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Calcium channel β subunit heterogeneity: functional expression of cloned cDNA from heart, aorta and brain

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Complemetary DNAs encoding three novel and distinct β subunits (CaB2a, CaB2b and CaB3) of the high voltage activated (L-type) calcium channel have been isolated from rabbit heart. Their deduced amino acid sequence is homologous to the β subunit originally cloned from skeletal muscle (CaB1). CaB2a and CaB2b are splicing products of a common primary transcript (CaB2). Northern analysis and specific amplification of CaB2 and CaB3 specific cDNAs by polymerase chain reactions showed that CaB2 is predominantly expressed in heart, aorta and brain, whereas CaB3 is most abundant in brain but also present in aorta, trachea, lung, heart and skeletal muscle. A partial DNA sequence complementary to a third variant of the CaB2 gene, subtype CaB2c, has also been cloned from rabbit brain. Coexpression of CaB2a, CaB2b and CaB3 with α1heart enhances not only the expression in the oocyte of the channel directed by the cardiac α1 subunit alone, but also effects its macroscopic characteristics such as drug sensitivity and kinetics. These results together with the known α1 subunit heterogeneity, suggest that different types of calcium currents may depend on channel subunit composition.

Key words: β subunit/calcium channel/calcium channel subtypes/ molecular cloning/oocyte expression

Introduction

High voltage activated (L-type) calcium channels are virtually ubiquitous and are the major pathway for voltage-gated calcium entry in heart and most kinds of smooth muscle (Tsien *et al.*, 1991). In these tissues calcium currents are differentially modulated by guanosine nucleotide-binding proteins (Brown and Birnbaumer, 1990) and phosphorylation (Trautwein and Hescheler, 1990). However, the sites of the channel responsible for this modulation are unknown and might reside in one of the four subunits α1, α2, β or γ, which form the oligomeric channel complex (Hofmann *et al.*, 1990; Catterall, 1991). Complementary DNAs of these subunits have been cloned from skeletal muscle and their amino acid sequences deduced (Tanabe *et al.*, 1987; Ellis *et al.*, 1988; Ruth *et al.*, 1989; Bosse *et al.*, 1990; Jay *et al.*, 1990). So far only α1 and α2 have been identified in cardiac and smooth muscle but not β and γ (Mikami *et al.*, 1989; Biel *et al.*, 1990, 1991; Koch *et al.*, 1990). As very similar α1 and α2 exist in both tissues, β and γ may be responsible for tissue specific channel function and modulation. We have now isolated complementary DNAs from three novel and distinct β subunits (CaB2a, CaB2b and CaB3) which are expressed in different abundance in heart, smooth muscle and brain. Like the skeletal muscle β subunit CaB2 and CaB3 modulate channel activity directed by the cardiac α1 subunit alone, suggesting a similar subunit composition of calcium channels in these tissues.

Results and Discussion

Isolation of cDNAs encoding β subunits of the high voltage activated calcium channel from rabbit heart

A directional oligo(dT) primed cDNA library was constructed from rabbit heart poly(A)$^+$ RNA and 3×10^5 transformants were probed with three radiolabelled cDNA fragments covering the complete protein coding region of the skeletal muscle β subunit. Nine independent full-length cDNA clones were obtained which could be assigned to three different molecular species designated CaB2a (six clones), CaB2b (two clones) and CaB3 (1 clone) on the basis of restriction enzyme mapping and sequence analysis. Figure 1 shows the deduced primary structures of the cloned cDNAs encoding rabbit β subunits CaB2a, CaB2b and CaB3 which were deduced by using the reading frame corresponding to the amino acid sequence of the skeletal muscle counterpart (CaB1) (Ruth *et al.*, 1989). CaB2a, CaB2b and CaB3 are composed of 606, 632 and 477 amino acids with calculated M_rs of 68 177, 70 943 and 53 814, respectively. Amino acid sequence comparison reveals 71.0% (CaB2a/CaB1), 71.5% (CaB2b/CaB1), 66.6% (CaB3/CaB1), 64.7% (CaB2a/CaB3), 64.9% (CaB2b/CaB3) and 97.2% (CaB2a/CaB2b) sequence homology between the four β subunits. CaB2a and CaB2b are identical in the coding and noncoding nucleotide region except for the 5′ end of the nucleotide sequence containing the predicted amino termini of the two proteins. The nucleotide sequence of CaB3 is completely different from CaB2a and CaB2b whereas based on amino acid sequence there is considerable similarity between the three proteins. An analysis of the three amino acid sequences for local hydropathicity (Kyte and Doolittle, 1982) revealed the absence of either a typical membrane spanning domain or of a signal sequence. Secondary structure analysis (Chou and Fasman, 1978) predicts a high degree of α-helical content. Four α-helical domains noted for CaB1 (Ruth *et al.*, 1989) are relatively well conserved in CaB2a, CaB2b and CaB3, including an eight amino acid repeat which starts and ends with leucine or isoleucine and is interspersed by negatively charged residues. The function of these regions is unknown, but it is assumed that they play a role in divalent cation binding (Ruth *et al.*, 1989).

