

Structure and Functional Expression of α_1 , α_2 , and β Subunits of a Novel Human Neuronal Calcium Channel Subtype

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Summary

The primary structures of human neuronal α_1 , α_2 , and β subunits of a voltage-dependent Ca^{2+} channel were deduced by characterizing cDNAs. The α_1 subunit (α_{1D}) directs the recombinant expression of a dihydropyridine-sensitive L-type Ca^{2+} channel when coexpressed with the β (β_2) and the α_2 (α_{2b}) subunits in *Xenopus* oocytes. The recombinant channel is also reversibly blocked by 10–15 μM ω -conotoxin. Expression of the α_{1D} subunit alone, or coexpression with the α_{2b} subunit, did not elicit functional Ca^{2+} channel activity. Thus, the β_2 subunit appears to serve an obligatory function, whereas the α_{2b} subunit appears to play an accessory role that potentiates expression of the channel. The primary transcripts encoding the α_{1D} , α_2 , and β subunits are differentially processed. At least two forms of neuronal α_{1D} were identified. Different forms of α_2 and β transcripts were also identified in CNS, skeletal muscle, and aorta tissues.

Introduction

The primary pathway by which Ca^{2+} enters excitable cells is through voltage-dependent Ca^{2+} channels present in cellular membranes (Bean, 1989). Multiple subtypes of these channels have been identified (Hess, 1990), the best characterized of which is the rabbit skeletal muscle dihydropyridine (DHP)-sensitive Ca^{2+} channel, consisting of four tightly coupled subunits, α_1 , α_2 , β , and γ (Campbell et al., 1988). Each of these subunits has been characterized by cDNA cloning (Tanabe et al., 1987; Ellis et al., 1988; Ruth et al., 1989; Jay et al., 1990). Recent evidence suggests that different α_1 subunits are encoded by a gene family comprising at least five distinct genes, some of which are expressed in several tissues (Ellis et al., 1988; Mikami et al., 1989; Perez-Reyes et al., 1990; Snutch et al., 1990). The gene encoding the α_1 subunit expressed in rabbit skeletal muscle directs the recombinant expression of a functional DHP-sensitive Ca^{2+} channel in cultured myotubes of *mdg* mice and in mouse L cells (Tanabe et al., 1988; Perez-Reyes et al., 1989). A second gene, encoding α_1 subunits expressed in rabbit cardiac and lung tissues, directs the synthesis of

DHP-sensitive Ca^{2+} channels in *Xenopus* oocytes (Mikami et al., 1989; Biel et al., 1990). In contrast, a third α_1 subunit gene, expressed in rabbit brain, directs the synthesis of Ca^{2+} channels that are insensitive to both DHPs and ω -conotoxin GVIA (ω -CgTx) when coexpressed with the rabbit skeletal muscle α_2 and β subunits in *Xenopus* oocytes (Mori et al., 1991). These expression studies in oocytes demonstrated that the α_1 subunit forms the pore through which Ca^{2+} enters the cell. The functional expression of α_1 subunits encoded by the two remaining genes has not yet been reported.

The entry of Ca^{2+} through voltage-dependent Ca^{2+} channels in neurons controls diverse functions, such as neurotransmitter release, excitability, and differentiation (Tsien et al., 1988). On the basis of biophysical and pharmacological characterizations, four subtypes of neuronal voltage-dependent Ca^{2+} channels have been proposed (Llinás et al., 1989; Swandulla et al., 1991). Although specific neuronal functions have been ascribed to different Ca^{2+} channel subtypes, the analysis has been difficult due to the coexistence of multiple subtypes in individual cells (Miller, 1987; Bean, 1989; Hess, 1990; Swandulla et al., 1991). One important step in defining subtype-function relationships is the cloning and expression of each neuronal subtype as a pure population. We report the complete amino acid sequence and functional expression of three subunits of a human neuronal L-type voltage-dependent Ca^{2+} channel: an α_1 subunit (designated α_{1D}), an α_2 subunit (designated α_{2b}), and a β subunit (designated β_2). A description of the nomenclature used to designate the different Ca^{2+} channel subunits is provided in the Experimental Procedures. We also report tissue-specific processing of the α_2 and β transcripts.

Results

Cloning and Characterization of Three Human Neuronal Voltage-Dependent Ca^{2+} Channel Subunits

We previously reported the isolation of cDNAs that encode the α_1 , α_2 , β , and γ subunits of the rabbit skeletal muscle DHP-sensitive, L-type Ca^{2+} channel (Ellis et al., 1988; Jay et al., 1990). These subunit cDNAs were used as probes to isolate related human neuronal cDNAs as described in the Experimental Procedures. The primary structures of the human neuronal α_{1D} , α_{2b} , and β_2 subunits (Figure 1; see Figure 3 and Figure 4) were deduced from these cDNA sequences.

α_{1D} Subunit

The primary structure of the human α_{1D} subunit (Figure 1) comprises 2161 amino acids, yielding a calculated molecular weight of 245,163. The α_{1D} sequence is most similar (96.3% deduced amino acid sequence identity) to the previously reported 188 amino acid

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Figure 1. α_{1D} Nucleotide and Deduced Amino Acid Sequences

The 5' untranslated sequence is negatively numbered. Positive numbering begins at the first nucleotide of the proposed initiating codon. The number of the nucleotide and amino acid residue is given at the end of each line. The proposed transmembrane segments S1-S6 in each of the repeats I-IV are shown (brackets).

GATTCAACTTCGACAACGTCCTCTCTGCTATGATGGCCTCTTCAACAGTCTCCACAGTTGAGGGCTGGCTGCCTGCTGTATAAAQCC
D F N F D N V L S A M M A L F T Y S T F E G W P A L L Y K A 3330
1110

ATCQAQCTCGAAATGGAGAACAATCGGCCCAATCTACAACCACCCGCTGGAGATCTCCACATCTCATCTACATCATCATTGTAQCT
I D S H G E N I Q P I Y N H R V E [B I F F I I Y I I Y A 3420
1140

TTCTTCATGATGAAACATCTTGTGGCTTGTCTACATCGTACATTTCAGGAACAAGGAGAAAAAGAGTATAAGAACGTGTQAGCTGGACAAA
F F M M M I F V G F V I V T F Q E Q G E K E Y K N C E L D K 3510
1170

AATCAQCGTCAQGTGTGGATACGCCCTGGAGAGATACATCCCCAAAACCCCTACCAAGTACAAGTCTGGTAC
N Q R Q C V E Y A L K A R P L R R Y I P K N P Y Q Y K F W Y 3600
1200

GTGGTGAACCTTCGCGCTTCAATACATGATGGCTCTCATGTCACACACTCTGCTGGCCATGCAGCACTACGAGCAGTCC
V V H S S [P F E Y M M F V L I M L N T L C L A M Q H Y E Q S 3690
1230

AAGATGTTCAATGATGCCATGGACATTCTGAAACATGGCTTCACCGGGGTTTCAACCGTCAGAGATGGTTTGGAAAGTCATCGCATTAAAG
K M F N D A M D I L N M V F T Q V F T V E M V L K V I A F K 3780
1260

CCTAAGGGTATTAGTGACCCCTGGAAACACGTTGACTCCCTCATCGTAATCQGAGCATTATAAGACGTGGCCCTCAGCQAAGCAGC
P K G Y F S D A W N T F D S L I V I G S I I D V A L B E A D 3870
1290

CCAACGAAAGTGGAAATGTCCTGTCACCTGGAGCTGGAAAGAGCATAAGATCTCCATCACCTTTTCGCTT
P T E S E N V P V P T A T P Q N S E E S N R I S [T F F R L 3960
1320

TTCCGAGTGTGCGATTGGTGAAGCTTCTCAAGCAGGGGGAAAGGCATCCGGACATTGCTGTGGACTTTTATTAAGTCTTCAGGCCTC
F R V M R L V K L L B R G I E Q I R T L L W T F I K F F Q A L 4050
1350

