

## Tissue-specific expression of high-voltage-activated dihydropyridine-sensitive L-type calcium channels

Martin BIEL<sup>1,2</sup>, Roger HULLIN<sup>1,2</sup>, Stefanie FREUNDNER<sup>1</sup>, Dafna SINGER<sup>3</sup>, Nathan DASCAL<sup>3</sup>, Veit FLOCKERZI<sup>1</sup> and Franz HOFMANN<sup>2</sup>

<sup>1</sup> Institut für Medizinische Biochemie, Medizinische Fakultät, Universität des Saarlandes, Homburg, Federal Republic of Germany

<sup>2</sup> Institut für Pharmakologie und Toxikologie, Technische Universität München, Federal Republic of Germany

<sup>3</sup> Sackler School of Medicine, Department of Physiology and Pharmacology, Tel Aviv University, Israel

(Received February 11 / April 26, 1991) – EJB 91 0210

The cloning of the cDNA for the  $\alpha_1$  subunit of L-type calcium channels revealed that at least two genes (CaCh1 and CaCh2) exist which give rise to several splice variants. The expression of mRNA for these  $\alpha_1$  subunits and the skeletal muscle  $\alpha_2/\delta$ ,  $\beta$  and  $\gamma$  subunits was studied in rabbit tissues and BC<sub>3</sub>H1 cells. Nucleic-acid-hybridization studies showed that the mRNA of all subunits are expressed in skeletal muscle, brain, heart and aorta. However, the  $\alpha_1$ -,  $\beta$ - and  $\gamma$ -specific transcripts had different sizes in these tissues. Smooth muscle and heart contain different splice variants of the *CaCh2* gene. The  $\alpha_1$ ,  $\beta$  and  $\gamma$  mRNA are expressed together in differentiated but not in proliferating BC<sub>3</sub>H1 cells. A probe specific for the skeletal muscle  $\alpha_2/\delta$  subunit did not hybridize to poly(A)-rich RNA from BC<sub>3</sub>H1 cells. These results suggest that different splice variants of the genes for the  $\alpha_1$ ,  $\beta$  and  $\gamma$  subunits exist in tissues containing L-type calcium channels, and that their expression is regulated in a coordinate manner.

Voltage-activated calcium channels comprise a group of very similar yet distinct proteins or protein complexes that differ in electrophysiological properties, modulation by phosphorylation and in their relative sensitivity to organic-calcium-channel blockers [1–4]. The principal transmembrane subunit of an L-type calcium channel is the receptor for calcium channel blockers or  $\alpha_1$  subunit, and when purified from rabbit skeletal muscle this protein (165 kDa) is associated with a 55-kDa protein ( $\beta$ ), a 32-kDa protein ( $\gamma$ ) and a disulfide-linked dimer of 130/28 kDa ( $\alpha_2/\delta$ ) [5–8]. The primary structures of the  $\alpha_1$  [9–11],  $\alpha_2/\delta$  [10, 12],  $\beta$  [13] and  $\gamma$  proteins [14, 15] have been deduced by cloning their cDNA and  $\alpha_1$ -like proteins have been identified in a variety of tissues from different species on the basis of sequence homology with their skeletal muscle counterpart [16–23].

However, only the expression of full-length cDNA of the  $\alpha_1$  subunits from rabbit heart [16] and lung [17] in *Xenopus laevis* oocytes results in dihydropyridine-sensitive calcium

channels. The transient expression of the skeletal muscle  $\alpha_1$  subunit in myoblasts from dysgenic mice [24] is complicated by the presence of endogenous Ca<sup>2+</sup> currents. Its expression in murine L-cells [11] leads to the appearance of a dihydropyridine-sensitive Ca<sup>2+</sup> current, which activates about ten times slower than Ca<sup>2+</sup> currents in skeletal muscle, and phenotypic cell lines of skeletal muscle, such as BC<sub>3</sub>H1 cells. The  $\alpha_2/\delta$  and  $\beta$  subunits are missing in murine L-cells [25] and it has been suggested that to acquire skeletal muscle properties,  $\alpha_1$  must be associated with one or more of the other subunits. In this case a common regulatory mechanism controlling the expression of calcium channel  $\alpha_1$  subunits and other  $\alpha_2/\delta$ -,  $\beta$ - and  $\gamma$ -like proteins might exist, and mRNA for all subunits should be present in cells containing functional L-type calcium channels. Therefore, in this investigation, the expression of  $\alpha_1$ ,  $\alpha_2/\delta$ ,  $\beta$  and  $\gamma$  mRNA was studied in parallel by blot hybridization analysis using poly(A)-rich RNA from various rabbit tissues and BC<sub>3</sub>H1 cells, as well as subunit-specific RNA probes. It is shown that splice variants of the genes for these subunits are expressed in each tissue, and that in BC<sub>3</sub>H1 cells only expression of the  $\alpha_1$ ,  $\beta$  and  $\gamma$  mRNA, but not of  $\alpha_2/\delta$  mRNA, is regulated in a coordinate manner.

## MATERIALS AND METHODS

### Materials

<sup>35</sup>S-labeled deoxyadenosine [ $\alpha$ -thio]triphosphate [<sup>35</sup>S]-dATP (1.370 Ci/mmol), [ $\alpha$ -<sup>32</sup>P]dCTP (3.000 Ci/mmol), [ $\alpha$ -<sup>32</sup>P]GTP (800 Ci/mmol), (+)-isopropyl-4-(2,1,3-benzoxadiazol-4-yl)-1,4-dihydro-2,6-dimethyl-5-[<sup>3</sup>H]methoxycarbo-

Correspondence to V. Flockerzi, Institut für Medizinische Biochemie, Medizinische Fakultät, Universität des Saarlandes, W-6650 Homburg, Federal Republic of Germany

Abbreviations. (+)PN200–110, (+)-isopropyl-4-(2,1,3-benzoxadiazol-4-yl)-1,4-dihydro-2,6-dimethyl-5-methoxycarbonylpyridine-3-carboxylate; PCR, polymerase chain reaction; CaCh1, rabbit skeletal muscle calcium channel  $\alpha_1$  subunit; CaCh2A, rabbit heart calcium channel  $\alpha_1$  subunit; CaCh2B, rabbit lung calcium channel  $\alpha_1$  subunit.