Cardiac but not smooth muscle L-type calcium currents

are modulated by cAMP-dependent phosphorylation (Kameyama *et al.*, 1985; Hartzell *et al.*, 1991; Welling *et al*, 1991). However, the phosphorylation site responsible for this regulation is unknown. The skeletal muscle β subunit can be phosphorylated *in vitro* by cAMP- and cGMP-dependent protein kinase, protein kinase C, casein kinase

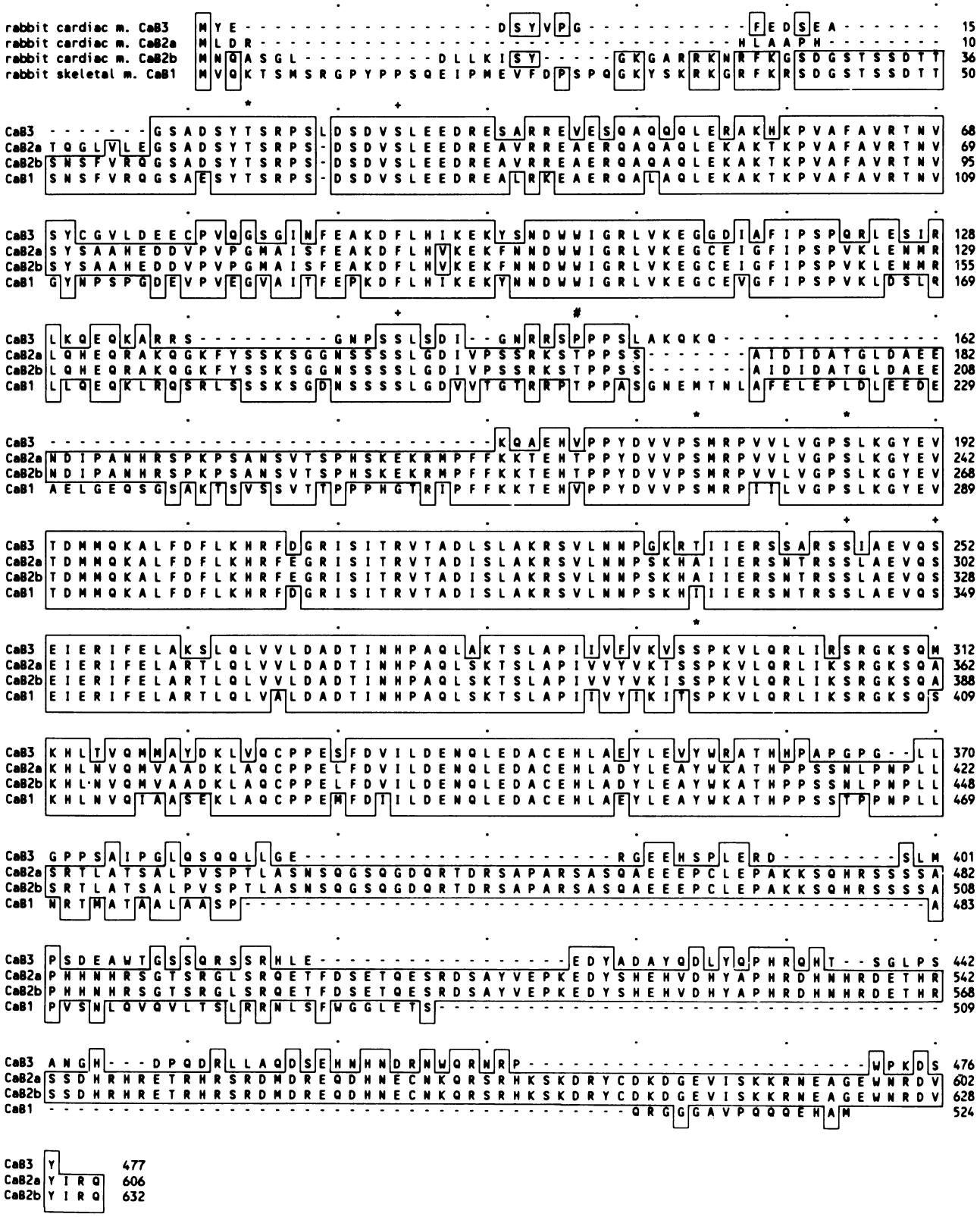


Fig. 1. The amino acid sequence (CaB2a, CaB2b and CaB3) of the rabbit cardiac β subunits (as predicted from the cloned cDNAs; the nucleotide sequences to be deposited in the EMBL Data Library) with that of the skeletal muscle β subunit (CaB1). Regions of sequence identity are enclosed in boxes. Gaps in the sequences are represented by dashes. The potential sites for phosphorylation are indicated above the line with the following symbols: cAMP-dependent protein kinase (#), protein kinase C (*), casein kinase II (+).

