Genomic Structure and Functional Expression of a Human $\alpha_2/\delta$ Calcium Channel Subunit Gene (CACNA2)

Lothar Schleithoff, Gerhard Mehrke, Bettina Reutlinger, and Frank Lehmann-Horn

Abteilung für Angewandte Physiologie, Universität Ulm, Ulm, Germany

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CACNA2 encodes the $\alpha_2/\delta$ subunit of the human voltage-gated calcium channels and is located in the candidate region of malignant hyperthermia susceptibility type 3 (MHS3). We determined the structural organization of CACNA2 by isolation of overlapping genomic DNA clones from a human phage library. The gene consists of at least 40 exons, 2 of which are alternatively spliced, spanning more than 150 kb of genomic DNA. Exons range from 21 to 159 bp, and introns range from 98 bp to at least more than 20 kb. We constructed a full-length cDNA and cloned it into a mammalian expression vector. Cotransfection of the CACNA2 cDNA with $\alpha_{1A}$ and $\beta_4$ cDNA into HEK293 cells led to the expression of Q-type calcium currents. The $\alpha_2/\delta$ subunit enhanced the current density 18-fold compared to cells transfected with only $\alpha_{1A}$ and $\beta_4$ cDNA. The sequence analysis provides the basis for comprehensive mutation screening of CACNA2 for putative MHS3 individuals and patients with other channelopathies. © 1999 Academic Press

INTRODUCTION

Voltage-gated calcium channels regulate the entry of extracellular $\text{Ca}^{2+}$ ions into the cytoplasm where they participate in a variety of calcium-dependent processes including transmitter release, muscle contraction, and secretory processes (Berridge, 1997). Multiple gene families encoding the various subunits have been identified (Hofmann et al., 1994). They consist of five distinct polypeptides, $\alpha_1$, $\alpha_2/\delta$, (or briefly $\alpha_2$ as the two subunits are encoded by the same gene), $\beta$, and $\gamma$ (Nastainczyk et al., 1990). For each polypeptide, several isoforms have been identified. All $\alpha_1$ isoforms (e.g., $\alpha_{1A}$, brain; $\alpha_{1C}$, heart; $\alpha_{1S}$, skeletal muscle) are channels as they possess an ion-conducting pore, whereas all others are auxiliary subunits ($\alpha_2$, $\beta_1$–$\beta_4$, and $\gamma$). For $\alpha_2$, two splice variants have been reported, $\alpha_{2A}$ (skeletal muscle, aorta smooth muscle) and $\alpha_{2B}$ (brain) (Greenberg, 1997).

The best characterized calcium channels are the dihydropyridine sensitive L-type calcium channels of heart ($\alpha_{1C}$, $\alpha_{2A}$, $\beta_1$) and skeletal muscle consisting of $\alpha_{1S}$, $\alpha_{2A}$, $\beta_1$, and $\gamma$ and the P/Q-type calcium channel of brain that is composed of $\alpha_{2A}$, $\alpha_{2B}$, $\beta_4$, and possibly stargazin as $\gamma_2$ (Ellis et al., 1998; Letts et al., 1998). Each subunit is encoded by a separate gene, with the exception of the $\alpha_2/\delta$ primary transcript, with the $\delta$ subunit arising as a result of proteolytic cleavage of the C-terminal end of the $\alpha_2/\delta$ polypeptide (De Jongh et al., 1990). The extracellularly located $\alpha_2$ protein is anchored by disulfide bonds to the $\delta$ subunit that contains a single transmembrane domain (Jay et al., 1991; Felix et al., 1997). Although only a single gene for the $\alpha_2/\delta$ subunit has been identified, cDNA cloning and PCR analysis have revealed different splice variants expressed in various tissues. In humans the brain expresses $\alpha_{2B}$, which differs only by an insertion of 7 amino acids and a deletion of a 19-amino-acid segment between the first and the second hydrophobic domains (Kim et al., 1992) [CACNA2; for nomenclature see Lory et al. (1997)]. The gene has been mapped to chromosome 7q11.23–q21.1 between the polymorphic markers D7S524 and D7S524. Previously, susceptibility to malignant hyperthermia (MH), an anesthesia-related crisis, has been linked in a large German pedigree to D7S549 also flanked by D7S675 and D7S524. Physical mapping places D7S549 within 110–380 kb of CACNA2 (Iles et al., 1994). This type of MH susceptibility is named MHS-3 (McKusick, 1997).