Enzymes. T4 DNA Ligase (EC 6.5.1.1); reverse transcriptase (EC 2.7.7.49); DNA polymerases, Klenow enzyme, sequenase (EC 2.7.7.7); SP6 RNA polymerase (EC 2.7.7.6), alkaline phosphatase (EC 3.1.3.1); type II restriction endonucleases (EC 3.1.21.4).

Table 1. Recombinant antisense constructs

Source	Plasmid	Subunit	Antisense probe positions	Reference
Skeletal muscle	pCaA1a	$\alpha_1$	1006–5314	9
	pCA2a	$\alpha_2/\delta$	2550–3528	16
	pCaB1a	$\beta$	–22–1608	13
	pCaG1a	$\gamma$	–48–680	14
Lung	pCaCa	$\alpha_1$	2199–4335	17
	pATL	$\alpha_1$	–118–32	17
Heart	pATH	$\alpha_1$	–191–134	16

nylpyridine-3-carboxylate (+)[<sup>3</sup>H]PN200–110, 85.9 Ci/mmol] were purchased from DuPont-New England Nuclear. CsCl was from Gibco BRL Life Technologies. All enzymes were from Boehringer-Mannheim except Sequenase II™ and Taq polymerase which were from United States Biochemical GmbH and Perkin-Elmer GmbH, respectively. Guanidine isothiocyanate was from Fluka Chemie AG, salmon sperm DNA, polyvinylpyrrolidone and Ficoll were from Sigma Chemicals. Seakem GTG-agarose was from FMC Bioproducts. Bacto-agar, yeast extract and bacto-tryptone were from Difco. Glass-fiber filters GF/C were obtained from Whatman. All other chemicals were of the highest quality available.

#### Methods

**RNA-blot-hybridization analysis.** For the preparation of antisense RNA probes, seven recombinant plasmids were constructed carrying the SP6 promoter [26] and the nucleotide sequences of calcium channel subunits from skeletal muscle, lung and heart (Table 1). The skeletal muscle and lung sequences were obtained by subcloning the respective cDNA [9, 13, 14, 16, 17]. The full-length cardiac  $\alpha_1$  cDNA was cloned using the nucleotide sequence of Mikami et al. [16]. Briefly, the *Xho*I–*Sph*I fragment (the *Xho*I site is located in the oligodeoxynucleotide linker 5'-CCGCTCGAGGTCGAC-3' attached to position –191; the fragment runs to position 1488) and the *Bsp*HI–*Sac*II (positions 3553–4116) fragment of the cardiac  $\alpha_1$  cDNA [16] obtained by polymerase chain reaction (PCR) amplification of a random-primed cDNA library from rabbit heart and the *Sph*I–*Bsp*HI (positions 1473–3542) fragment from pSCaL [17] were ligated with the 5.3-kb *Xho*I–*Sac*II fragment from pSCaL [17] to yield the pSP72 recombinant plasmid pCaH. As in [16], the cloned cardiac cDNA induces the formation of a high-voltage-activated dihydropyridine-sensitive L-type calcium channel when expressed in *Xenopus* oocytes. The antisense RNA probes were synthesized *in vitro* with [ $\alpha$ -<sup>32</sup>P]GTP using the linearized plasmids as templates for transcription with SP6 polymerase. RNA-blotting analysis was carried out essentially according to [27] except that Biodyne nylon membranes (Pall) were used and that hybridization was performed at 65°C in the presence of 50% formamide and 0.5% SDS. For blot-hybridization analysis, total RNA was prepared from tissues of adult rabbits or from BC<sub>3</sub>H1 cells by the guanidinium thiocyanate method and poly(A)-rich RNA, as in [13, 14]. The yield of poly(A)-rich RNA during oligo(dT)-cellulose chromatography was 5–7% for all preparations.

#### Cell culture

BC<sub>3</sub>H1 (ATCC 1443) cultures were grown in Dulbecco's modified Eagle's medium containing 20% fetal bovine serum,