CCGTATGTGGCCCTCCCTCATGCCATGCTGGTCTCATCTATGCCATTGGCATQCAGAGATGGGGAAAGTGGCCATGAGAGATAAC
P Y V A L L I A M L F F I Y A V I G M Q M F J G K V A M R D H 4140
1380

AACAGATCAATAGGAACAATACTCCAGCTGGCTGCTGCTCTCAGGTGCAAGGAGGGCTGGAGAG
N Q I N R N N F Q T F P Q A V L L F R C A T Q E A W Q E 4230
1410

ATCATGCTGGCTCTCCAGGGAAAGCTCTGTGACCCCTGAGTCAGATTACAACCCCOGGAGGAGCATACTGAGGAGCAACTTQCC
I M L A C L P G K L C D P E S D Y N P Q E E H T C Q S N F A 4320
1440

ATTGTCTATTTCATCGATTTACATGCTGTGCAATTCTGATCATCAATCTGTTTGTGGCTGTATCATGATAATTTCGACTATCTG
I V Y F I S F Y M L C A F L I I N L F V A Y I M D N F D Y L 4410
1470

ACCCGGGACTGGCTTATTTGGGGCTCAGATTAGATAATTCAAAAGATAATGGCTGAGGAGGGAGGAG
T R D W S I L O P H H L D E F K R I W S E Y D P E A K G R I 4500
1500

AAACACCTTGATGTGGTCACTGCTGACGCACTCCAGCTGGGGTTGGGAGGTTATGTCACACAGGGTAGCAGCAGAG
K H D V V T L L R R I Q P L O F Q K L C P H R V A C K R 4590
1530

TTAGTTGCCATGAAACATGCCCTCAACAGTGACGGGACAQTGTTAATGCAACCCCTGGTTGGCTCGAACCGCTCTAAQATC
L V A M N M P L N S D G T V M F N A T L F A L V R T A L K I 4680
1560

AAGACCGAAGGGAACTGGAGCAAGCTAATGAAAGACTTCGGGCTGTGATAAAAGAAATTGGAGAAAACCAGCATGAAATTACTGAC
K T E G N L E O A N E E L R A V I K K I W K K T S M K L L D 4770
1590

CAAGTTGCTCCAGCTGGTGTGATGAGGTAACCGTGGGGAGTTCTATGCCACTTCTGATACAGGACTACTTAGGAAATTCAAG
Q V Y P P A G Q D D E V T Y Q F Y A T F L I Q D Y F R K F K 4860
1620

AAACGGAAAGAACAGGACTGGGAAAGTACCCCTGCGAGAACACCAATTGCCCTACAGGGCGGAGATTAGGACACTGCATGACATT
K R K E Q G L V Q K Y P A K N T T I A L Q A G L R T L H D I 4950
1650

GGGCCAGAAATCCGGCGTCTATATCGTGATTGCAAGATGACGAGCCTGAGGAAACAAACGAGAAAGAGATGATGTQTTCAA
G P E I R R A I S C D L Q D D E P E E T K R E E E D D V F K 5040
1680

AGAAATGGTGCCCTGCTTGAAACCATGTCATGTTAATGATAGGAGAGATCCCTCAQCGAGCCAATACCCACCGTCCC
R N G A L L G N H V N H V N B D R R D B L Q Q T N T T H R P 5130
1710

CTGCATGTCCAAAGGGCTCAATTCCACCTGCAAGTGATACTGAGAAACCGCTGTTCTCCAGCAGGAAATTCCGQGTGTCTCATACCAT
L H V Q R P S I P P A S D T E K P L F P P A G N S V C H N H 5220
1740

CATAACCATAATTCCATGQAAGGCAAGTCCACCAATGCCATCAAAATGCAATATGCTCAAQCTGCCATGQAAAG
H N H N S I Q K Q V P T S T N A N L N N A M S K A A H Q K 5310
1770

CGGCCCAQCATGGGAACCTTGAGCATGTTGAAATGGCATCATCTTCCACAAAGCATGACGGGAGCCTCAGAGAAGGTCAGT
R P S I G N L E H V S E N O H H S S H K H D R E P Q R R S S 5400
1800

GTGAAAAGAACCCGCTATTATGAAACTTACATTAGGTCCAGCTCAGGGAGATGAAACAGCTCCCAACTTGTGCGGGAGACCCAGAGATA
V K R T R Y Y E T Y I R S D G D E Q L P T I C R E D P E I 5490
1830

CATGGCTATTTCAGGGACCCCCACTGCTGGGGAGCAGGGAGTATTTCAGTAGTGTGGAGATGCTACAGGGAGTACAGCAGCTCGCCCCACCTGG
H Q Y F R D P H C L Q E Q E Y F S S E E C Y E D D S B P T W 5580
1860

AGCAGGGCAGAAACTATGGCTACTACAGCAGATAACCCAGGGAGAACATGCACTCTGAGAGGGCCCGAGGCTACCATCATCCCCAACQATC
S R Q N Y G Y Y S R Y P G R N I D S E R P R G Y H H P Q Q F 5670
1890

TTGGAGGAGCATGACTCGCCCTGGCTATGATTACCGGAGATCTCCAAAGGAGACGCCACTACCTCCACCCAGCATCCCACCGGAG
L E D D D S P V C Y D S R R S P R R L L P P T P A S H R R 5760
1920

TCCCTCTCAACTTGAGTQCCCTGCAGCCAGAGCAGCCAGGAAGGGTCCCGTCTCTCCCATCTTCCACATCGCACGGCCCTGCC
S S F N F E C L R R Q G S S O E E V P S S P I F P H R T A L P 5850
1950

CTGACTCTAATGCAAGAACAGATCATGGAGCTGGCCAGCTAGATTCAAGTAAAGCCAGAGTACTCACCAGGACTACTCGACCCGGCTCG
L H L M Q Q I M A V A Q G L D S S K A Q K Y S P S H S T R S 5940
1980

TGGGCCACCCCTCAGCAACCCCTCCACCGGGACTGGACACCCGATCTACACCCCTGATCCAAGTGGAGCACTGAGAGGCCCTGGAC
W A T P P A T P P Y R D W T P C Y T P L I Q V E Q S E A L D 6030
2010

CAGGTGAAACGGCAGCCCTGGCTCCCTGACCCGAGCTCTGGTACACAGACGAGGCCACATCTTACCCGGACTTTCACACCAAGCCAGC
Q V N Q S L P S L H R B S W Y T D E P D I S Y T F T P A S 6120
2040

CTGACTGTCCCCAGCAQCTTCCGGAAACAAAACAGCGACAAAGCAGAGGGAGTGGCGACAGCTTGGTGGAGGGAGTCTGATATCCGAAGGC
L T V P S S F R N K N S D K Q R S A D S L V E A Y L I S E G 6210
2070

TTGGGACGCTATGCAAGGGACCCAAAATTGTGTCAGCAACAAAACAGCAAAATCGCTGATGCCCTGTGACCTCACCATCGACGAGATGGAG
L Q R Y A R D P K F V S A T K H E I A D A C D L T I D E M E 6300
2100

AGTCAQCCAGCACCCCTGCTTAATGGGAGCAGCTGGCTCCCGAGCCAACGGGAGTGGGGCCCTCTCACACCCGGAGACTATGAGCTA
S A A S T L L N G N V R P R A N O D V G P L S H R Q D Y E L 6390
2130

CAGGACTTTGGTCTGGCTACAGCGACGAGAGCCAGACCTGGGGAGGGAGTGGAGGGACCTGGGGAGTGGAGAGGAAAGTGGCTCATAGT
Q D F G P G Y S D E E P D P G R D E E D L A D E M I C I T T 6480
2160

TTGTAQCCCCCAGCGAGGGAGACTGGCTGGCCCTGAGTGGGGCGAGAGAGGCAAGGGGAGTGGAGAGGAAAGTGGCTCATAGT
L * 6570
2161

AGQCACTAGTGGAGATAATATCAATTAAATTAAGACTTTGTATAAGAGATGTCATGCCCTCAAGAACGCCATAACCTGGTAGGAAACAGG
TCCCAAGCGGGTGGAGCAAGTACCATGQGCCAGCTGGCCAGCTGGAGGAAACAGCAGGGCCGCCCTCAGAGGAGATGGGTGAG 6660
6750