Malignant hyperthermia is an autosomal dominant, pharmacogenetic disorder of skeletal muscle that manifests itself as a potentially lethal crisis triggered by volatile anesthetics and depolarizing muscle relaxants. A pathologically high increase of the myoplasmic calcium concentration during exposure to the triggering agents (Iaizzo et al., 1988) underlies the MH susceptibility (MHS), resulting in muscle contracture and hy-
permetabolism with subsequent acidosis, hypoxia, hypercapnia, and hyperkalemia. In skeletal muscle, calcium release is initiated by a depolarization of the transverse tubular membrane and by a fast conformational change of intracellular loops of the α1 subunit of the voltage-gated L-type calcium channel that opens the ryanodine receptor (RYR1), which is situated in the membrane of the sarcoplasmic reticulum (SR). This signal transmission between tubular and SR membrane is referred to as excitation–contraction coupling (Catterall, 1991). MHS-causing mutations have been identified in RYR1, the calcium release channel of the SR [MHS-1 (Manning et al., 1998)], as well as in the α1s subunit of the L-type calcium channel [MHS-5 (Monnier et al., 1997)]. The α2 subunit of this L-type calcium channel seems to be a reasonable candidate as a carrier of MHS-causing mutations.

To provide the basis for a mutation screening, we determined the complete structural organization of the human CACNA2 gene. We further constructed a full-length cDNA for functional coexpression of the human (skeletal muscle) α2 cDNA and show that this construct enhances functional expression of calcium channels. Functional expression of a human (skeletal muscle) α2 cDNA has not been reported yet. However, expression of a rabbit skeletal muscle α2 cDNA (and an accessory β cDNA) resulted in an increase of the α1c calcium current. The rabbit α2 subunit also accelerated inactivation kinetics and shifted the steady-state inactivation and activation curves in the hyperpolarizing direction (Singer et al., 1991; De Waard and Campbell, 1995). Since α1s, the skeletal muscle isoform, and its accessory subunits βs and α2 cannot be properly expressed in nonmuscular cell systems, we have chosen a brain-specific form of calcium channel consisting of α1A and βs subunits with the here described first coexpression of the full-length transcript of CACNA2 that encodes the human α2δβ subunit, α2δ.

MATERIALS AND METHODS

Genomic library screening. To prepare a genomic library, DNA from human whole blood cells partially digested with Sau3AI was cloned into the BamHI site of Lambda GET (Nehls et al., 1994). Vector arms were purified by sucrose gradient centrifugation after digestion with XhoI and BamHI to eliminate stuffer contamination of the library (Sambrook et al., 1989). Genomic DNA fragments were also size selected prior to cloning. Fragments were ligated to vector arms and packaged in vitro using Gigapack Gold extracts (Stratagene) and plated on Escherichia coli C600 bacteria.

Plaques were transferred to nylon filters and screened with a full-length DNA probe generated from CACNA2 cDNA. Screening was performed by a standard hybridization technique using cDNA probes radiolabeled by [α-32P]dCTP with a random-primed DNA labeling kit (Pharmacia) according to the manufacturer’s instructions.

Plasmid subcloning. After three rounds of plaque purification, phage DNA of positive clones was converted to plasmid by infecting E. coli strain BNN 132 (Elledge et al., 1991), which expresses cre-recombinase. The plasmid was purified using the Qiagen plasmid kit (Qiagen Inc.). To determine the insert size, the plasmid versions of Lambda GET recombinants were cut with KpnI, which flanks the sites and releases the 4.5-kb plasmid backbone.

Exon mapping by PCR. To determine the distance between exons, genomic DNA and positive clones containing part of the CACNA2 locus were used as templates for long PCR amplification. To obtain the long PCR fragments required, Taq extender (Stratagene) and the Expand Long Template PCR system (Boehringer Mannheim) were used. The standard PCR mix in a final volume of 50 μl consisted of 50 ng DNA, 30 pmol of each primer, based on known cDNA sequences, 500 μM dNTPs, 1× Taq buffer, and 2 to 4 units of Taq polymerase. The amplification protocol consisted of 30 cycles at 94°C for 45 s, 58°C for 45 s, and 72°C for 10–15 min.