II and calmodulin-dependent protein kinase (Curtis and Catterall, 1985; Flockerzi *et al.*, 1986; Jahn *et al.*, 1989). One of the two consensus sequences (Kennelly and Krebs, 1991) of cAMP-dependent protein kinase (Thr205) (De Jongh *et al.*, 1989) is conserved in CaB2a (Arg-Lys-Ser-Thr165) and CaB2b (Arg-Lys-Ser-Thr191) and might be responsible for the regulation of the channel by the kinase. No cAMP-dependent phosphorylation site exists in CaB3. The cardiac β subunits contain four potential protein kinase C sites (Ser/Thr-X-Lys/Arg) and four sites for casein kinase II (Ser/Thr-X-X-Asp/Glu) which occur at identical positions in CaB1, CaB2a, CaB2b and CaB3 (Figure 1).

CaB2a, CaB2b and CaB3 modulate calcium channel activity directed by cardiac α1 expressed in oocytes from Xenopus laevis

To determine the modulatory roles of the novel β subunits cRNAs of cardiac α1 (Singer *et al.*, 1991) and of CaB2a, CaB2b and CaB3 were synthesized *in vitro* and injected into *Xenopus* oocytes. Four or five days after cRNA injection currents were measured using the two electrode voltage clamp method, in a solution containing 40 mM $BaCl_2$ since this results in a larger current through the calcium channel (Figure 2). Coexpression of the three β subunits with cardiac α1 consistently enhanced the expressed currents with CaB3 being more effective in expressing higher currents than CaB2a or CaB2b (Table I). I_{Ba} was potentiated by Bay K 8644 (0.5 μM) in all cases containing the α1 subunit (Table I), but with different efficiency, all β subunit containing

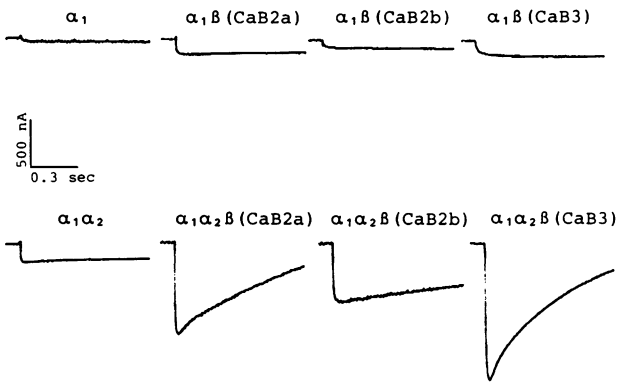


Fig. 2. Properties of I_{Ba} obtained by steps from −100 to 0 mV in oocytes injected with mRNA of the subunit combinations indicated.

combinations being less sensitive. CaB2a and CaB2b alter channel properties conferring acceleration of channel activation kinetics (Table I) with CaB2b being the more effective β subunit type. Combination of the three β subunits and the skeletal muscle α2 caused a more than additive increase in the amplitude of I_{Ba} (Figure 2 and Table I) and the current decay rate (Figure 2), whereas current rise time is similar to the current from α1+α2 alone (Table I).

Tissue specific expression of CaB1, CaB2 and CaB3

The tissue distribution of the mRNA for CaB1, CaB2 and CaB3 was analysed by Northern blot analysis (Figure 3). As noted earlier (Ruth *et al*, 1989; Biel *et al.*, 1991) CaB1-specific transcripts (Figure 3a) of 1.6, 1.9 and 3.0 kb are present in skeletal muscle. The 3.0 kb transcript is also present in brain. In heart, CaB2 (Figure 3b) hybridizes to two major mRNA species of 4.9 and 2.7 kb and to a less abundant 2.2 kb transcript. In aorta, brain, lung and trachea, CaB2 recognizes slightly larger transcripts (3.0 and 5.2 kb) than in heart, yet the third species detected in brain and aorta is the same size as the cardiac 2.7 kb transcript. CaB3 specific transcripts (Fig 3c) are predominantly expressed in brain and to a minor extent in aorta, trachea and lung. In heart and skeletal muscle CaB3 hybridizes to two low abundant 2.7 and 3.0 kb mRNA species. Species of the same size are also present in aorta, trachea and lung. In brain, CaB3 hybridizes to 2.4 and 2.7 kb transcripts.