GAGGGCAGACCTGCCCTGCCCATGTCAGATGGGACTGTGGAGATGAGCTGCTCTCCCATGACAGGGGAGCAGGCCACCCAACT 6840
6930

GAAGGAGCATGGCGGGGGGGTQCAAGGGGGAGTAAAGGTGAGCATACACCCCTGCTGTCAGTCTACCGCATCGGTCTAGCATATC
AGTCACTGGGCAACATATCCATTTCCTTCCCAAAACTACCTGCGCTCTGGTGTGGAGGGAGCTGGCTCATAGTCTGAGATGGCTCATAGT
GTAAGTAAGTCAGAACCCAGCTACCAAGTGGATATTGGGAGGGCAATGGGACCTCATAAATAAGGTTTCTGTGATGTGACGCCAGTTAC
ATAAGAGAAATATCAC 7020
7110

L * 7125

partial rat brain class D cDNA (Snutch et al., 1990). The translation initiation site was assigned to the first methionine codon that appears downstream of an in-frame nonsense codon. Interestingly, 7 methionine codons appear at the beginning of the putative coding sequence, followed by 2 lysine codons and an eighth methionine codon; none of these methionine codons are contained within the consensus sequence for eucaryotic initiation codons (Kozak, 1987). This series of methionine codons was confirmed by direct sequence analysis of cloned polymerase chain reaction (PCR) products derived from reactions performed on human neuroblastoma IMR32 cell cytoplasmic RNA, as described in the Experimental Procedures.

The predicted structure of the α_{1D} subunit consists of four repeating domains, each domain comprised of five hydrophobic segments (S1, S2, S3, S5, and S6) and one positively charged segment (S4), suggesting the same transmembrane topology as described previously for Ca^{2+} channel α_1 subunits and Na^+ channels (Numa and Noda, 1986; Tanabe et al., 1987; Mikami et al., 1989; Biel et al., 1990; Koch et al., 1990; Mori et al., 1991). Based on this proposed topology, the α_{1D} subunit has 3 of 12 potential N-glycosylation sites (Bause, 1983) assigned to the extracellular side and nine of ten potential cAMP-dependent phosphorylation sites (Glass et al., 1986) and 22 of 26 potential protein kinase C phosphorylation sites (Woodgett et al., 1986) assigned to the cytoplasmic side of the cellular membrane (Figure 2).

The α_{1D} cDNA clone $\alpha 1.136$ was found to encode an incompletely processed transcript containing two exons encoding the IS6 transmembrane domain, designated α_{1D} exon A and α_{1D} exon B. The deduced amino acid sequences are MNDAMGFELPWVYFVSL-VIFGSFFVLNLVLGVLSG and VNDAIGWEWPWVYFV-SLIILGSFFVLNLVLGVLSG, respectively, which share 83% identity. Exon A was present in clone $\alpha 1.144$, which was used for the construction of the full-length α_{1D} cDNA used in the present study (Figure 1).

The deduced amino acid sequences of two different α_1 subunits, the rabbit cardiac (Mikami et al., 1989) and the rabbit brain BI-2 (Mori et al. 1991), previously expressed in *Xenopus* oocytes, are shown aligned with the human α_{1D} sequence (Figure 2). The amino acid sequence identity of α_{1D} to these sequences is significant: 70.3% and 40.5% for the cardiac and BI-2 sequences, respectively. The sequence identity is well conserved through the four repeating domains, 79.7% and 50.5% for the α_{1D} -cardiac and the α_{1D} -BI-2 pairs, respectively. Most noteworthy is the divergence of the α_{1D} and cardiac sequences compared with the BI-2 sequence through the putative DHP-binding region (Regulla et al., 1991). In this region, the α_{1D} and cardiac DHP-sensitive forms differ by a single amino acid (Ser-1490) as does the rabbit skeletal muscle sequence (Ala-1404), whereas the BI-2 DHP-insensitive form has 18 amino acid substitutions in this region (Figure 2). This evidence, together with the results of the expression studies reported here (see below), supports the proposed identity of the DHP-binding region.

α_{2b} Subunit

The primary structure of the human brain α_{2b} subunit (Figure 3) consists of 1091 amino acids, yielding a calculated molecular weight of 123,182. The amino acid sequence homology is 97.1% identical to the rabbit skeletal muscle α_{2a} subunit sequence (Figure 3) and has essentially an identical predicted topography and secondary structure (Ellis et al., 1988; Jay et al., 1991), with the exceptions of a 19 amino acid deletion in the human sequence compared with the rabbit sequence (α_{2a} residues Pro-507 to Gln-525) and a 7 amino acid insertion in the human sequence compared with the rabbit sequence (α_{2b} residues Lys-602 to Asp-608). The 16 potential glycosylation sites that were identified in the rabbit skeletal muscle α_{2a} subunit (Jay et al., 1991) also are conserved in the human α_{2b} sequence. Previous studies suggest that posttranslational processing of the rabbit skeletal α_{2a} subunit results in a heterogeneous population of δ peptides, all of which begin at Ala-935 (Jay et al., 1991). The human brain α_{2b} sequence has two conservative amino acid substitutions at this proposed cleavage site, Val-923 and Glu-924 replacing Ala-935 and Asp-936, respectively (Figure 3).

β_2 and β_3 Subunits

The primary structure of the human brain β_2 subunit (Figure 4) comprises 478 amino acids and has a calculated molecular weight of 52,934. The amino acid sequence homology is 96.9% identical to the rabbit skeletal muscle β_1 subunit sequence (Figure 4). The β_2 subunit has essentially an identical topography and secondary structure as predicted for the rabbit skeletal muscle β_1 subunit (Ruth et al., 1989) except that the deduced human β_2 sequence has a deletion of 45 amino acids (Ala-217 to Lys-261). The lack of this region in β_2 removes the second α helical domain proposed for the rabbit skeletal muscle β_1 subunit (Ruth et al., 1989). Thirteen of sixteen potential phosphorylation sites identified in the rabbit skeletal muscle β_1 subunit (Ruth et al., 1989) are conserved in the human β_2 sequence (Figure 4). Two sites are changed due to amino acid substitutions (β_1 Ser-179 and Ser-182), and the third is removed by the 45 amino acid deletion (β_1 Ser-238).

Another form of β , designated β_3 , which has the same deduced 45 amino acid deletion, was identified in the hippocampus cDNA library. Clone $\lambda\beta 4$ encodes the β_3 cDNA and diverges from β_2 after nucleotide 1332. The β_3 cDNA extends another 429 nucleotides with no translation stop codon identified (data not shown). A GT splice donor is not present at the point of divergence between the β_2 and β_3 sequences. A complete characterization of β_3 is in progress.

Tissue-Specific Processing of the α_2 and β Transcripts and Distribution of α_{1D} , α_2 , and β mRNAs

PCR analysis and hybridization with oligonucleotides derived from α_{2a} - or α_{2b} -specific regions (the 19 amino acid region or the 7 amino acid region, respectively; Figure 3) demonstrated that the human skeletal

Human Neuronal Calcium Channel Subtype 75

Figure 2. Alignment of Deduced Amino Acid Sequences of α_1 Subunits.

The amino acid sequences of the human neuronal Ca^{2+} channel α_{1D} (Hum 1D), the rabbit cardiac α_1 (Card; Mikami et al., 1989), and the rabbit brain α_1 (Bl-2; Mori et al., 1991) are shown in single-letter code. The numbering begins with the proposed initiating methionine. The number of the amino acid residue at the end of each line is given. Identical residues at one position in two of the three sequences are shown as white letters on black background. The putative transmembrane segments S1–S6 in each of the repeats I–IV are shown (brackets). The putative DHP-binding regions (N1, N2, A2; Regulla et al., 1991) are shown (underlined). Potential N-glycosylation (o), cAMP-dependent phosphorylation (<), and protein kinase C phosphorylation (>) sites are shown. Potential targets of either kinase are labeled (+).