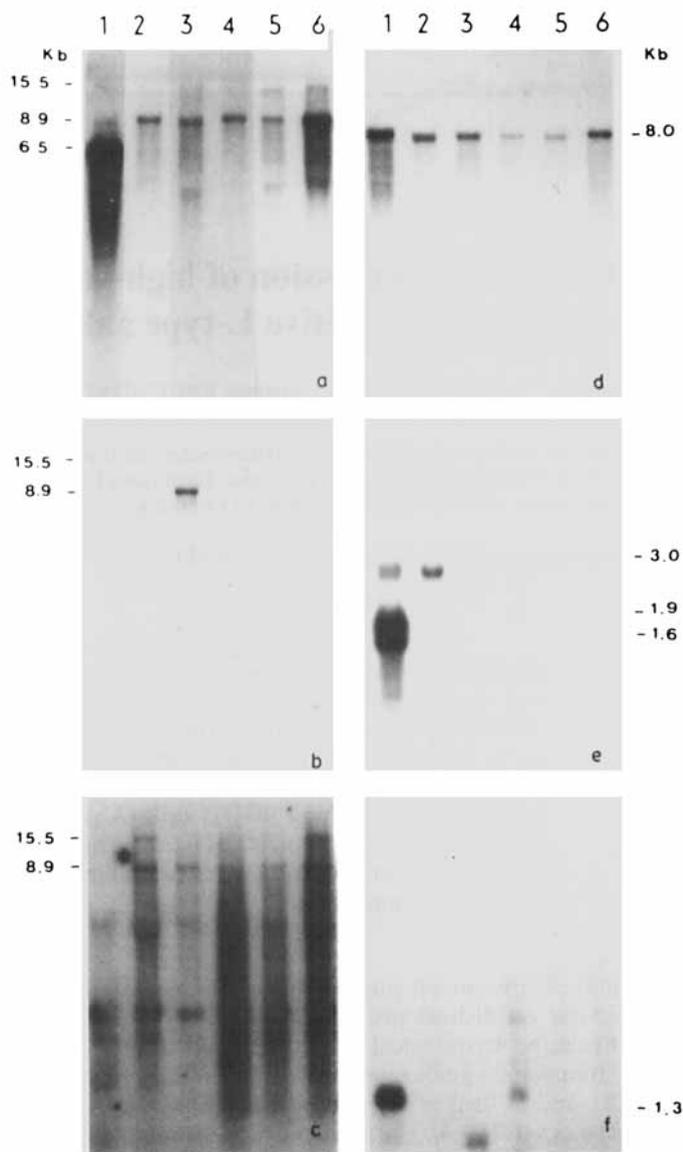


Fig. 1. Autoradiograms of blot hybridization analysis of poly(A)-rich RNA from adult rabbit tissues. Samples of 10  $\mu$ g poly(A)-rich RNA from skeletal muscle (lane 1), brain (lane 2), heart (lane 3), aorta (lane 4), trachea (lane 5) and lung (lane 6) were electrophoresed on 1.2% agarose gels. Autoradiographic exposure was 16 h (a, lanes 2, 5 and 6; d, lanes 1–3; b and f), 3 days (a, lanes 1, 3 and 4; d, lanes 4–6; e) and 20 days (c). The size markers used were *Escherichia coli* rRNA and the RNA ladder purchased from Gibco BRL Life Technologies. The RNA probes used were specific for the  $\alpha_1$  subunits from heart and lung (a), heart (b), lung (c) and for the skeletal muscle  $\alpha_2/\delta$  (d),  $\beta$  (e) and  $\gamma$  (f) subunits. The specific activities of the six probes used were 0.8–2.2  $\times 10^9$  dpm/ $\mu$ g

penicillin (100 U/ml) and streptomycin (100  $\mu$ g/ml), as described in [28]. The medium was changed every 2 days. To minimize differentiation, BC<sub>3</sub>H1 cells were passaged when no more than 60% confluent. P1-, P2-, and P3-designate proliferating BC<sub>3</sub>H1 cells were used which were 10–20%, 30–50% and 60–80% confluent, respectively. Differentiation was induced by reduction of the fetal bovine serum concentration to 0.5%. For binding assays and RNA preparation, cells were washed twice with Hanks' balanced salt solution, collected by scraping, sedimented and frozen at –80°C.

Table 2. *CaCh2A*-derived [16] and *CaCh2B*-derived [17] oligodeoxynucleotide primers used for cDNA synthesis and PCR

Primer	Sequence	Subunit	Positions
1	5'-GTGTTTCAGGAAGACCAG-3'	CaCh2B	1672 to 1688
2	5'-TAAAACGTAAAGTATTACTAAAACCTC-3'	CaCh2A	-191 to -165
3	5'-GCACTGCCCATCAGCTTG-3'	CaCh2B	186 to 203
4	5'-TGGCGCCGCTCGCTCTGTT-3'	CaCh2B	-312 to -294
6	5'-CATGGGTCTCTGTGGAGTGACTAA-3'	CaCh2B	1442 to 1467

### [<sup>3</sup>H]PN200-110 binding to BC<sub>3</sub>H1 cells

Cell pellets were thawed in 5mM Tris/HCl, pH 7.4 containing 0.1 mM phenylmethylsulfonyl fluoride, 1 mM phenanthroline, 100 mM iodoacetamide, 1 mM benzamidine, 1μM pepstatin A, 1 μg/ml antipain and 1 μg/ml leupeptin (protease inhibitor mix), homogenized and centrifuged at 1000xg for 20 min. The supernatant was then centrifuged at 42000xg for 40 min at 4°C. The pellet was rehomogenized in 50 mM Tris/HCl, pH 7.4 containing the protease inhibitor mix. Equilibrium binding of (+)[<sup>3</sup>H]PN200-110 was performed as described previously [29] in the presence of 25 μg protein and 1 nM (+)[<sup>3</sup>H]PN200-110 at 22°C for 45 min. Nonspecific binding was determined in the presence of 2 μM unlabelled PN200-110.

### Miscellaneous methods

Oligodeoxynucleotide synthesis was run by standard protocol supplied by the manufacturer on an automated single-column synthesizer. PCR were performed as in [14] and the amplified cDNA were subcloned and sequenced on both strands [30]. Cloning procedures, if not stated otherwise, were carried out as described [31]. Protein concentrations were determined according to [32].

## RESULTS

### Tissue specific expression of calcium channel subunits

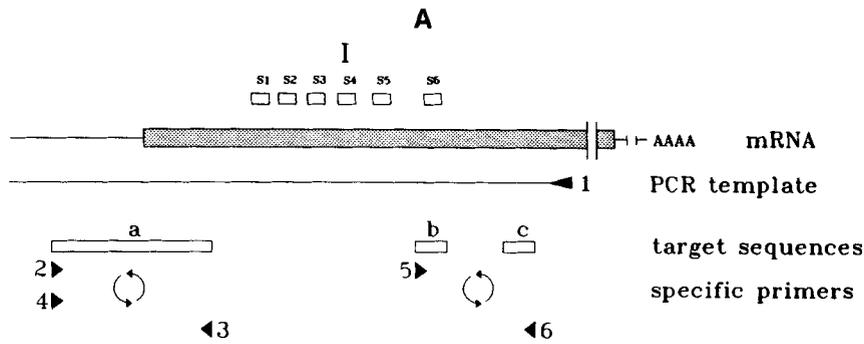
The tissue-specific distribution of mRNA specific for the calcium channel  $\alpha_1$ ,  $\alpha_2/\delta$ ,  $\beta$  and  $\gamma$  subunits was analyzed by RNA-blot analysis of poly(A)-rich RNA from several rabbit tissues using subunit-specific antisense RNA probes. In Fig. 1a, a probe derived from domains 3 and 4 of CaCh2 hybridized to a poly(A)-rich RNA species in skeletal muscle of 6.5 kb, which is identical to the skeletal muscle  $\alpha_1$  subunit mRNA described previously [9-11]. A less-abundant mRNA species, with an estimated size of about 8.9 kb, is also present in skeletal muscle. In contrast, 8.9-kb, but not 6.5-kb, transcripts were detected in poly(A)-rich RNA from brain, heart, aorta, trachea and lung. In addition, weakly hybridizing transcripts of about 15.5 kb were observed in these tissues. The intensity of the  $\alpha_1$  hybridization signals varied among the different tissues, being most intense in skeletal muscle and lung.

To investigate the expression of transcripts related to those encoding the lung and cardiac muscle  $\alpha_1$  subunits, specific RNA probes derived from their 5' nucleotide sequences were used (Fig. 1b and c). For the cardiac-channel-specific probe, a prominent 8.9-kb and a much weaker 15.5-kb transcript were detected in poly(A)-rich RNA from cardiac muscle (Fig. 1b). However, no signals were detected in other tissues, even after two weeks autoradiographic exposure (data not shown). The

probe derived from the lung  $\alpha_1$  sequence hybridized to a number of RNA species in all tissues, including 8.9-kb and 15.5-kb transcripts in brain, heart, aorta, trachea and lung (Fig. 1c).

