the 2.6-kb *Eco*RI(3,689)/*Eco*RI(6,293) fragment from λCBA240 and labelled by the nick translation method [29]. The procedures used were as described previously [13]. Autoradiography was at −70°C for 3 days (A) or 7 days (B) with an intensifying screen. The equal loading of total RNA was confirmed by the uniform levels of a ~2,000 nucleotide mRNA in each lane when the filter was re-probed with a β-actin cDNA (data not shown). An RNA ladder (Bethesda Research Laboratories) was used for size markers (in kilobases).

identities between the BII-1/BI-2 and BII-2/BI-2 pairs) than to those of the other calcium channels (38, 40, 41 and 42% amino acid identities between the BII-1/cardiac, BII-2/cardiac, BII-1/skeletal and BII-2/skeletal pairs, respectively). The regions corresponding to the four internal repeats are relatively well conserved, whereas the remaining regions, all of which are assigned to the cytoplasmic side of the membrane, are less well conserved, except for the short segment between repeats III and IV (III–IV loop). The putative cytoplasmic region between repeats II and III (II–III loop) of the BII calcium channel (423 amino acids residues) and the BI calcium channel (522 residues) is more than three-times larger than the II–III loop of the cardiac (130 residues) and skeletal muscle (121 residues) calcium channels. The II–III loop of the skeletal muscle calcium channel is an important determinant of skeletal-type excitation–contraction coupling [26] and may interact directly with the foot region of the ryanodine receptor [27]. It is also possible that the II–III loop of the brain calcium channels may interact directly with intracellular proteins in neurons.

RNA preparations from different rabbit tissues (Fig. 3A) and from different regions of rabbit brain (Fig. 3B) were subjected to blot hybridization analysis with a BII calcium channel cDNA probe. Two major hybridizable RNA species of ~10,500 and ~11,000 nucleotides, which may be attributable to alternative RNA splicing, were found abundantly in the brain (Fig. 3A, lane 3), whereas no hybridizable RNA species were detected in the skeletal muscle, heart, stomach and kidney (Fig. 3A, lanes 1,2,4 and 5). These RNA species differ in size from the rabbit brain RNA species hybridizable with the cardiac calcium channel cDNA [4] (~8,900 and ~15,500 nucleotides in length) and with the BI channel cDNA [11] (~9,400 nucleotides), which demonstrates the existence of multiple calcium channel mRNA species in this tissue. In the brain, the cerebral cortex, hippocampus and corpus striatum show much higher mRNA levels (Fig. 3B, lanes 2,3 and 4) than the olfactory bulb,

midbrain, cerebellum and medulla-pons (Fig. 3B, lanes 1,5,6 and 7). Interestingly, the level of two BII mRNA species (~10,500 and ~11,000 nucleotides) were approximately equal in the hippocampus and corpus striatum (Fig. 3B, lanes 3 and 4). In contrast, only an ~11,000-nucleotide RNA species was found in the cerebral cortex (Fig. 3B, lane 2) and only an ~10,500-nucleotide RNA species was found in the cerebellum (Fig. 3B, lane 6).

In summary, we have isolated and characterized a cDNA encoding a novel brain calcium channel. Our results show that voltage-dependent calcium channels can be classified into at least two main subfamilies, according to the degrees of amino acid sequence homology between calcium channel pairs. One subfamily consists of the L-type calcium channels from skeletal muscle, heart, smooth muscle, pancreas and brain, and the other subfamily consists of the BI calcium channel, which may represent P-type, and the BII calcium channel. The L-type calcium channels are highly homologous in the III–IV loop, which is important for the inactivation [22], as well as in the DHP-binding region adjacent to segment S6 of repeat IV [3], whereas the BI and BII calcium channels show significant sequence divergence in these regions. The spatial distribution of BII calcium channel mRNA in the brain is markedly different from that of BI calcium channel mRNA. BII calcium channel mRNA is abundant in the cerebral cortex, hippocampus and corpus striatum, while BI calcium channel mRNA [11] is expressed predominantly in the cerebellum. Notably, the distribution of BII calcium channel mRNA seems to correlate well with the distribution of ω-CgTx binding in rat brain [30,31]. Thus, it is conceivable that the BII calcium channel represents N-type calcium channels. Characterization of the BII calcium channel in heterologous expression systems will enable functional classification and elucidate functional roles of the BII calcium channel.

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