-24 GC GGGGGGAGGGGGCATTGATCTCGATCGCGAAG -1

Hum	M A A G C L L A L T L T L F Q S L L I G P S S E E P F P	1
Rab	R P W W A W L I	4
>		
Hum	TCGGCCGTCACTATCAAATCATGGGTGGATAAGATGCAAGAAGACCTTGTACACTGGCAAAAACAGCAAGTGGAGTCATCAGCTTGTT	174
Rab	Y A V T I K S W V D K M Q E D L V T L A K T A S G V N Q L V	34
H		
Hum	GATATTTATGAGAAAATATCAAGATTTGATACTGTGGAAACCAAAATAATGCACGCCAGCTGGTAGAAAATTGCAGCCAGGGATATTGAGAAA	264
Rab	E I Y E K Y Q D L Y T V E P N N A R Q L V E I A A R D I E K	64
>		
Hum	GTTCTGAGCAACAGATCTAAAGCCCTGGTGAAGCCTGGCATTGGAAAGCGGAGAAAAGTTCAAGCAGCTCACCAGTGGAGAGAAGATTTGCA	354
Rab	A L S N R S K A L V S L A L E A E K V Q A A H Q W R E D F A	94
R		
Hum	GGCAATGAAGTTGCTACTACAATGCAAGGATGATCTCGATCCTGAGAAAAATGACAGTGAGCCAGGCAGCAGAGGATAAAACCTGTT	444
Rab	V N E V V Y Y N A K D D L D P E K N D S E P G S Q R I K P V	124
>		
Hum	ATCATTGAAGATGCTAATTTGGACGACAAATATCTTATCAGCACGCAGCAGTCCATATTCTACTGACATCTATGAGGGCTCAACAAATT	534
Rab	K I E D A N F G R Q V S Y Q H A A V H I P T D I Y E G S T I	154
D		
Hum	CTGTAAATGAACCTCAACTGGACAAGTGCCTTAGATGAAGTTTCAAAAAGAATCGCGAGGAAGACCCCTTCAATTGTGGCAGGTTTTT	624
Rab	L L N E L N W T S A L D E V F K K N R E E D P S L L W Q V F	184
D		
Hum	AGCAGTGCACGGCTAGCTCGATATTATCCAGCCTCACCATGGGTTGATAATAGTGAACCTCAAATAAGATTGACCTTATGATGTA	714
Rab	G S A T G L A R Y Y P A S P W V D N S R T P N K I D L Y D V	214
>		
Hum	CGCAGAACGACATGGTACATCCAAGGAGCTGCATCTCTAAAGACATGCTTATTCTGGTGGATGTGAGTGGAAAGTGTAGTGGATTGACA	804
Rab	R R R P W Y I Q G A A S P K D M L I L V D V S G S V S G L T	244
>		
Hum	GTAAACTGATCCGAACATCTGTCCTCGAAATGTTAGAAACCCCTCAGATGATGATTCTGTGAAATGTAGCTCATTTAACAGCAATGCT	894
Rab	V K L I R T S V S E M L E T T L S D D D D F V N V A S F N S N A	274
>		
Hum	TAGGATGTAAGCTGTTTCAGCACCTTGTCCAAGCAATGTAAGAAATAAAAAGTGTGAAAGACGCGGTGAAATAATCAGCAACAGCAA	984
Rab	L D V S C F Q H L V Q A N V R N K K V L K D A V H N N I T A K	304
>		
Hum	AGAATTACAGATTATAAGAAAGGGCTTGTAGTTTGCTTTGAACAGCTGCTTAATTATAATGTTCCAGAGCAAACGTCAATAAGATTATT	1074
Rab	K I T D Y K K G F S F A F E Q L L N Y N V S R A N C N K I I	334
>		
Hum	CTGCTATTACCGGATGGAGGAGAAGAGAGAGCCCTGGAGATTTAACAAATAACATAAGATAAAAAGTACGTGTATTCAAGGTTTCA	1164
Rab	L L F T D G G E E R A Q E I F N K Y N K D K K V R V F R F S	364
T		
Hum	ATGGTCAACACAATTATGAGAGAGGACTTACAGTGGATGGCTGTGAAACAAAGGTTATTATTATGAAATTCTTCATGGTGCA	1254
Rab	I G Q H N Y E R G P I Q W M A C E N K G Y Y Y E I P S I G A	364
D		
Hum	ATAAGAATCAAACTCAGGAATATGGATGTTGGGAAGACCAATGGTTTAGCAGGAGACAAAGCTAAGCAAGTCCAATGGACAAAT	1344
Rab	K R I N T Q E Y L D V L G R P M V L A G D K A K Q V Q W T N	424
>		
Hum	GTGTACCTGGATGCATTGGAACTGGACTTGTCAATTACTGGAACTCTTCCGGTCTCACACATAACCGGCCAATTGAAAGATAAGACAAAC	1434
Rab	E Y L D A L E L G L V I T G T L P V F N I T G Q F E N K T N	454
>		
Hum	ATAAAAGAACAGCTGATTCTGGTGTGATGGGAGTAGATGTGCTTTGGAAAGATATTAAAAGACTGACACCACGTTTACACTGTGCC	1524
Rab	S K N Q L I L G V M G V D V S L E D I K R L T P R F T L C P	484
+>		
Hum	CATGGTATTACTTGCATCGATCTTAATGGTTATGTTTATTACATCCAATCTCAGCAGAAG.....	1590
Rab	R G Y Y F A I D P N G Y V L L H P N L Q P K P I G V G I P T	506
>		
HumACCCCCAAATCTCAGGAGCCAGTAACATTGGATTTCCTGTATGCAGAGTTAGAGAAT	1647
Rab	I N L R K R R P N V O N P K S Q E P V T L D F L D A E L E N	525
>		
Hum	GATATTAAGTGGAGATTGCAAATAAGATGATTGATGGGAAAGTGGAGAAAAACATTCAAGACTCTGGTAAATCTCAAGATGAGAGA	1737
Rab	D I K V E I R N K M I D G E S G E K T F R T L V K S Q D E R	555
S		
>		
Hum	TATATTGACAAAGAACAGGACATACACATGGACACCTGTCAATGGCACAGATTAC...AGTTGGCCTTGTATTACCAACCTACAGT	1824
Rab	Y I D K G N R T Y T W T P V N G T D Y S L A L V L P T Y S	584
S		
>		
Hum	TTTACTATATAAAAGCCAAACTAGAAGAGACAATAACTCAGGCCAGATCAAAAAGGGCAAAATGAAGGATTCTGGAAACCCCTGAAGCCA	1914
Rab	F Y Y I K A K L E E T I T Q A R S K K G K M K D S E T L K P	614
I		
>		
Hum	GATAATTGGAGAAATCTGGCTATACATTCTAGCACCAGAGATTACTGCAATGACCTGAAATATCGGATAATAACACTGAATTCTT	2004
Rab	D N F E E S G Y T F L A P R D Y C N D L K I S D N N T E F L	644
S		
>		
Hum	TTAAATTCACCGAGTTATTGATAGAAAACCTCCAAACAAACCCATCATGTAACCGCGGATTGATTAATAGAGTCTTGTGATGCAGGC	2094
Rab	L N F N E F I D R K T P N N P S C N A D L I N R V L L D A G	674
T		
>		

Figure 3. Determined cDNA Sequence of α_{2b} and Alignment of the Deduced Amino Acid Sequence with the Deduced Rabbit Skeletal Muscle α_{2a} Sequence

For the rabbit α_{2a} sequence (Rab; Ellis et al., 1988), only the amino acid differences compared with the human α_{2b} sequence (Hum) are shown. The 5' untranslated sequence is negatively numbered. Positive numbering begins at the first nucleotide of the proposed initiating codon. The number of the nucleotide and amino acid residue is given at the end of each line. Negative numbers for amino acids designate residues contained in the proposed signal sequence (Ellis et al., 1988) beginning with the initiating methionine (-24 and -26). The positive numbering begins at the NH₂-terminal residue (glutamic acid) of the mature protein. Amino acid sequence differences and insertions/deletions are identified by the black boxes. Potential N-glycosylation (o), cAMP-dependent phosphorylation (<), and protein kinase C phosphorylation (>) sites are shown. The potential target of either kinase is labeled (+).