The probe of the skeletal  $\alpha_2/\delta$  subunit hybridized to an 8.0-kb transcript in poly(A)-rich RNA from each of the tissues examined (Fig. 1d). In addition, a weakly hybridizing  $\alpha_2$  7.0-kb transcript was observed in these tissues. The  $\beta$ -subunit-derived probe hybridized to three poly(A)-rich RNA species of skeletal muscle with estimated sizes of 1.6, 1.9 and 3.0 kb. mRNA species of similar size to the 3.0-kb species appear to be present in brain and aorta. A less-abundant mRNA population of about 4.8 kb, which was detected only as a faint band after long autoradiographic exposure, is present in heart. The  $\gamma$ -subunit probe hybridized to a 1.3-kb poly(A)-rich RNA species from skeletal muscle [14, 15]. Weakly hybridizing  $\gamma$  transcripts of 1.0 kb (heart), and 1.3 kb and 1.8 kb (aorta) were observed. In brain, a 1.5-kb signal was clearly visible after 5 day autoradiographic exposure, but not after 16 h (compare Fig. 3B, d, lane 6, with Fig. 1f, lane 2). No  $\alpha_1$ -,  $\alpha_2/\delta$ -,  $\beta$ - and  $\gamma$ -specific hybridization was observed with poly(A)-rich RNA and total RNA from HeLa cells, murine L-cells and *Paramecium tetraurelia*.

*Characterization of tissue specific expression of  $\alpha_1$  subunits by PCR.* The Northern-blot experiments indicated that CaCh2A is expressed only in heart, whereas CaCh2B seems to be expressed in heart, brain, aorta, lung and trachea (Fig. 1b and c). To confirm the existence and differential expression of mRNA for type CaCh2A and CaCh2B by an independent method, CaCh2A- and CaCh2B-specific sequences were amplified by two sets of PCR from heart, lung and trachea. This method has an important advantage over Northern-blot analysis of RNA in that the exact sequence expressed in a given tissue can be determined. The general location of the sequence spanned by the primers used (Table 2) is depicted as a, b and c in Fig. 2A. These regions of the CaCh2 gene have been described as alternative splicing products [17]. Template cDNA were constructed with poly(A)-rich RNA from rabbit heart, lung and trachea using oligodeoxynucleotide primer 1 which covers a region which is conserved between the cardiac (CaCh2A) and lung (CaCh2B) isoforms. For PCR covering the amino-terminal region (site a) pairs of oligonucleotides were used, which were derived from the 5' sequence of CaCh2A (primer 2) and CaCh2B (primer 4) and a region which is identical in both isoforms (primer 3). Clones amplified from cardiac cDNA with primers 2 and 3 had an identical sequence to that obtained by Mikami et al [16]. For example, the DNA sequence of rabbit clone 7 is 100% identical to that of the rabbit cardiac calcium channel (CaCh2A, Fig. 2B). However, using the same primer pair, but with cDNA from lung and trachea, did not lead to any specific amplification. In contrast, PCR initiated by primers 4 and 3 with cDNA from heart, lung and trachea yield specific amplification products in all cases. Sequencing by the dideoxy-chain-termination

**B**Aminotermminus (a)

```

rabbit cardiac (CaCh2A) : MLRALVQPATPAYQPLPSHLSAETESTCKGTVVHEAQLNH
rabbit lung (CaCh2B) : M.....-NENTRMYI
rabbit cardiac clone 7 : M-----
rabbit cardiac clone 41 : M.....-NENTRMYI
rabbit lung clone 205 : M.....-NENTRMYI
rabbit trachea clone 67 : M.....-NENTRMYI

```

```

CaCh2A : FYISPGGSNYGSPRPAHANMNANAAAGLAPEHIPTPGAALSWQAAIDAARQAKLMGS
CaCh2B : PEENHQ-----
cl 7 : -----
cl 41 : PEENHQ-----
cl 205 : PEENHQ-----
cl 67 : PEENHQ-----

```

**C**Segment IS6 (b) and consecutive region (c)

```

rabbit cardiac (CaCh2A) : MQDAMGYELPWVYFVSLVIFGSFFVLNLVLGVLGSEFSKE
rabbit lung (CaCh2B) : VN--V-RDW--I---T-I-I-----
rabbit cardiac clone 22 : VN--V-RDW--I---T-I-I-----
rabbit lung clone 2 : VN--V-RDW--I---T-I-I-----
rabbit trachea clone 13 : VN--V-RDW--I---T-I-I-----

```

```

CaCh2A : REKAKARGDFQKLRKQQLLEEDLKGVDWITQAEDIDPENEDGMDDEEKPRN.....
CaCh2B : -----RGTPA
cl 22 : -----RGTPA
cl 2 : -----RGTPA
cl 13 : -----RGTPA

```

```

CaCh2A : .....M
CaCh2B : GLHAQKKGKFAWFSHSTETHV
cl 22 : GLHAQKKGKFAWFSHSTETHV
cl 2 : GLHAQKKGKFAWFSHSTETHV
cl 13 : GLHAQKKGKFAWFSHSTETHV

```

Key  
---, identity  
..., gap

Fig. 2. Diagram of part of the general structure of L-type calcium channels (A) and amino acid sequences of PCR products (B,C) obtained from first-strand cDNA derived from rabbit heart, lung and trachea. (A) A representation of part of the  $\alpha_1$  subunit of L-type calcium channels showing relative positions of the transmembrane segments (S) 1–6 of domain I. The hatched box represents the protein coding-region. Triangles indicate relative positions of oligodeoxynucleotide primers 1–6 used for primer extension (1) and PCR (2–6). a–c Target sequences which are not identical in the  $\alpha_1$  subunit cDNA cloned from rabbit heart [16] and lung [17]. (B, C) Results of PCR using specific primers derived from CaCh2A (2, 3) and CaCh2B (3–6). The deduced amino acid sequences are compared with that of the rabbit cardiac muscle isoform (CaCh2A)

method revealed that the clone amplified from cardiac cDNA (rabbit cardiac clone 41) is identical to the sequence of CaCh2B, except that it lacks 42 bp within the sequence conserved in CaCh2A and CaCh2B. The sequences of the clones obtained with cDNA from lung (clone 205) and trachea (clone 67) were 100% identical to that of CaCh2B.