DNA sequencing. Double-stranded sequencing of plasmid DNA was carried out using the dideoxy nucleotide method and performed according to the instructions specified in the ALFexpress Auto read sequencing kit (Pharmacia) on an ALFexpress sequencer.

Construction and transfection of the expression plasmids. Using two primers, a 1-kb fragment of CACNA2 was amplified from a human skeletal muscle tissue cDNA. This fragment was used as a probe for the screening of a human skeletal muscle cDNA library (Clontech). Screening was performed as described above. The inserts of positive phages were cut out with EcoRI and subcloned into pT7T3 19U (Pharmacia). Identification of inserts was checked by sequencing with vector primers flanking the cloning site. A BamHI/AWI-NI fragment of one large clone that contained the full-length human α2 cDNA was subcloned into the BamHI/EcoRV site of the pcDNA3 vector (Invitrogen) after blunt ending of the AlwNI site. HEK293 cells were transfected with α2, α1s, and βs cDNA using the standard calcium phosphate precipitation technique. The cells were grown in MEM, supplemented with 10% fetal calf serum (Gibco BRL).

Electrophysiological recordings and data analysis. Ca2+ and Ba2+ currents were measured using the whole-cell mode of the patch clamp method at room temperature. For data acquisition, an Axodamp 2000 amplifier (Axon Instruments, USA) connected to a PC equipped with the Pclamp6 acquisition package (Axon Instruments) was used. The extracellular solution contained (in mM): CsCl, 112; N-methyl-D-glucamine chloride, 104; MgCl2, 1; glucose, 5; Hepes, 10. The pH was adjusted to 7.4 using HCl. The patch pipettes were filled with an internal solution containing (in mM): CsCl, 112; MgCl2, 1; Hepes, 5; MgATP, 1; EGTA, 10 (pH 7.4, adjusted with CsOH). Recordings were filtered at 3 kHz using a 4-pole Bessel filter and digitized at 40 kHz. Holding potentials of –100 mV were used to record ionic currents throughout the study. Steady-state inactivation of ionic currents was measured by 3-s prepulses and applying the depolarizing protocol from the same membrane potential (–100 mV) each time. Recordings were capacity-corrected. From the holding potential, four scaled hyperpolarizing prepulses were given, and the sum of the prepulses was subtracted from each recording.

Statistical analysis and curve fitting were carried out using the Pclamp6 software package, Origin (Microcal, Northampton, MA), or SigmaPlot (Jandel). All grouped data are reported as mean ± standard error of the mean (SEM).

RESULTS

Exon/Intron Structure

To define the genomic structure of CACNA2, a genomic library of human blood cell DNA constructed in Lambda GET was screened with different fragments from the CACNA2 cDNA. About 2 x 105 clones were screened, and approximately 200 independent clones were analyzed. Positive clones containing part of CACNA2 were converted to plasmids by infecting E. coli strain BNN132, which expresses cre-recombinase (Elledge et al., 1991). Exon/intron boundaries were determined by sequencing plasmids using oligonucleo-


### TABLE 1

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*Note: Exon sequences are indicated as uppercase letters, and intron sequences are in lowercase letters.

*Approximate length was determined by PCR and restriction digestion mapping.

The genomic sequences corresponding to the 3' end of exons were determined by intron spanning primers. In some cases the genomic sequence can be alternatively spliced (Kim et al., 1992). We have included both in our gene structure map as exons 19 (57 bp) and 24 (21 bp). One of the alternatively spliced exons was not accounted for in the original human CACNA2 cDNA (Williams et al., 1992). We have added these 19 amino acids, which are encoded by exon 19 and are inserted after Lys-510 only in the skeletal muscle splice product. The sizes of intervening introns were determined either by restriction enzyme digestion mapping or by PCR amplification of genomic DNA clones using intron spanning primers. In some cases