Hum	TTTACAAATGAACTTGTC	AAAATTACTGGAGTAAGCAGAAAAAATATCAAGGGAGT	GAAAGCACGATTGTTGTACTGATGGTGGATT	2184
Rab	F T N E L V Q H Y W S K Q K N I K G V K A R F V V T D G Q I			704
Hum	ACCAGAGTTTATCCCAAAGAGGCTGGAGAAAAATTGGCAAGAAAACCCAGAGACATATGAGGACAGCTCTATAAAAAGGAGCCTAGATAAT			2274
Rab	T R V Y P K E A G E N W Q E N P E T Y E D S F Y K R S L D N			734
Hum	GATAACTATGTTTCACTGCTCCCTACTTTAACAAAAGTGGACCTGGTGCCTATGAATCGGGCATTATGGTAAGCAAGCTGTAGAAATA			2364
Rab	D N Y V F T A P Y F N K S G P G A Y E S G I M V S K A V E I			764
Hum	TATATTCAAGGGAAACTTCTTAAACCTGCAGTTGGAAATTAAATTGATGTAATTCTGGATAGAGAATTTCACCAAAACCTCAATC			2454
Rab	Y I Q G K L L K P A V V G I K I D V N S W I E N F T K T S I			794
Hum	AGAGATCCGTGTGCTGGTCCAGTTGTGACTGCAAAAGAAAACAGTGCAGTAATGGATTGTGTGATCTGGATGATGGTGGGTTCTCTG			2544
Rab	R D P C A G P V C D C K R N S D V M D C V I L D D G G F L L			824
Hum	ATGGCAAATCATGATGATTATACTAATCAGATTGGAAAGATTTTTGGAGAGATTGATCCCAGCTTGATGAGACACCTGGTAATATATCA			2634
Rab	M A N H D D Y T N Q I G R F F G E I D P S L M R H L V N I S			854
Hum	GTTTATGCTTTAACAAATCTTATGATTATCAGTCAGTATGTGAGCCCGGTGCTGCACCAAAACAAGGAGCAGGACATCGCTCAGCATAT			2724
Rab	V Y A F N K S Y D Y Q S V C E P G A A A P K K Q G A G H R S A Y			887
Hum	GTGCCATCAQTAGCAGACATATTACAAATTGGCTGGTGGGCCACTGCTGCTGCCTGGTCTATTCTACAGCAGTTCTCTTGAGTTGACC			2814
Rab	V P S V A D I L Q I G W W A T A A A A W S I L Q Q F L L S L T			914
Hum	TTTCCACGACTCCTTGAGGCAGTTGAGATGGAGGATGATGACTTCACGGCCTCCCTGTCCAAGCAGAGCTGCATTACTGAACAAACCCAG			2904
Rab	F P R L L E A V E M E D D D F T A S L S K Q S C I T E Q T Q			944
Hum	TATTTCTCGATAACGACAGTAATCATTCACTGGTGTATTAGACTGTGGAAACTGTTCCAGATCTTCTATGGAGAAAAGCTTATGAAC			2994
Rab	Y F F D N D S K S F S G V L D C G N C S R I F H G E K L M N			987
Hum	ACCAACTTAATATTCTATAATGGTGGAGAGCAAGGGACATGTCCATGTGACACACGACTGCTCATACAAGCGGAGCAGACTTCTGACGGT			3084
Rab	T N L I F I M V E S K G T C P C D T R L L I Q A E Q T S D G			1004
Hum	C CAAATCCTTGTGACATGGTTAAGCAACCTAGATACCGAAAAGGGCTGATGTCGCTTGTATAACAATGTCTGGAGGATTATACTGAC			3174
Rab	P N P C D M V K Q P R Y R K G P D V C F D N N V L E D Y T D			1034
Hum	T GTGGTGGTGTCTGGATAAAATCCCTCCCTGTGGTATATCATTGGAACTTCACTACTTTGGCTGGTATCTGGCAGCACACAC			3264
Rab	C G G V S G L N P S L W Y I G I Q F L L L W L V S G S R H			1064
Hum	CGGCTGTTATGACCTCTAAACCAAACTGCGATAGTTAACTCCAGACCCCTGCCAAACATGAGCCCTGCCCTCAATTACAGTAACGT			3354
Rab	H L L *			1067
Hum	AGGGTCAGCTATAAAATCAAGAACACATTAGCTGGGCTGTTCCATGGCATAACACTAACGCGCAGACTCCTAAGGCACCCACTGGCTGC			3444
ATGTCAGGGTGTCACTAAACGTGTGTAATGCTGCATCATCTATGTGTAACATCAAAGCAAATCTATACGTGTCTCTATTGG				3534
AAAATTGGCGTTTGTGTCATTGTTGGT				3566

muscle α_2 transcript is processed in a manner similar to the rabbit skeletal muscle transcript (540 bp bands; Figure 5A). Furthermore, the α_2 transcripts expressed in IMR32 cells and human CNS tissues (501 bp bands; Figure 5B) and the α_2 transcript expressed in aorta tissue (490 bp band; Figure 5B) are processed differentially to yield at least two additional α_2 transcript species, α_{2b} (Figure 3) and α_{2c} , respectively.

PCR analysis of β -specific RNAs showed that the β primary transcript is also processed in a tissue-specific manner. Analysis of human skeletal muscle RNA detected the 135 nucleotides absent in β_2 (Figure 4) and, thus, confirmed the presence of a distinct skeletal muscle β_1 transcript (Figure 5C). In addition to the β_1 form expressed in skeletal muscle and the β_2 and β_3 forms expressed in the CNS, another form, designated β_4 , was detected in aorta tissue having a 156 nucleotide deletion relative to the skeletal muscle β_1 transcript (Figure 5C).

To confirm the tissue-specific processing of the β subunit primary transcript, β -specific PCR products of human skeletal muscle and aorta were cloned, and the DNA sequence was determined. The deduced human skeletal muscle amino acid sequence is 92% identical to the rabbit skeletal muscle sequence from position Gly-210 to Lys-261 (Figure 4). The β_2 sequence has a proposed alternative exon (Ala-210 to Ser-216) that

probably corresponds to either the human skeletal muscle sequence Gly-210 to Leu-216 (GNEMTNL) or Arg-255 to Lys-261 (RIPFFKK). The deduced aorta sequence lacks the region between residues Ser-209 and Thr-217 (Figure 4).

PCR analysis performed on RNAs isolated from several human primary tissues and IMR32 cells identified an α_{1D} transcript in IMR32 cells and each of the human CNS tissues, but not in human skeletal muscle (Figure 5D). An α_2 transcript was detected in all RNAs analyzed (Figures 5A and 5B), as was a β transcript (Figure 5C).

Functional Expression in Xenopus Oocytes

The expression of the human neuronal α_{1D} , α_{2b} , and β_2 subunits was studied in Xenopus oocytes. mRNAs encoding each subunit were synthesized in vitro and were injected into oocytes either alone or in various combinations. The oocytes then were examined for inward Ba^{2+} currents (I_{Ba}) mediated by voltage-dependent Ca^{2+} channels.