For PCR covering sites b and c, pairs of oligonucleotide primers were derived from the 5' and 3' ends of sites b and c,

respectively, of the CaCh2B sequence. DNA were amplified from heart, lung and trachea. Sequences of clones obtained from heart (rabbit cardiac clone 22), lung (clone 2) and trachea (clone 13) were 100% identical to that of CaCh2B (Fig. 2C).

*Expression of calcium channel transcripts in BC<sub>3</sub>H1 cells.* The results of the nucleic acid hybridization studies indicated that mRNA of various  $\alpha_1$ ,  $\alpha_2/\delta$ ,  $\beta$  and  $\gamma$  subunits are coexpressed in each tissue, suggesting that all subunits may con-

tribute to calcium channel function. In this case, their mRNA expression should be regulated in a coordinate manner. A model system to study this regulation is the clonal BC<sub>3</sub>H1 cell line. It is derived from a mouse brain tumor and possesses many properties characteristic of muscle [28]. In addition, differentiated, but not proliferating, BC<sub>3</sub>H1 cells express voltage-activated dihydropyridine-sensitive and dihydropyridine-insensitive calcium currents, whose electrophysiological properties are different from those found in smooth muscle, cardiac muscle and neurons and resemble best those found in skeletal muscle cells [33–35]. Accordingly, differentiated BC<sub>3</sub>H1 cells express calcium-channel-blocker-binding sites (Fig. 3A, lane D), whereas these sites are barely detectable in subconfluent, proliferating cells (P1–P3). To determine the expression of the mRNA of the calcium channel proteins, total RNA and poly(A)-rich RNA was isolated from proliferating subconfluent and differentiated BC<sub>3</sub>H1 cells. As shown in Fig. 3B,a,  $\alpha_1$  transcripts were barely detectable in proliferating cells (Fig. 3B,a, lanes 2–4). However, after switching subconfluent BC<sub>3</sub>H1 cells to serum-deprived medium there was a marked induction of a 6.5-kb transcript (Fig. 3B,a, lane 5). This  $\alpha_1$  specific transcript was also induced when cells in growth medium reached confluence (data not shown). The size of this transcript is identical to that of the skeletal muscle  $\alpha_1$  subunit (Fig. 3B,a, lane 1), but different to various transcripts observed in rabbit brain (Fig. 3B,a, lane 6). In addition, a 4.0-kb transcript was observed in BC<sub>3</sub>H1 cells, which has been described previously [35] and has been attributed to nonspecific hybridization to the leading edge of 28S ribosomal RNA. For the  $\beta$  subunit, a 1.9-kb transcript was detected in total RNA from differentiated, but not from proliferating, cells (Fig. 3B,b, lane 5), whereas no  $\alpha_2/\delta$ -specific transcripts were detectable (Fig. 3B,c, lanes 2–5). In contrast, a transcript specific for the  $\gamma$  subunit was detected in total RNA from each of the cells examined (Fig. 3B,d, lanes 2–5). The intensity of this signal increase being most prominent in differentiated cells. Its size is similar to that of the  $\gamma$  transcript in skeletal muscle (Fig. 3B,d, lane 1), but smaller than that in brain (Fig. 3B,d, lane 6). Taken together, these results demonstrate, that  $\alpha_1$ ,  $\beta$  and  $\gamma$  mRNA are expressed together in differentiated but not in proliferating BC<sub>3</sub>H1 cells, suggesting that expression of their mRNA, but not of  $\alpha_2/\delta$  mRNA, is regulated in a coordinate manner.

## DISCUSSION

In this investigation, the expression of the calcium channel  $\alpha_1$ ,  $\alpha_2/\delta$ ,  $\beta$  and  $\gamma$  mRNA was studied in various rabbit tissues and BC<sub>3</sub>H1 cells by Northern-blot analysis and PCR. It is shown that  $\alpha_1$ -,  $\alpha_2/\delta$ -,  $\beta$ - and  $\gamma$ -specific transcripts exist together in skeletal muscle, brain, heart and aorta, (b) that alternate splicing products for the CaCh2 gene are tissue-specifically expressed and (c) that in BC<sub>3</sub>H1 cells induction of a functional receptor for calcium channel blockers parallels the expression of  $\alpha_1$ -,  $\beta$ - and  $\gamma$ -specific mRNA, suggesting that their expression is developmentally regulated. At least three  $\alpha_1$  specific transcripts exist with estimated sizes of about 6.5, 8.9 and 15.5 kb. The 6.5-kb transcript represents the mRNA of the skeletal muscle calcium channel  $\alpha_1$  subunit [9–11]. In the nomenclature used here, this transcript is derived from gene 1 (CaCh1) and predominantly expressed in skeletal muscle (Fig 1a, lane 1), although expression in other tissues has been described [10, 18]. The size of the second transcript which is present in skeletal muscle, brain, heart, aorta, trachea