Characterization of CaB2- and CaB3-specific transcripts expressed in aorta and brain by polymerase chain reactions

In order to demonstrate that the species detected in the Northern blot of aorta and brain mRNA were derived from the same genes as the cDNAs cloned from heart, specific cDNAs were synthesized by polymerase chain reactions using specific primer pairs for CaB2 (primers 1 and 2) and CaB3 (primers 3 and 4) and oligo(dT) primed cDNAs from rabbit aorta and brain as templates. The general localization of the sequences spanned by the primers used is depicted in Figure 4a. Specifically amplified sequences were subcloned and several of the clones obtained were sequenced on both strands (Figure 4b). Clones amplified from aorta cDNA with primers 1 and 2 had an identical sequence to CaB2 cloned from heart. For example, the DNA sequence of rabbit aorta clone 3 is exactly the same as that of CaB2. Using the same primer pair but cDNA from brain yields specific amplification of two similar but not identical classes

Table I. The cardiac β subunits affect the expressed cardiac α_1 subunit

Subunit combination	I_{Ba} (nA)	Increase in I_{Ba} by Bay K (%)	90% ttp[a] (ms)
α_1	−11 ± 2 (31,8)	414 ± 56 (15,8)	43 ± 3 (3,2)
α_1CaB2a	−209 ± 16 (23,6)	138 ± 15 (18,6)	31 ± 4 (23,6)
α_1CaB2b	−69 ± 6 (27,6)	152 ± 10 (15,6)	21 ± 2 (21,6)
α_1CaB3	−343 ± 55 (8,2)	197 ± 32 (5,2)	51 ± 6 (8,2)
$\alpha_1\alpha_2$	−290 ± 31 (38,9)	368 ± 46 (25,7)	19 ± 0.7 (27,9)
$\alpha_1\alpha_2$CaB2a	−2683 ± 287 (26,6)	196 ± 54 (10,6)	17 ± 0.7 (22,6)
$\alpha_1\alpha_2$CaB2b	−889 ± 125 (32,6)	234 ± 30 (14,4)	19 ± 0.8 (27,6)
$\alpha_1\alpha_2$CaB3	−1554 ± 274 (8,2)	147 ± 17 (8,2)	23 ± 2 (8,2)

The current amplitudes were measured at 0 mV in a solution containing 40 mM $BaCl_2$. The entries are mean ± SEM; numbers of oocytes and frogs are indicated in parentheses.
[a]90% ttp, the time by which I_{Ba} reaches 90% of its peak amplitude.

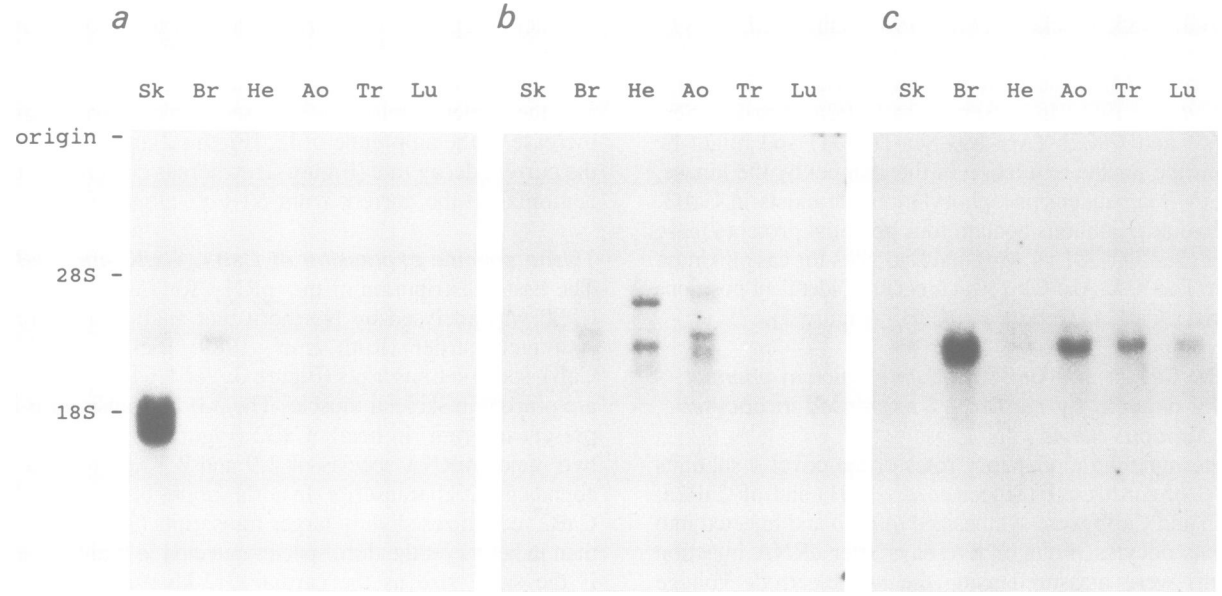


Fig. 3. Autoradiograph of blot hybridization analysis of poly(A)$^+$ RNA from different adult rabbit tissues with CaB1 (**a**), CaB2 (**b**) and CaB3 (**c**) specific probes. Samples of 10 μg poly(A)$^+$ RNA from skeletal muscle (Sk), brain (Br), heart (He), aorta (Ao), trachea (Tr) and lung (Lu) were electrophoresed on 1.2% agarose gels. The size markers used were *E.coli* rRNA and the RNA ladder purchased from Gibco BRL Life Technologies. The specific activities of the three probes used were $0.5-2.4\times10^9$ d.p.m./μg.