Oocytes coinjected with the α_{1D} , α_{2b} , and β_2 mRNAs expressed sustained I_{Ba} upon depolarization ($162 \pm 121 \text{ nA}$, $n = 46$) that typically showed little inactivation during test pulses ranging from 140–700 ms (Figure 6A). A series of voltage steps revealed currents that appeared at approximately -30 mV and peaked at approximately 0 mV (Figure 6B). Application of the DHP

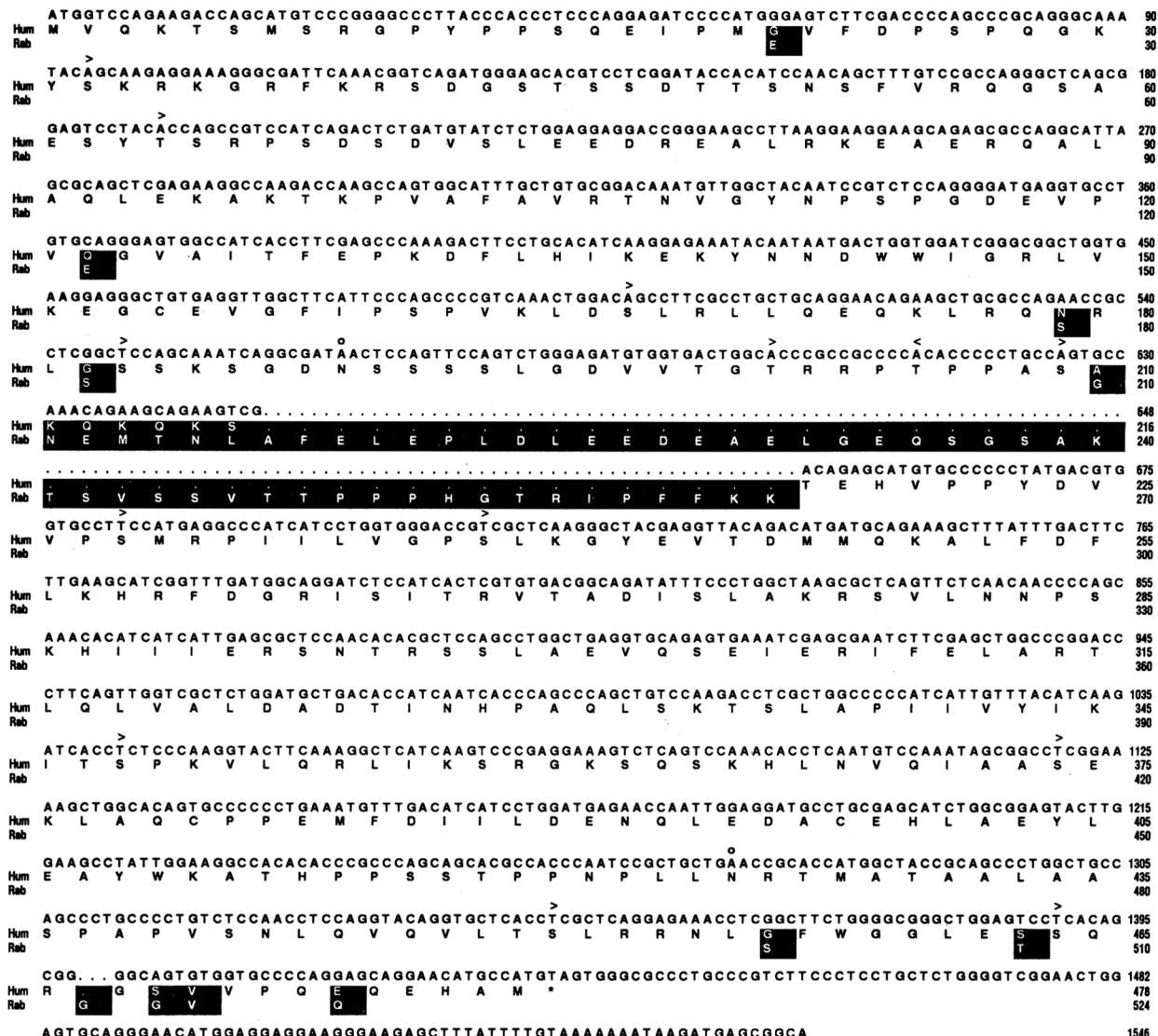


Figure 4. Determined cDNA Sequence of β_2 and Alignment of the Deduced Amino Acid Sequence with the Deduced Rabbit Skeletal Muscle β_1 Sequence

For the rabbit β_1 sequence (Rab; Ruth et al., 1989), only the amino acid differences compared with the human β_2 sequence (Hum) are shown. See legend of Figure 3 for description of symbols and numbering.

Ca^{2+} channel agonist Bay K 8644 increased the magnitude of the $\alpha_{1D}\alpha_{2b}\beta_2$ -mediated currents, prolonged the "tail" currents present upon repolarization of the cell, and induced a hyperpolarizing shift in current activation (Figures 6A and 6B). Application of the DHP Ca^{2+} channel antagonist nifedipine blocked a substantial fraction of the I_{Ba} in oocytes coinjected with α_{1D} , α_{2b} , and β_2 ($91\% \pm 6\%$, $n = 7$; Figure 6C). Much of the I_{Ba} recovered when the holding potential was shifted from -50 mV to -90 mV (data not shown), consistent with the voltage-dependent block by nifedipine (Bean, 1984; Sanguinetti and Kass, 1984). A residual inactivating component of I_{Ba} typically remained after nifedipine application. Consistent with previous studies on neuronal L-type Ca^{2+} channels (Fox et al., 1987), the $\alpha_{1D}\alpha_{2b}\beta_2$ -mediated I_{Ba} was blocked completely by 50 μM Cd^{2+} , but only approximately 15% by 100 μM Ni^{2+} .

The $\alpha_{1D}\alpha_{2b}\beta_2$ -mediated I_{Ba} was blocked weakly ($54\% \pm 29\%$, $n = 7$) and reversibly by relatively high concentrations (10 – 15 μM) of ω -CgTx (Figure 6D). Bay K 8644 was first applied to the cell in order to determine the effect of ω -CgTx on the DHP-sensitive current component that was distinguished by the prolonged tail currents. Both the test currents and the accompanying tail currents were blocked progressively within 1–3 min after application of ω -CgTx, but both recovered partially as the ω -CgTx was flushed from the bath.

The contribution of the α_{2b} and β_2 subunits to the $\alpha_{1D}\alpha_{2b}\beta_2$ -mediated current was assayed by expression of the α_{1D} subunit alone or in combination with either the β_2 subunit or the α_{2b} subunit. Oocytes injected with only the α_{1D} mRNA produced no discernable I_{Ba} upon depolarization ($n = 10$). Oocytes coinjected with the α_{1D} and β_2 mRNAs expressed I_{Ba} (108 ± 39 nA, $n = 4$) that resembled the $\alpha_{1D}\alpha_{2b}\beta_2$ -mediated currents,

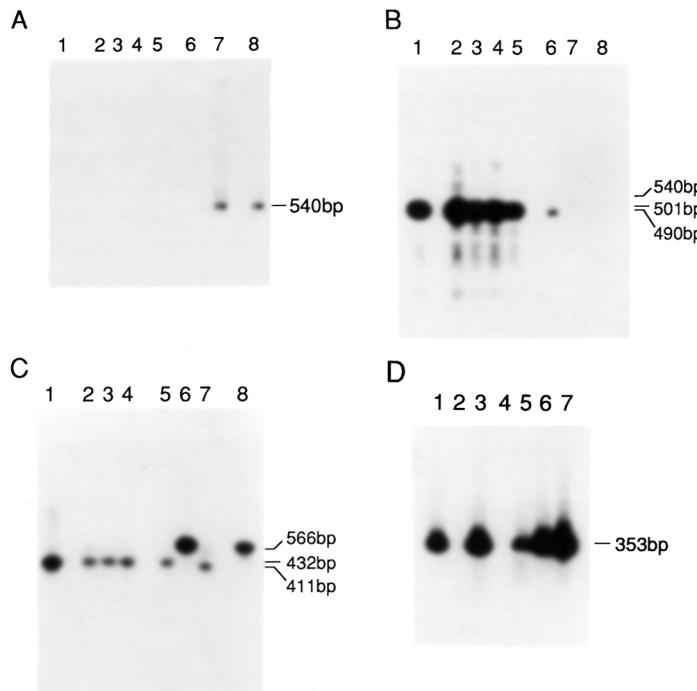


Figure 5. Autoradiographs of PCR Products Showing Distribution of Voltage-Dependent Ca^{2+} Channel Subunit Transcripts and Alternative Splicing of the α_2 and β Transcripts

IMR32 cytoplasmic RNA and human primary tissue poly(A)⁺ RNAs were used as templates to synthesize cDNA prior to PCR analysis.