The ATG start codon and the signal peptide are included in exon 1. Exon 40 contains the termination codon followed by 494 bp of the 3' untruncated region where no adenylation signal was found. Exons 37-40 encode the putative proteolipid, which is proteolytically cleaved from the protein. The membrane-spanning part of the proteolipid is encoded by a single exon (exon 40). The CACNA2 gene contains two sequences that can be alternatively spliced (Kim et al., 1992). In our analysis we found that both were distinct exons (see Fig. 1b). We have included them both in our gene structure map as exons 19 (57 bp) and 24 (21 bp). One of the alternatively spliced exons was not accounted for in the original human CACNA2 cDNA (Williams et al., 1992). We have added these 19 amino acids, which are encoded by exon 19 and are inserted after Lys-510 only in the skeletal muscle splice product.
FIG. 1. (A) Genomic organization of the human CACN2A gene. The location of exons is shown with respect to the size of the introns. The exact sizes of the first and last exons are not known. Position of exons is shown by numbered filled boxes. Open rectangles indicate alternative spliced exons 19 and 24. Hatched boxes represent partially sequenced exons containing 5' and 3' untranslated sequences. Heavy lines represent the relative position of studied genomic phage clones (GET). (B) (Top) Localization of the two differentially spliced exons. (Bottom) cDNA sequence and deduced amino acid sequence of the two exons.
intron length could not be detected due to extended size.

Screening for Mutations and Isolation of cDNA

Knowledge of the intron sequences flanking the exons enabled us to develop primer pairs for amplification of exons from genomic DNA from patients of the MHS family linked to the above-mentioned region on chromosome 7q (Iles et al., 1994). We analyzed both the DNA of the patient who had suffered from the MH crisis and that of additional affected members of this family. However, so far no mutation has been detected in CACNA2. Sequence analysis revealed neither differences in the coding region of the gene nor differences in sequences flanking exon/intron boundaries. We could not detect any sequence alteration in the first 300 bases of the putative promoter region. The complete promoter region remains to be analyzed, and the possibility of an intrinsic mutation must be considered. Our sequence analysis identified errors in the published (Accession No. M76559) human CACNA2 cDNA (these are corrected in Table 2), which altered amino acid residues but did not affect numbering. Furthermore, a silent mutation at nucleotide position 1038 (a T to C transition in exon 39) was found that did not segregate with the MH phenotype in the family. This nucleotide transition affects the third base of a proline codon. The change eliminates a BfaI site and can be used as an intragenic polymorphism. It can easily be detected by BfaI digestion of the PCR product obtained from amplification of exon 39 with flanking primers.

Electrophysiological Characterization of the Gene Product

For expression studies we inserted the full-length muscle CACNA2 cDNA into the pcDNA3 vector. The human skeletal muscle \( \alpha_2 \) cDNA (human \( \alpha_{2A} \)) when coexpressed in HEK293 cells with the brain-specific \( \alpha_{1A} \) subunit did not lead to measurable currents. Yet the addition of \( \beta_2 \) subunits led to quite large ion currents of greater than 2 nA (Fig. 2). The mean current density was 65 pA/pF \( \pm 18 \) (n = 12). For comparison, the combination of \( \alpha_{1A}, \beta_4 \), and an \( \alpha_2 \) from rabbit brain \( (= \alpha_{2B}) \) resulted in a significantly lower current density of 41 pA/pF \( \pm 9 \) (n = 32). In contrast, after transfection of the cells with the cDNA of the \( \alpha_{1A} \) and \( \beta \) subunit without an \( \alpha_2 \) subunit the mean current density amounted to 3.4 pA/pF, and the expression efficiency was low: less than 10% of the transfected cells showed calcium currents when tested. Coexpression of \( \alpha_{1A}, \beta_4 \), and \( \alpha_{2A} \) subunits raised the expression rate to about 25%.

The \( \alpha_{2A} \) subunits influenced the voltage dependence of activation and inactivation. They induced a shift in hyperpolarizing direction in activation (7 mV) and inactivation (4 mV) (see Table 3; Fig. 3), but the change in current inactivation was not statistically significant. We further looked at the gating-related channel characteristics. The addition of the \( \alpha_{2A} \) subunits accelerated the inactivation kinetics. To analyze this action in detail, we estimated the time course of the voltage-dependent inactivation of the current during a 2-s activating pulse to \(+20\) mV by fitting the decaying current with a 2-exponential equation. A significant difference was seen on the slow component of the decay. \( \alpha_{2A} \) reduced the time constant \( \tau_2 \) by 34% (Table 3).