of clones. Rabbit brain clone 17 represents one class of clones, which are identical to CaB2 isolated from heart. Sequencing by the dideoxy chain termination method (Sanger *et al.*, 1977) of rabbit brain clone 35, which represents the second class of clones, CaB2c, revealed that it is identical with the sequence of CaB2 with the exception of 114 bp missing at exactly the same position as CaB3 when aligned with CaB2 (Figure 4). In addition it contains a 16 bp segment adjacent to this deletion which differs between the CaB2 sequence and this clone but has 87.5% of residues identical with CaB3. Polymerase chain reactions initiated by primers 3 and 4 with cDNAs from aorta and brain yielded specific amplification products in all cases. The sequences of the clones obtained from aorta (clone 22) and brain (clone 18) were identical to that of CaB3. These results, in combination with the results from the Northern blots, provide strong evidence that the CaB2 and CaB3 mRNAs are expressed in heart, brain and aorta.

The present study demonstrates by molecular cloning the existence of calcium channel β subunits in cardiac muscle, aorta and brain and that these subunits are derived from at least two distinct genes, CaB2 and CaB3. Like the skeletal muscle protein (Lacerda *et al.*, 1991; Mori *et al.*, 1991; Singer *et al.*, 1991; Varadi *et al.*, 1991) these subunits modulate channel activity directed by the cardiac $\alpha1$ subunit alone, suggesting a fundamental structural relatedness of the oligomeric calcium channel complexes present in these tissues and in skeletal muscle. This modulatory effect is unique to each subunit and may be due to the extent of protein expression in the oocyte and/or linkage to the cardiac $\alpha1$. This is in accordance with the finding that CaB1, CaB2 and CaB3 mRNAs are expressed tissue-specifically and might interact with different types of $\alpha1$ subunits. In fact, distinct molecular species of $\alpha1$ subunits have been identified in these tissues (Biel *et al.*, 1990, 1991; Koch *et al.*, 1990; Mikami *et al.*, 1989; Mori *et al.*, 1991). Thus different types of calcium currents observed in excitable tissues and their distinct regulation by guanine nucleotide-binding proteins and phosphorylation may depend on channel composition.

Materials and methods

cDNA library

A directional, oligo(dT) primed cDNA library was constructed using RNase H-free Moloney murine leukaemia virus reverse transcriptase and 10 μg of poly(A)$^+$ RNA from rabbit heart. 22.5 ng of the size selected (>2 kb) cDNA from heart was cloned into pSPORT1 (Gibco BRL Life Technologies). After electroporation in *Escherichia coli* XL1-blue (Stratagene) $\sim3\times10^5$ clones were obtained. Screening with radiolabelled cDNA fragments covering the complete coding region of the skeletal muscle β subunit (Ruth *et al.*, 1989) yielded nine positive clones, which could be divided into three classes (CaB2a, CaB2b and CaB3) by restriction enzyme mapping: (i) CaB2a, clones pBH19, 20, 22, 13, 5, 7; (ii) CaB2b, clones pBH 27, 17; and (iii) CaB3, clone pBH23. Recombinant DNA was propagated in B1 and B2 host vector systems under S1 containment conditions according to German law. The cDNAs obtained were sequenced on both strands.

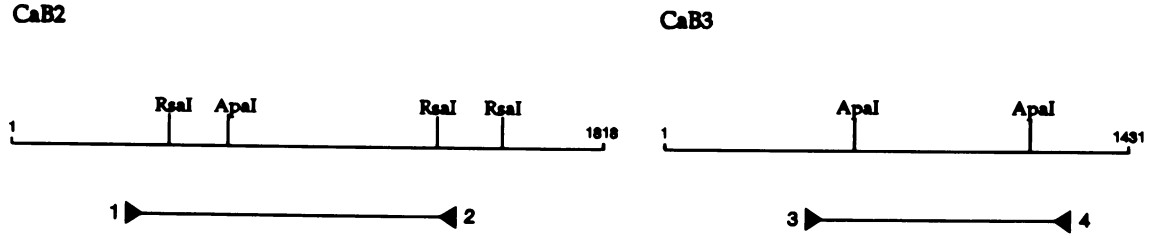
In vitro transcription and expression of RNA in Xenopus oocytes