(A and B) PCR products of pHBCaCH $\alpha_2\beta$, a human brain α_{2b} cDNA clone (lane 1), IMR32 cells (lane 2), hippocampus and basal ganglia (lane 3), habenula (lane 4), thalamus (lane 5), aorta (lane 6), skeletal muscle (lane 7), and p $\alpha_2.15\Delta 5'$, a rabbit skeletal muscle α_{2a} clone (lane 8), were hybridized with (A) an α_{2a} oligonucleotide (nucleotides 1597–1619 corresponding to Pro-507 to Thr-514; Ellis et al., 1988) or (B) an α_{2b} oligonucleotide (nucleotides 1876–1896 corresponding to Lys-602 to Asp-608). The PCR reactions were primed with human α_{2b} oligonucleotides, nucleotides 1455–1479, and the complement of nucleotides 1931–1955. An approximately equal mass of DNA was present in each lane. The sizes of the 490 bp, 501 bp, and 540 bp α_2 -specific PCR products derived from human aorta, IMR32 cell and CNS tissues, and skeletal muscle RNAs, respectively, were further

confirmed by electrophoresis through a 1% agarose/2% NuSieve composite gel. The weaker hybridization of the human α_{2b} -derived oligonucleotide with the 490 bp aorta and 540 bp skeletal muscle PCR products further supports their difference from the α_{2b} transcript. Each band observed also hybridized with an α_2 tissue nonspecific probe, nucleotides 1601–1626 (data not shown). (C) PCR products of pHBCaCH β_1 , a human brain β_2 cDNA clone (lane 1), IMR32 cells (lane 2), hippocampus and basal ganglia (lane 3), habenula (lane 4), thalamus (lane 5), skeletal muscle (lane 6), aorta (lane 7), and pRSKmCaCH β_2 , a rabbit skeletal muscle β_1 cDNA clone (lane 8), were hybridized with a β_2 oligonucleotide, nucleotides 755–784. The PCR products were primed with β_2 oligonucleotides, nucleotides 541–560, and the complement of nucleotides 953–972. (D) PCR products of pVDCCIII(A), an α_{1D} cDNA clone (lane 1), human genomic DNA (lane 2), IMR32 cells (lane 3), skeletal muscle (lane 4), hippocampus and basal ganglia (lane 5), habenula (lane 6), and thalamus (lane 7), were hybridized with an α_{1D} oligonucleotide, nucleotides 164–187. The PCR products were primed with α_{1D} oligonucleotides, nucleotides –39 to –18, and the complement of nucleotides 201–314.

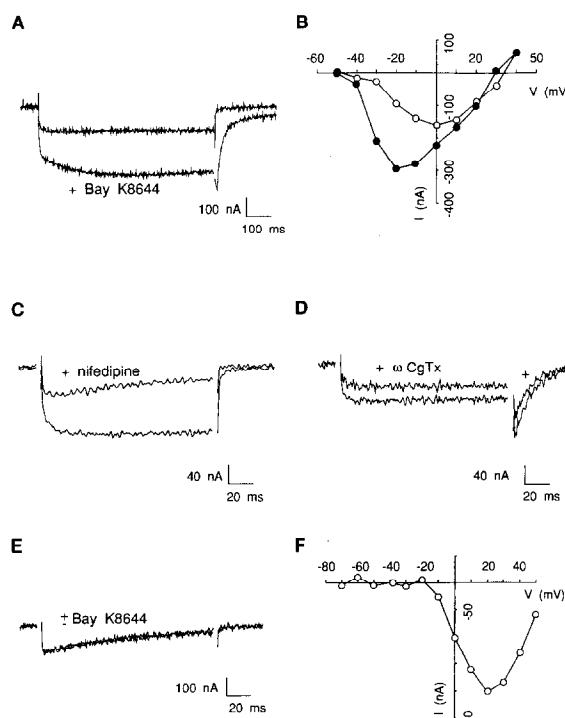


Figure 6. Functional Expression of α_{1D} , α_{2b} , and β_2 in Xenopus Oocytes

(A) I_{Ba} recorded before and after application of Bay K 8644 (1 μM) in an oocyte injected with α_{1D} , α_{2b} , and β_2 mRNAs. Test pulse, –10 mV; holding potential, –50 mV.

(B) Peak current-voltage relations before (open circles) and after (closed circles) application of Bay K 8644 for the α_{1D} , α_{2b} , and β_2 mRNA-injected cell of (A). Holding potential, –50 mV.

(C) Currents before and after (+) application of nifedipine (5 μM) in an oocyte injected with α_{1D} , α_{2b} , and β_2 mRNAs. Current traces are signal averages of three traces before and three traces after application of nifedipine. Test pulse, 0 mV; holding potential, –50 mV.

(D) Currents in the absence and presence (+) of ω -CgTx (10 μM) in an oocyte injected with α_{1D} , α_{2b} , and β_2 mRNAs. Bay K 8644 (1 μM) was present throughout. Current traces are signal averages of three traces before and three traces after application of ω -CgTx for approximately 1.5 min. Test pulse, 0 mV; holding potential, –50 mV.

(E) Currents before and after application of Bay K 8644 (1 μM) in an oocyte injected with α_{2b} and β_2 mRNAs. Superimposed current traces are signal averages of four traces before and four traces after application of Bay K 8644. Test pulse, 20 mV; holding potential, –90 mV.

(F) Peak current-voltage relation for the α_{2b} and β_2 mRNA-injected cell of (E). Holding potential, –90 mV.

although the magnitude of the current was, on average, smaller. Two of four oocytes injected with $\alpha_{1D}\beta_2$ responded to Bay K 8644 application similarly to the $\alpha_{1D}\alpha_{2b}\beta_2$ -mediated currents, whereas the remaining two showed no response. Three of five oocytes coinjected with the α_{1D} and α_{2b} mRNAs displayed very small currents (15–30 nA) and were unresponsive to Bay K 8644.

To ensure that the currents observed in the $\alpha_{1D}\alpha_{2b}\beta_2$ -injected oocytes were mediated by the α_{1D} subunit, expression of the β_2 or α_{2b} subunits alone or both together was assayed. Oocytes injected with the α_{2b} mRNA displayed no detectable I_{Ba} ($n = 5$). Surprisingly, oocytes injected with β_2 mRNA displayed I_{Ba} upon depolarization (54 ± 23 nA, $n = 5$), and $\alpha_{2b}\beta_2$ -injected oocytes displayed I_{Ba} (Figure 6E) approximately 50% larger than the I_{Ba} of β_2 -injected oocytes (81 ± 60 nA, $n = 21$). Oocytes injected with the β_2 mRNA or the α_{2b} and β_2 mRNAs together displayed I_{Ba} that typically was observed first at -30 mV and that peaked at 10 – 20 mV (Figure 6F). Macroscopically, the β_2 - and $\alpha_{2b}\beta_2$ -induced currents were indistinguishable.

In contrast to the $\alpha_{1D}\alpha_{2b}\beta_2$ -mediated currents, the β_2 and $\alpha_{2b}\beta_2$ currents showed both a significant inactivation during the test pulse and a strong sensitivity to the holding potential. The I_{Ba} observed in oocytes coinjected with α_{2b} and β_2 mRNAs usually inactivated markedly during a 140 ms pulse (Figure 6E). Changing the holding potential of oocytes coinjected with the α_{2b} and β_2 mRNAs from -90 mV to -50 mV reduced the I_{Ba} $81\% \pm 15\%$ ($n = 11$). In contrast, I_{Ba} measured in oocytes coinjected with the $\alpha_{1D}\alpha_{2b}\beta_2$ mRNAs was reduced $24\% \pm 16\%$ ($n = 11$) when the holding potential was changed from -90 mV to -50 mV.