### DISCUSSION

A growing number of diseases are recognized as being caused by mutations in genes encoding voltage-gated ion channels of skeletal muscle (Hoffman et al., 1995). Naturally occurring mutations in channel genes or antibodies directed against channel proteins have also been found for other excitable tissues such as the heart and the brain as well as unexcitable organs such as the kidney (Greenberg, 1997; Lehmann-Horn and Jurkat-Rott, 1999). Mutations in calcium channels or channel subunits cause malignant hyperthermia susceptibility types 1 and 5 (Monnier et al., 1997; Manning et al., 1998), hypokalemic periodic paralysis, and various forms of hemiplegic migraine, episodic ataxias, and epilepsies (Jurkat-Rott et al., 1994; Ptacek et al., 1994; Terwindt et al., 1998).

In a large German pedigree, malignant hyperthermia susceptibility (MHS type 3) was found to be tightly linked to the gene on chromosome 7q encoding the \( \alpha_{2/\delta} \) subunit of voltage-gated calcium channels (Iles et al., 1994; McKusick, 1997). To perform a mutation screening with MHS susceptible individuals, we performed a detailed analysis of the sequence and genomic structure of the CACNA2 gene. Yet, in the MH patients we tested up to now, we did not identify a mutation in the coding region of CACNA2 or in sequences flanking the exon/intron boundaries. Still, the promoter region remains to be analyzed, and the possibility of an intrinsic mutation must be considered. Independent of the molecular genetic results for this family, CACNA2 remains a prime candidate for MH for susceptible individuals of other families that show no mutation in the RYR1 or the calcium channel \( \alpha_1 \) subunit of skeletal muscle. The genomic structure reported here allows for improved screening for mutations in those cases of MHS.
other diseases that are caused by an altered cell excitability, particularly where no mRNA will be available.

The expression studies were intended to prove the functional integrity of our isolated gene product and to test for the modifying capabilities of the $\alpha_{2A}$ subunit on calcium currents. The skeletal muscle variant of the voltage-gated calcium channel consisting of the pore-forming subunit $\alpha_{1S}$ cannot be properly expressed in heterologous expression systems. Therefore we used the combination of our isolated form $\alpha_{2A}$ with the brain-specific type $\alpha_{1A}$ and $\beta_4$ subunits for our expression studies, thus forming P/Q-type calcium channels. The physiological properties of the channels (P- or Q-type) hereby are determined by the type of the auxiliary subunits and the type of splice variant of the $\alpha_{1A}$ subunit. With our combination we obtained currents that were similar to the Q-type. The characterization of the $\alpha_2$ influence on this brain-specific type of calcium channel may contribute to an understanding of how aberrant $\alpha_2$ subunits may induce neurological disorders.

The most obvious manifestation of the interaction of $\alpha_2$ with $\alpha_{1A}$ subunits was the expression of high-amplitude currents in the transfected cells. Even though the $\alpha_{1A}$ subunits theoretically should be able

<table>
<thead>
<tr>
<th>Properties</th>
<th>$\alpha_{1A}/\beta_4$</th>
<th>$\alpha_{2A}/\beta_4/\alpha_{2A}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peak current (pA)</td>
<td>$65 \pm 18$ (16)</td>
<td>$810 \pm 78$ (12)*</td>
</tr>
<tr>
<td>Potential for half-activation (mV)</td>
<td>$19 \pm 2$ (18)</td>
<td>$11 \pm 0.9$ (32)*</td>
</tr>
<tr>
<td>Potential for steady-state half-inactivation (mV)</td>
<td>$-4 \pm 5$ (6)</td>
<td>$-8 \pm 1.2$ (13)</td>
</tr>
<tr>
<td>Time constant of decay $\tau_1$ (ms)</td>
<td>$244 \pm 38$ (6)</td>
<td>$217 \pm 30$ (12)</td>
</tr>
<tr>
<td>Time constant of decay $\tau_2$ (ms)</td>
<td>$2640 \pm 190$ (6)</td>
<td>$1750 \pm 157$ (12)*</td>
</tr>
</tbody>
</table>

Note. Numbers of experiments in parentheses.