RNAs specific for the cardiac $\alpha1$ and the skeletal muscle $\alpha2$ subunits were synthesized *in vitro* using Asp718-cleaved pCaH (Biel *et al.*, 1991; Singer *et al.*, 1991) and *Sal*I-cleaved pCaA2 (Singer *et al.*, 1991) as templates. Capped β-specific *in vitro* RNA was synthesized using T7 RNA polymerase from clone pBH17 (CaC2b), pBH19 (CaB2a) and pBH23 (CaB3) after linearizing with *Not*I. Translation was optimized by removing the total 5′ untranslated sequence from pBH23 and replacing it with the sequence TCGACGGTACCGCCGCCACC, which includes the consensus sequence for initiation of translation in vertebrates (Kozak, 1987). In each experiment, 20–30 defolliculated oocytes were injected with $\alpha1$ cRNA, $\alpha1+\beta$ cRNAs or $\alpha1+\alpha2+\beta$ cRNAs at 1:1 and 1:1:1 stoichiometry, respectively (3.3–5 ng of each subunit at a concentration of 0.5 mg/ml). After 3 or 4 days of incubation at 22°C the oocytes were injected with $\sim$200 pmol EGTA 1 day before testing the currents. I_{Ba} was measured at 22°C in 40 mM Ba^{2+}, 2 mM K^+, 60 mM Na^+ and 5 mM HEPES (pH 7.4). The two-electrode voltage clamp, data aquisition and subtraction procedures were as described by Singer *et al.* (1991).

Northern blot analysis

Rabbit poly(A)$^+$ RNA was separated on 1.2% agarose gels in 10 mM sodium phosphate (pH 7.4), transferred to Biodyne nylon membranes (Pall)