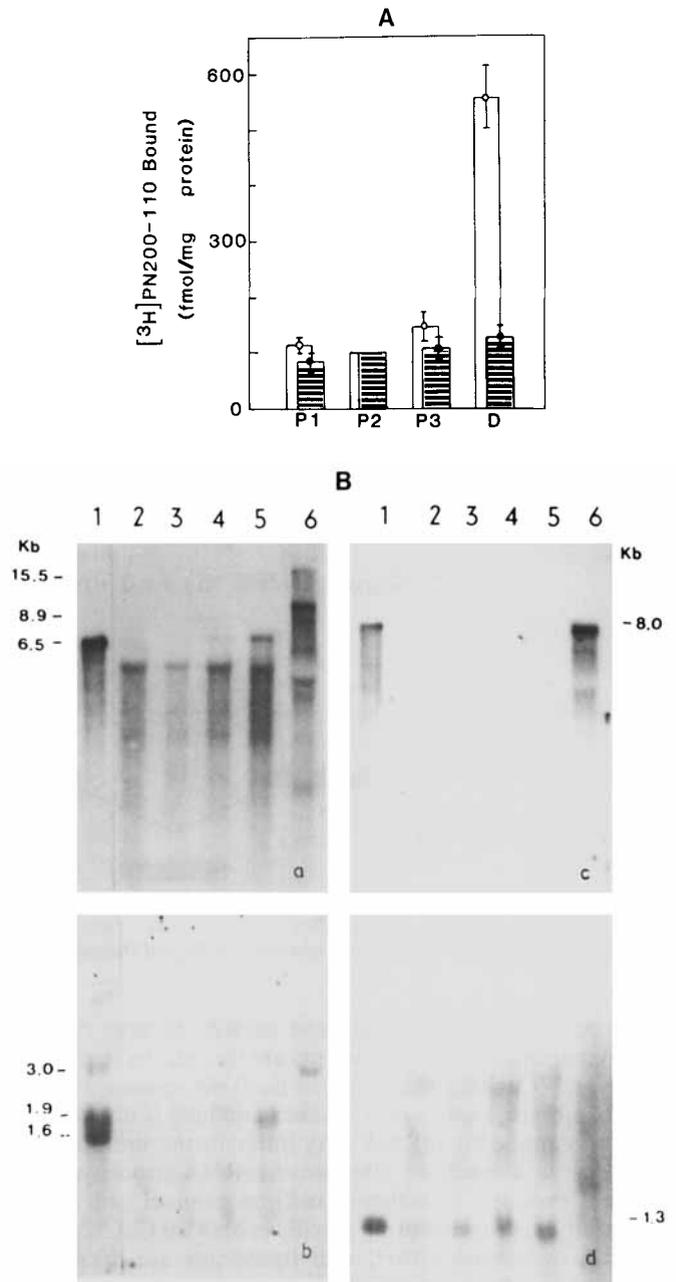


Fig. 3. Induction of dihydropyridine binding sites (A) and calcium channel subunits mRNAs (B). (A) Development of [<sup>3</sup>H]PN200-110-binding site density in proliferating (P1–P3) and differentiated (D) BC<sub>3</sub>H1 cells. Open and hatched columns represent total and nonspecific binding, respectively. Bars indicate mean  $\pm$  SEM from six different experiments performed in triplicate. (B) Samples of 2  $\mu$ g poly(A)-rich RNA from rabbit skeletal muscle (lane 1) and brain (lane 6), 5  $\mu$ g of poly(A)-rich RNA (a, c) and 20  $\mu$ g total RNA b, d) from proliferating (lanes 2–4) and differentiated (lane 5) BC<sub>3</sub>H1 cells, respectively, were electrophoresed on 1% agarose gels. Autoradiographic exposure was 16 h (a, lanes 1 and 6; b, lanes 1 and 6; d, lane 1), 5 days (a, lanes 2–5; b, lanes 2–5; c, d, lanes 2–6) at  $-70^{\circ}\text{C}$  with an intensifying screen. The RNA probes used were specific for the skeletal muscle  $\alpha_1$ ,  $\beta$ ,  $\alpha_2/\delta$  and  $\gamma$  subunits (a–d, respectively). The specific activities of the four RNA probes used were  $0.7\text{--}2.6 \times 10^9$  dpm/ $\mu$ g.

and lung is consistent with the size of the cloned cDNA of the  $\alpha_1$  subunits from heart (7036 nucleotides) [16] and lung (7374 nucleotides) [17], considering that both cDNA lack conven-

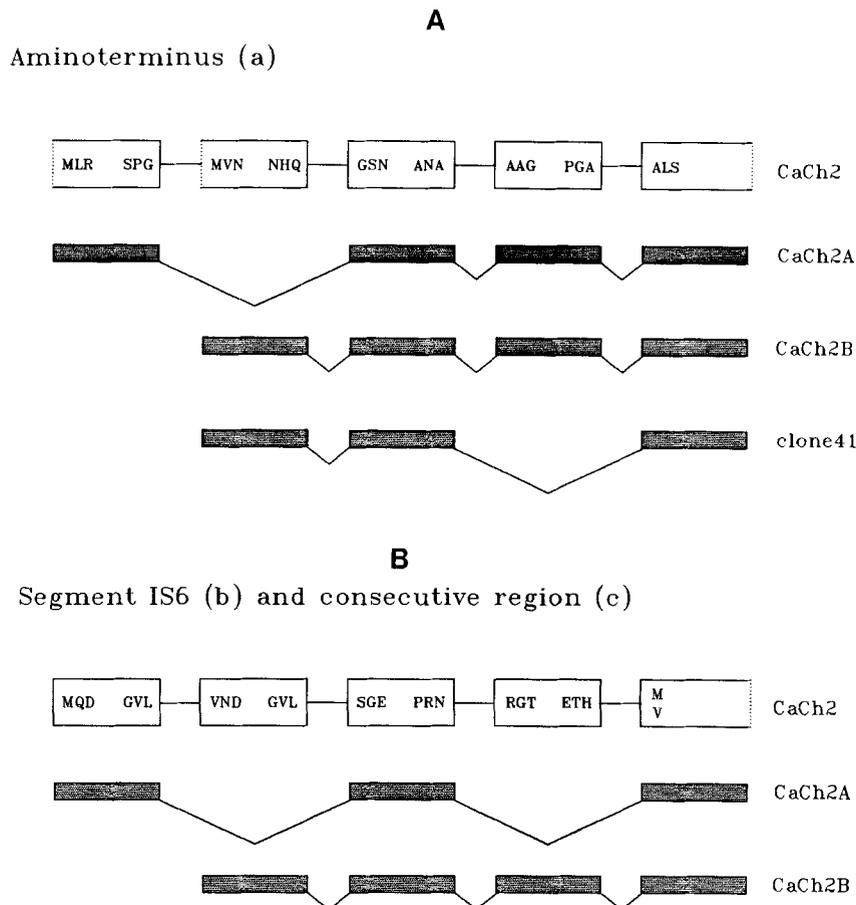


Fig. 4. Models of alternate splicing events that give rise to nucleotide sequences of CaCh2 encoding different amino-termini (A), IS6 segments and consecutive regions (B). Boxes represent exons and include amino acids encoded at their 5'- and 3' ends

tional polyadenylation signals and poly(A) tails at their 3' ends. Accordingly, these transcripts are thought to be derived from gene 2 (CaCh2). The nature of the RNA species of about 15.5 kb in brain, heart, aorta, trachea and lung is not known. These less-abundant mRNA may differ in the untranslated sequences as a result of alternative mRNA processing, or represent products of different calcium channel genes. Hybridization experiments using specific probes for CaCh2A and CaCh2B, demonstrate that both transcripts are present in heart whereas only the CaCh2B transcript is expressed in brain, heart, aorta, trachea and lung. This tissue distribution suggests that the CaCh2A transcript is exclusively expressed in cardiac myocytes, whereas the CaCh2B transcript might be present in airway and vascular smooth muscle cells which are present in brain, heart, aorta, trachea and lung. The skeletal muscle differs from the other tissues since it contains  $\alpha_1$ -specific transcripts of 8.9 kb, although no hybridization signals of this size were found with the CaCh2A- and CaCh2B-specific probes. This may be due either to the lower vascularisation of this tissue or to the presence of additional splice variants of the CaCh2 gene.