* Significant differences ($P < 0.05$).
FIG. 3. Effects of $\alpha_{2A}$ subunits on currents through $\alpha_{1A}$-type channels. (A) Currents evoked by a depolarizing pulse to +20 mV (upper trace) of a cell transfected with $\alpha_{1A}$, $\beta_4$ (middle trace) and $\alpha_{1A}$, $\beta_4$ and $\alpha_{2A}$ (lower trace). (B) Steady-state inactivation. The steady-state inactivation was determined by examining the amplitude of the current elicited by a test pulse to 20 mV after prepulses to various potentials. The data were fitted by a Boltzmann function: $\frac{I}{I_{max}} = \frac{1}{1 + \exp[(V_{hold} - V_{0.5})/k]}$. $V_{0.5}$ was estimated as $-2.4$ and $-5.6$ mV; the slope factor $k$ = 5.5 and 3.5, respectively. (C) Conductance plot. The conductance ($G$) was calculated according to $G(V) = \frac{I_v}{(V - V_{rev})}$, where $I_v$ is the peak $I_{Ca}$ as a function of the test potential, and $V_{rev}$ is the reversal potential. Results of fitting procedures are summarized in Table 2. The symbols in A and B correspond to data for $\alpha_{1A}$, $\beta_4$ (●) and $\alpha_{1A}$, $\beta_4$, $\alpha_{2A}$ (■).
to form a functional ion pore, in expression systems the accessory subunits \( \alpha_2 \) and \( \beta \) are necessary to form a population of channels in the cell membrane that develop measurable currents. There is only one report on the expression of reasonable amounts of “pure \( \alpha_{1A} \)” current in Xenopus oocytes after the injection of very large amounts of cRNA (De Waard and Campbell, 1995).

In the combination \( \alpha_{1A} \) plus \( \beta \), the currents were barely measurable, and in accordance with previous reports (Mori et al., 1991), we were not able to record any current on \( \alpha_{1A} \) plus \( \alpha_2 \) transfected cells without an additional \( \beta \) subunit. We suppose that the effect of current enhancement by the \( \alpha_2 \) subunit, in our system by a factor of almost 20, is analogous to cardiac L- and neuronal E-type calcium channels, where it has been shown that the auxiliary subunits facilitate membrane insertion (Gao et al., 1999). In those combinations it has also been shown that the gating characteristics of the channels are modified (Bangalore et al., 1996; Shistik et al., 1995; Qin et al., 1998). In our case, the interaction of the \( \alpha_2A \) with \( \alpha_{1A} \) subunits led to a hyperpolarizing shift in activation and steady-state inactivation as well as in an acceleration of the inactivation kinetics. In sum, the regulatory action of the \( \alpha_{2A} \) subunit results in a gain of function of the channels, a drastic increase in current amplitudes, and an activation threshold at lower voltages.

The marked influence of the \( \alpha_2 \) subunit on channel properties makes it plausible that even minor changes in the \( \alpha_2 \) subunit affecting the interaction with the \( \alpha_1 \) subunit could lead to abnormal channel function and disease. The functional interaction of the \( \alpha_2 \) subunit with the pore forming unit is also underscored by the fact that the anticonvulsant drug gabapentin exerts its pharmacological action by binding to the \( \alpha_2 \) subunit (Gee et al., 1996).

In addition to its effects on high-voltage-activated calcium channels, it seems that the \( \alpha_2 \) subunit has a significant influence on the low-voltage-activated T-type currents (Wyatt et al., 1998). The overexpression of \( \alpha_2 \) modified the properties of different native voltage-gated calcium channels including T-type channels, whereas the \( \beta \) subunits had only minor effects, supporting the hypothesis that native T-type channels are associated with \( \alpha_2 \). T-type channels are important in heart and are also found in sensory neurons and in brain, e.g., cerebellar Purkinje cells or thalamic neurons, where they are involved in neuronal firing regulation. An involvement of T-type channels has also been proposed in mechanisms of genetic forms of epilepsy, e.g., absence epilepsy (Futatsugi and Riviello, 1998). Therefore even if no mutations during the screening of MHS patients could be found in the CACNA2 gene, the gene still should be considered as a candidate for other muscular or neuronal inherited diseases.

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