a



b

```
rabbit heart CaB2:        GTACCTAGTTCCAGAAAATCTACACCTCCATCATCTGCTATAGACATAGATGCTACTGGCTTAGATGCAGAAGAAAATGATATTCCAGCAAACCACCGCTCCCCT
rabbit aorta clone  3:  <----------------------------------------------------------------------------------------------------
rabbit brain clone 17:  <----------------------------------------------------------------------------------------------------
rabbit brain clone 35:  <-----------------------------------------AGC-G-AGC-G-------------------------------------------------
rabbit heart CaB3:        ------G-CAA-C--CGC--CC-T--------TCTA--C-AGC-G-AGC-A-------------------------------------------------
rabbit aorta clone 22
rabbit brain clone 18
CaB2 (deduced aa seq.):   V  P  S  S  R  K  S  T  P  P  S  S  A  I  D  I  D  A  T  G  L  D  A  E  E  N  D  I  P  A  N  H  R  S  P
CaB2c (deduced aa seq.):                                    *  K  Q  K  Q
CaB3 (deduced aa seq.):   -------  G  N  -  R  -  P  -  -  -  L  -  K  Q  K  Q  -------------------------------------------------

CaB2:  AAACCCAGTGCAAACAGTGTAACGTCACCCCACTCCAAAGAGAAAAGAATGCCCTTCTTTAAGAAGACAGAGCACACTCCTCCGTATGATGTGGTGCCCTCCATGCGACCCGTGGTCCTCGTG
cl  3: ----------------------------------------------------------------------------------------------------------------------
cl 17: ----------------------------------------------------------------------------------------------------------------------
cl 35: ----------------------------------------------------------------------ATC-----------------------------------------------
CaB3:  ----------------------------------------------------------------------C--G----A---GT---C-----C--------------------G--T-----G--G---
cl 22:
cl 18:
CaB2:  K  P  S  A  N  S  V  T  S  P  H  S  K  E  K  R  M  P  F  F  K  K  T  E  H  T  P  P  Y  D  V  V  P  S  M  R  P  V  V  L  V
CaB2c:                                                          -  S  *
CaB3:  ----------------------------------------------------------  Q  A  -  -  V  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -

CaB2:  GGCCCTTCCCTGAAGGGGTATGAGGTCACAGATATGATGCAGAAAGCGCTGTTTGATTTTTTAAAACACAGATTTGAAGGGCGGATATCCATCACAAGGGTCACTGCTGATATCTCGCTTGCC
cl  3: ----------------------------------------------------------------------------------------------------------------------
cl 17: ----------------------------------------------------------------------------------------------------------------------
cl 35: ----------------------------------------------------------------------------------------------------------------------
CaB3:  --G--C--------A--C--C----------C--------A--G--C--C--C--C--CC-C-----T--G--C--T--CA----C--------CC-T-----C-----CC----C--G---
cl 22:<--G--C--------A--C--C----------C--------A--G--C--C--C--C--CC-C-----T--G--C--T--CA----C--------CC-T-----C-----CC----C--G---
cl 18:<--G--C--------A--C--C----------C--------A--G--C--C--C--C--CC-C-----T--G--C--T--CA----C--------CC-T-----C-----CC----C--G---
CaB2:   G  P  S  L  K  G  Y  E  V  T  D  M  M  Q  K  A  L  F  D  F  L  K  H  R  F  E  G  R  I  S  I  T  R  V  T  A  D  I  S  L  A
CaB3:   -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  D  -  -  -  -  -  -  -  -  -  -  -  L  -  -  -

CaB2:  AAACGCTCAGTGTTAAACAACCCCAGTAAGCATGCAATAATAGAAAGATCCAACACTAGGTCAAGCTTAGCGGAAGTTCAGAGTGAAATTGAAAGGATCTTTGAACTTGCAAGGACACTGCAG
cl  3: ----------------------------------------------------------------------------------------------------------------------
cl 17: ----------------------------------------------------------------------------------------------------------------------
cl 35: ----------------------------------------------------------------------------------------------------------------------
CaB3:  --G--A--C---C-C-----T--TG-C---AGGA-C--C--T--GC-C---TCTG-CC-C--C---A-C--C-----A--------G-----GC-C--T--C--G--G--C-AAT-C------
cl 22: --G--A--C---C-C-----T--TG-C---AGGA-C--C--T--GC-C---TCTG-CC-C--C---A-C--C-----A--------G-----GC-C--T--C--G--G--C-AAT-C------
cl 18: --G--A--C---C-C-----T--TG-C---AGGA-C--C--T--GC-C---TCTG-CC-C--C---A-C--C-----A--------G-----GC-C--T--C--G--G--C-AAT-C------
CaB2:   K  R  S  V  L  N  N  P  S  K  H  A  I  I  E  R  S  N  T  R  S  S  L  A  E  V  Q  S  E  I  E  R  I  F  E  L  A  R  T  L  Q
CaB3:   -  -  -  -  -  -  -  -  G  -  R  T  -  -  -  -  -  S  A  -  -  -  I  -  -  -  -  -  -  -  -  -  -  -  -  -  -  K  S  -  -

CaB2:  TTGGTAGTCCTTGATGCGGATACTATTAACCATCCAGCTCAACTTAGTAAAACCTCTTTGGCCCCTATTGTAGTGTATGTGAAGATTTCTTCTCCTAAGGTTTTACAAAGGCTAATAAAATCT
cl  3: ----------------------------------------------------------------------------------------------------------------------
cl 17: ----------------------------------------------------------------------------------------------------------------------
cl 35: ----------------------------------------------------------------------------------------------------------------------
CaB3:  C----G--G--G-----C--C--C--C-----C-----A--G--GGCC--G-----GC----T--C--CA-C--C-T---C--AG-C-----A--G-----CC-C---C-T--C--CCGC--C
cl 22: C----G--G--G-----C--C--C--C-----C-----A--G--GGCC--G-----GC----T--C--CA-C--C-T---C--AG-C-----A--G-----CC-C---C-T--C--CCGC--C
cl 18: C----G--G--G-----C--C--C--C-----C-----A--G--GGCC--G-----GC----T--C--CA-C--C-T---C--AG-C-----A--G-----CC-C---C-T--C--CCGC--C
CaB2:   L  V  V  L  D  A  D  T  I  N  H  P  A  Q  L  S  K  T  S  L  A  P  I  V  V  Y  V  K  I  S  S  P  K  V  L  Q  R  L  I  K  S
CaB3:   -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  A  -  -  -  -  -  -  -  I  -  F  -  -  V  -  -  -  -  -  -  -  -  -  -  R  -

CaB2:  CGAGGGAAATCTCAAGCTAAACACCTTAATGTCCAGATGGTAGCAGCTGATAAACTGGCTCAGTGTCCTCCAGAATTGTTTGATGTGATCTTAGATGAGAACCAGCTTGAGGATGCTTGTGAG
cl  3: ----------------------------------------------------------------------------------------------------------------------
cl 17: ----------------------------------------------------------------------------------------------------------------------
cl 35: ----------------------------------------------------------------------------------------------------------------------
CaB3:  --G--A--G--A--GATG--G-----G-CA--A--A---A-G--GTA---C--G----TG--------A-----G-CA-----------C-G-------------G--A-----C-----A
cl 22: --G--A--G--A--GATG--G-----G-CA--A--A---A-G--GTA---C--G----TG--------A-----G-CA-----------C-G-------------G--A-----C-----A
cl 18: --G--A--G--A--GATG--G-----G-CA--A--A---A-G--GTA---C--G----TG--------A-----G-CA-----------C-G-------------G--A-----C-----A
CaB2:   R  G  K  S  Q  A  K  H  L  N  V  Q  M  V  A  A  D  K  L  A  Q  C  P  P  E  L  F  D  V  I  L  D  E  N  Q  L  E  D  A  C  E
CaB3:   -  -  -  -  -  M  -  -  -  T  -  -  -  M  -  Y  -  -  -  V  -  -  -  -  -  S  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -

CaB2:  CATCTTGCCGACTATCTGGAGGCCTACTGGAAGGCCACCCATCCTCCCAGCAGCAATCTCCCCAACCCTCTCCTTAGCCGTAC
cl  3: ---------------------------------------------------------------------------------->
cl 17: ---------------------------------------------------------------------------------->
cl 35: ---------------------------------------------------------------------------------->
CaB3:  --CT-G-----G--C--C----TG------CG---------C-AC--AGC-CC-GGG-CTGGG-------------GG---CCCC
cl 22: --CT-G-----G--C--C----TG------CG---------C-AC--AGC-CC-GGG-CTGGG-------------GG---C>
cl 18: --CT-G-----G--C--C----TG------CG---------C-AC--AGC-CC-GGG-CTGGG-------------GG---C>
CaB2:   H  L  A  D  Y  L  E  A  Y  W  K  A  T  H  P  P  S  S  N  L  P  N  P  L  L  S  R
CaB3:   -  -  -  E  -  -  -  V  -  -  R  -  -  -  H  -  A  P  G  P  G  -------  -  -  G  P
```