Further evidence for the presence of alternate splice products of the CaCh2 gene comes from PCR covering the 5' nucleotide sequence encoding the amino-terminus (Fig. 2B, site a) and the membrane spanning region 6 of domain I (Fig. 2C, site b), which are different in the CaCh2A and CaCh2B sequences [17] and an insertion within the predicted cytoplasmic linker between domains I and II (Fig. 2C, site c) which is present in CaCh2B, but not in CaCh2A [17]. The

amplified sequences covering site a in Fig. 2B are identical with either the CaCh2A or the CaCh2B sequence. However, the CaCh2A sequence is only amplified from heart, whereas the CaCh2B sequence is not only present in lung but also in trachea and heart. This tissue-specific expression of alternate splicing products of CaCh2 is in agreement with the results of the Northern-blot experiment shown in Fig. 1b and c. In addition, a third splice variant was identified (clone 41) which is identical to CaCh2B except that it lacks 42 bp within the sequences that are identical in CaCh2A and CaCh2B. The amplified sequences covering predicted transmembrane segment 6 of domain I (Fig. 2C, site b) and the consecutive region (site c) are identical with the CaCh2B subtype. Fig. 4 illustrates models for the formation of the alternatively spliced forms of CaCh2 within site a, and sites b and c. Both models assume the existence of three alternatively spliced exons and one intervening sequence of which two exons are of the alternate type and the third is either used or skipped. Although this kind of analysis was not extended to other regions of the CaCh2 sequence, there is the possibility that additional alternate splice products exist. In fact, another splice variant has been identified in partial CaCh2-derived cDNA clones covering the consecutive region of transmembrane segment 3 of domain IV [18]. The presence of regions of CaCh2 that are alternative splice products make it necessary to examine carefully whether the cDNA segments spliced together *in vitro* to reconstruct full-length cDNA do indeed coexist *in vivo* on the same mRNA molecule. In addition, it will be important not only to define which type of channel variant is expressed, but

also what, if any, functional correlate may be established between splice variants and type of current. The functions of the amino-terminus and of segment 6 of domain I described here have not been determined. However, in current topographical models the amino-terminus is localized intracellularly and cytoplasmic elements have been shown to be involved in both activation and inactivation gating in voltage-activated potassium [36] and sodium channels [37].

$\alpha_2$ -specific transcripts of 8.0 kb were detected in poly(A)-rich RNA from each of the tissues examined. In addition, weakly hybridizing  $\alpha_2$  7.0-kb transcripts were observed. The size of the cloned  $\alpha_2$  cDNA (about 7.8 kb including a 4.5-kb untranslated sequence) [10, 16], is consistent with an 8.0-kb  $\alpha_2$  mRNA, whereas the 7.0-kb  $\alpha_2$  mRNA may differ in the 3' untranslated sequence. Three  $\beta$ -subunit-specific transcripts exist in skeletal muscle, which may all differ in the coding and noncoding sequences as a result of alternative mRNA processing. The size of the 1.9-kb species is consistent with the size of the cloned cDNA (1835 nucleotides) [13]. Splice variants of the same  $\beta$ -subunit gene appear to be present in brain and aorta, whereas heart contains a faintly hybridizing transcript of 4.8 kb which might be derived from a second  $\beta$ -like gene.  $\gamma$ -subunit-specific transcripts of various sizes were observed in skeletal muscle, brain, heart and aorta. The lack of  $\beta$ - and  $\gamma$ -specific hybridization signals from lung and trachea may be due either to the lower concentration of  $\beta$ - and  $\gamma$ -subunit mRNA in these tissues, or to the presence of subunits with considerably different nucleotide sequences encoded by separate genes.

Apparently splice variants of the genes for the  $\alpha_1$ ,  $\alpha_2/\delta$ ,  $\beta$  and  $\gamma$  subunits coexist in tissues which contain L-type calcium channels. The correlation of their expression with that of functional channels was examined in the BC<sub>3</sub>H1 cell line, which has been used as a model for studying muscle cell differentiation and the regulation of muscle-related proteins. Removal of growth factors from the culture medium or allowing cultures to reach confluence induces BC<sub>3</sub>H1 cells to differentiate and to accumulate muscle-specific gene products [38]. In addition, it has been shown that differentiated BC<sub>3</sub>H1 cells express skeletal muscle voltage-activated calcium channels [33–35], and partial cDNA clones isolated from BC<sub>3</sub>H1 cells reveal a high degree of sequence similarity to the rabbit skeletal muscle  $\alpha_1$  nucleotide sequence [18]. In agreement with these results, the experiments shown in Fig. 3 indicate that BC<sub>3</sub>H1 express a skeletal-muscle-type  $\alpha_1$  subunit and that the time course for induction of a skeletal-muscle-type  $\alpha_1$ -,  $\beta$ - and  $\gamma$ -subunit mRNA is similar to that reported for the induction of the contractile protein mRNA and parallels the appearance of dihydropyridine-binding sites. Obviously, the expression of calcium channel  $\alpha_1$ ,  $\beta$  and  $\gamma$  subunits, but not of the  $\alpha_2/\delta$  subunit, is controlled by a common regulatory mechanism in these cells and contributes to the formation of a dihydropyridine-sensitive calcium channel. The availability, now, of molecular probes for the expression of the various calcium channel subunits should allow further dissection of channel components regulating calcium channel function and modulation.

We thank T. Tanabe and S. Numa for kindly providing pSPCA1, R. Nagel for expert technical assistance and H. Siepmann for the graphical work. This work was supported by *Deutsche Forschungsgemeinschaft*, *Thyssen Stiftung* and *Fond der Chemischen Industrie*.

## REFERENCES

1. Reuter, H. (1983) *Nature* 301, 969–974.
2. Tsien, R. W., Bean, B. P., Hess, P., Lansman, J. B., Nilius, B. & Nowicky, M. C. (1986) *J. Mol. Cell. Cardiol.* 18, 691–710.
3. Trautwein, W. & Hescheler, J. (1990) *Annu. Rev. Physiol.* 52, 257–274.
4. Brown, A. M. & Birnbaumer, L. (1990) *Annu. Rev. Physiol.* 52, 197–213.
5. Hofmann, F., Flockerzi, V., Nastainczyk, W., Ruth, P. & Schneider, T. (1990) *Curr. Top. Cell. Regul.* 31, 225–239.
6. Glossmann, H. & Striessnig, J. (1988) *Vitam. and Horm.* 44, 154–327.
7. Catterall, W. A., Seagar, M. J. & Takahashi, M. (1988) *J. Biol. Chem.* 263, 3535–3538.
8. Campbell, K. P., Leung, A. T. & Sharp, A. H. (1988) *Trends Neurosci.* 11, 425–430.
9. Tanabe, T., Takeshima, H., Mikami, A., Flockerzi, V., Takahashi, H., Kangawa, K., Kojima, M., Matsuo, H., Hirose, T. & Numa, S. (1987) *Nature* 328, 313–318.
10. Ellis, S. B., Williams, M. E., Ways, N. R., Brenner, R., Sharp, A. H., Leung, A. T., Campbell, K. P., McKenna, E., Koch, W. J., Hui, A., Schwartz, A. & Harpold, M. M. (1988) *Science* 241, 1661–1664.
11. Perez-Reyes, E., Kim, H. S., Lacerda, A. E., Horne, W., Wei, X., Rampe, D., Campbell, K. P., Brown, A. M. & Birnbaumer, L. (1989) *Nature* 340, 233–236.
12. De Jongh, K. S., Warner, C. & Catterall, W. A. (1990) *J. Biol. Chem.* 265, 14738–14741.
13. Ruth, P., Röhrkasten, A., Biel, M., Bosse, E., Regulla, S., Meyer, H. E., Flockerzi, V. & Hofmann, F. (1989) *Science* 245, 1115–1118.
14. Bosse, E., Regulla, S., Biel, M., Ruth, P., Meyer, H. E., Flockerzi, V. & Hofmann, F. (1990) *FEBS Lett.* 267, 153–156.
15. Jay, S. D., Ellis, S. B., McCue, A. F., Williams, M. E., Vedvick, T. S., Harpold, M. M. & Campbell, K. P. (1990) *Science* 248, 490–491.
16. Mikami, A., Imoto, K., Tanabe, T., Niidome, T., Mori, Y., Takeshima, H., Narumiya, S. & Numa, S. (1989) *Nature* 340, 230–233.
17. Biel, M., Ruth, P., Bosse, E., Hullin, R., Stühmer, W., Flockerzi, V. & Hofmann, F. (1990) *FEBS Lett.* 269, 409–412.
18. Perez-Reyes, E., Wei, X., Castellano, A. & Birnbaumer, L. (1990) *J. Biol. Chem.* 265, 20430–20436.
19. Koch, W. J., Hui, A., Shull, G., Ellinor, P. & Schwartz, A. (1989) *FEBS Lett.* 250, 386–388.
20. Snutch, T. P., Leonard, J. P., Gilbert, M. M. & Lester, H. A. (1990) *Proc. Natl. Acad. Sci. USA* 87, 3391–3395.
21. Koch, W. J., Ellinor, P. T. & Schwartz, A. (1990) *J. Biol. Chem.* 265, 17786–17791.
22. Huang, P., Temizer, D. & Quertermous, T. (1990) *FEBS Lett.* 274, 207–213.
23. Grabner, M., Friedrich, U., Knaus, H.-G., Striessnig, J., Scheffauer, F., Staudinger, R., Koch, W. J., Schwartz, A. & Glossmann, H. (1991) *Proc. Natl. Acad. Sci. USA* 88, 727–731.
24. Tanabe, T., Beam, K. G., Powell, J. A. & Numa, S. (1988) *Nature* 336, 134–139.
25. Kim, H. S., Wei, X., Ruth, P., Perez-Reyes, E., Flockerzi, V., Hofmann, F. & Birnbaumer, L. (1990) *J. Biol. Chem.* 265, 11858–11863.
26. Melton, D. A., Krieg, P. A., Rebagliati, M. R., Maniatis, T., Sinn, K. & Green, M. R. (1984) *Nucleic Acids Res.* 12, 7035–7056.
27. Thomas, P. S. (1980) *Proc. Natl. Acad. Sci. USA* 77, 5201–5205.
28. Olson, E. N., Caldwell, K. L., Gordon, J. I. & Glaser, L. (1983) *J. Biol. Chem.* 258, 2644–2652.
29. Ruth, P., Flockerzi, V., v. Nettelbladt, E., Oeken, J. & Hofmann, F. (1985) *Eur. J. Biochem.* 150, 313–322.
30. Sanger, F., Nicklen, S. & Coulson, A. B. (1977) *Proc. Natl. Acad. Sci. USA* 74, 5463–5467.
31. Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989) *Molecular cloning: a laboratory manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

32. Smith P. K., Krohn, R. I., Hermanson, G. T., Mallia, H. K., Gartner, F. H., Provenzano, M. D., Fujimoto, E. K., Goeke, N. H., Olson, B. J. & Klenk, D. C. (1985) *Anal. Biochem.* 150, 76–85.
33. Caffrey, J. M., Brown, A. M. & Schneider, M. D. (1987) *Science* 236, 570–574.
34. Rampe, D. E., Caffrey, J. M., Schneider, M. D. & Brown, A. M. (1988) *Biochem. Biophys. Res. Commun.* 152, 769–775.
35. Shih, H.-T., Wathen, M. S., Marshall, H. B., Caffrey, J. M. & Schneider, M.D. (1990) *J. Clin. Invest.* 85, 781–789.
36. Hoshi, T., Zagotta, W. N. & Aldrich, R. W. (1990) *Science* 250, 533–539.
37. Stühmer, W., Conti, F., Suzuki, H., Wang, X., Noda, M., Yahagi, N., Kubo, H. & Numa, S. (1989) *Nature* 339, 597–603.
38. Schubert, D., Harris, A. J., Devine, C. E. & Heinemann, S. (1974) *J. Cell. Biol.* 61, 398–413.