Key:

---, identity
___, gap
< >, limits of available sequence
* , identical with CaB2

all sequences are compared to that of the cardiac CaB2 β subunit type

Fig. 4. Amplified regions of CaB2 and CaB3 (**a**) and nucleotide and amino acid sequence alignment of cardiac CaB2 and CaB3 and PCR products derived from rabbit aorta and brain (**b**) (the nucleotide sequences to be deposited to the EMBL Data Library). (a) Representation of the coding regions of CaB2 and CaB3 including recognition sites for *RsaI* (CaB2) and *ApaI* (CaB3). Triangles indicate relative positions of oligodeoxynucleotide primers 1–4 used for PCR. Numbers refer to nucleotide sequences of CaB2a (CaB2) and CaB3. Primers 1 and 2 cover nucleotide sequences which are identical in CaB2a and CaB2b. (b) Results of PCR using specific primers derived from CaB2 (1 and 2) and CaB3 (3 and 4). The nucleotide sequences obtained are compared with those of the cardiac CaB2 and CaB3.

by diffusion and UV crosslinked to the filters. Northern blots were prehybridized for 1 h at 42°C in 50% formamide, 1×PE (50 mM Tris−HCl, pH 7.55, 0.1% sodium diphosphate, 1% SDS, 0.2% polyvinylpyrolidone, 0.2% Ficoll, 5 mM EDTA), 5×SSC and 150 μg/ml denatured salmon sperm DNA. Blots were hybridized with ^{32}P-labelled probes for 20 h at 42°C (DNA probes) and 65°C (RNA probe). Specific probes used for CaB1: an antisense RNA probe was synthesized *in vitro* with [α-^{32}P]GTP using the linearized recombinant plasmid pCaB1a (Biel *et al.*, 1991) as template for transcription with SP6 polymerase. CaB2: the *AluI*(210)−*AluI*(888) fragment from pBH19. CaB3: the *SmaI*(920)−*SmaI* (1096) fragment from pBH23. Both cDNA fragments were labelled by random priming. Blots were washed several times in 1×SSC, 0.1% SDS followed by 0.1×SSC, 0.1% SDS at 65°C.

Polymerase chain reactions

10 ng of oligo(dT) primed cDNA from aorta (reactions 1 and 2) and brain (reactions 3 and 4) and 50 pmol of the oligodeoxynucleotide primers 1 and 2 (reactions 1 and 3) and 3 and 4 (reactions 2 and 4), respectively, were incubated in 100 μl of 10 mM Tris−HCl, pH 8.3 containing 50 mM KCl, 1.5 mM $MgCl_2$, 100 μg/ml gelatin, dATP, dCTP, dGTP and TTP 0.2 mM each, and 2.5 U *Taq* polymerase. The mixture was incubated in a thermocycler for 40 cycles: 1 min at 94°C, 1 min at 50°C, and 1.5 min at 72°C and 8 min at 72°C in the last cycle, respectively. The gel resolved DNA products were cut with *RsaI* (reactions 1 and 3) and *ApaI* (reactions 2 and 4), subcloned and sequenced on both strands. Sequences of primers were as follows: 1, ACAGAGAGCAAAGCAAGGGAAAT, corresponding to nucleotides 399−421 of CaB2a (477−499 of CaB2b); 2, TCTCCTTG-AGAACCCTGTGAATT, complementary to nucleotides 1318−1340 of CaB2a (1396−1418 of CaB2b); 3, CGACGCTCCCCTCCTCCATCTC-TA, corresponding to nucleotides 448−471 of CaB3; 4, GTCCAGGCC-TCGTCTGAGGGCAT complementary to nucleotides 1201−1223 of CaB3.

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The sequence data reported here have been submitted to the EMBL/GenBank/DDBJ nucleotide sequence databases and are available under accession numbers X64297 (CaB2a), X64298 (CaB2b), X64299 (CaB2c) and X64300 (CaB3).