The $\alpha_{2b}\beta_2$ -mediated I_{Ba} was also pharmacologically distinct from the $\alpha_{1D}\alpha_{2b}\beta_2$ -mediated current. Oocytes coinjected with α_{2b} and β_2 mRNAs displayed I_{Ba} that was insensitive to Bay K 8644 ($n = 11$; Figure 6E). Nifedipine sensitivity was difficult to measure because of the holding potential sensitivity of both nifedipine and the $\alpha_{2b}\beta_2$ -mediated I_{Ba} . Nevertheless, two oocytes coinjected with the α_{2b} and β_2 mRNAs displayed measurable I_{Ba} (25–45 nA) when depolarized from a holding potential of -50 mV, and these currents were insensitive to nifedipine (5–10 μ M) application. The $\alpha_{2b}\beta_2$ -mediated I_{Ba} showed a sensitivity to heavy metals similar to the $\alpha_{1D}\alpha_{2b}\beta_2$ -mediated current.

Discussion

Distinct Neuronal Ca^{2+} Channel Subunits Comprise a Novel DHP-Sensitive Subtype

Our results demonstrate that the α_{1D} subunit mediates DHP-sensitive, high voltage-activated, long-lasting Ca^{2+} channel activity (Figure 6A). Significant functional expression in oocytes of the α_{1D} subunit is dependent on the coexpression of the β_2 subunit and is enhanced by coexpression with the α_{2b} subunit. The biophysical properties of activation and inactivation kinetics and voltage sensitivity of the channel formed

by the α_{1D} , α_{2b} , and β_2 subunits are generally consistent with previous characterizations of neuronal L-type Ca^{2+} channels (Bean, 1989; Hess, 1990; Swandulla et al., 1991).

Immunoprecipitation of a neuronal DHP receptor previously has revealed the presence of an α_1 , α_2 , and β subunit complex (Ahlijanian et al., 1990). As an initial step toward a detailed characterization of the multiple subtypes of neuronal voltage-dependent Ca^{2+} channels, we cloned and expressed the human neuronal α_{1D} , α_{2b} , and β_2 subunits. Characterization of these clones revealed that both the α_{1D} and β transcripts expressed in neuronal tissue are differentially processed. Alternatively spliced α_{1D} transcripts involve at least four regions: the IS6 region reported here, the cytoplasmic loop between IS6 and IIS1 (Hui et al., 1991; data not shown), the IVS3 region, and the extracellular loop between the IVS3 and IVS4 regions (Perez-Reyes et al., 1990). In addition, a recent report described a possible form of α_{1D} with a truncated carboxyl terminus, although the functional significance of this form is unknown (Hui et al., 1991). Minimally, three forms of the α_2 subunit exist (Figures 5A and 5B): α_{2a} , expressed in skeletal muscle (Ellis et al., 1988); α_{2b} , expressed in neuronal tissues; and α_{2c} , expressed in aorta. At least four forms of the β subunit also exist: β_1 , expressed in skeletal muscle; β_2 and β_3 , expressed in human brain tissue; and β_4 , detected in aorta (Figure 5C). Additional forms of the β subunit may also be expressed, as indicated by two β -specific transcripts identified in skeletal muscle (Ruth et al., 1989).

Recently, a rabbit brain α_1 subunit, designated BI, was cloned and expressed (Mori et al., 1991). Not only does this subunit differ structurally from the α_{1D} subunit (Figure 2), but the biophysical and pharmacological properties of the Ca^{2+} channel, formed by coexpression of the BI subunit with the rabbit skeletal muscle α_{2a} and β_1 subunits, differ from those of the human neuronal $\alpha_{1D}\alpha_{2b}\beta_2$ recombinant channel. The BI-mediated Ca^{2+} channel activity is insensitive to both DHPs and ω -CgTx and inactivates rapidly compared with the α_{1D} -mediated activity. The expression in *Xenopus* oocytes of both the BI-mediated and α_{1D} -mediated I_{Ba} requires the coexpression of a β subunit. Thus, two structurally and pharmacologically distinct α_1 subunits expressed in neuronal tissues require a β subunit for functional Ca^{2+} channel activity in oocytes, in contrast to the α_1 subunits expressed in cardiac (Mikami et al., 1989) and smooth muscle (Biel et al., 1990).

The α_1 subunits expressed in both cardiac and lung tissues are likely encoded by the same gene (Biel et al., 1990). This gene encodes mRNAs that direct the synthesis of DHP-sensitive Ca^{2+} channels in *Xenopus* oocytes with macroscopic biophysical properties similar to the $\alpha_{1D}\alpha_{2b}\beta_2$ channel (Mikami et al., 1989; Biel et al., 1990). However, the human neuronal $\alpha_{1D}\alpha_{2b}\beta_2$ DHP-sensitive channel has a current-voltage relation that is shifted by approximately -20 mV, and its tail currents are markedly prolonged after Bay K 8644 application compared with the cardiac and lung channel

types. A comparison of the single-channel properties might further distinguish these different DHP-sensitive L-type Ca^{2+} channels.

The β_2 Subunit Stimulates DHP-Insensitive I_{Ba} in Xenopus Oocytes

Our results suggest that the α_2 and β subunits expressed in skeletal muscle (α_{2a} and β_1) differ structurally (Figure 3; Figure 4; Figure 5) and possibly functionally from the α_2 and β subunits expressed in brain tissue (α_{2b} and β_2). Xenopus oocytes coinjected with the rabbit skeletal muscle α_{2a} and β_1 mRNAs apparently do not display I_{Ba} upon depolarization (Mori et al., 1991). This is in contrast to our observation that oocytes injected with the human neuronal β_2 mRNA alone or coinjected with the β_2 and α_{2b} mRNAs display significant I_{Ba} upon depolarization. Coexpression of the α_{2b} subunit enhances the I_{Ba} , but α_{2b} mRNA shows no activity when injected alone.

The Ca^{2+} channel expressed in $\alpha_{2b}\beta_2$ -injected oocytes has pharmacological and biophysical properties that resemble Xenopus oocyte endogenous voltage-dependent Ca^{2+} channels (Dascal et al., 1986). Similar to the skeletal muscle β_1 subunit (Ruth et al., 1989), the β_2 subunit lacks hydrophobic segments capable of forming transmembrane domains. Thus, it is unlikely that the β_2 subunit alone is forming an ion channel. It is more probable that a homologous α_1 subunit exists in oocytes comprising an endogenous Ca^{2+} channel and that the activity mediated by this α_1 subunit is enhanced by the expression of the β_2 subunit, similar to that observed for the α_{1D} and BI activities. Further information concerning the structure of the endogenous Xenopus oocyte Ca^{2+} channel is not yet available.

The Ca^{2+} channel stimulated by the presence of the β_2 subunit may contribute an inactivating, DHP-insensitive component of I_{Ba} to the total current in $\alpha_{1D}\alpha_{2b}\beta_2$ -injected oocytes, especially when recorded from strongly negative holding potentials. Recordings made from $\alpha_{1D}\alpha_{2b}\beta_2$ -injected oocytes at different holding potentials support this possibility and indicate that such contamination can be reduced, though not necessarily eliminated, by holding at -50 mV. The DHP-insensitive β_2 -mediated current may account for the residual inactivating I_{Ba} detected in $\alpha_{1D}\alpha_{2b}\beta_2$ -injected oocytes in the presence of nifedipine (Figure 6C).

ω -CgTx Interacts with the Neuronal DHP-Sensitive Ca^{2+} Channel

ω -CgTx blocks neuronal N-type Ca^{2+} channels irreversibly (Feldman et al., 1987; McCleskey et al., 1987). In contrast to this high affinity block, ω -CgTx blocks the $\alpha_{1D}\alpha_{2b}\beta_2$ channel reversibly with an affinity probably in the micromolar range, as indicated by the partial block with 10 – 15 μM ω -CgTx. Although preliminary experiments indicate that the $\alpha_{2b}\beta_2$ -mediated channel may be inhibited by ω -CgTx, block of Bay K 8644-induced tail currents in $\alpha_{1D}\alpha_{2b}\beta_2$ -injected oocytes demonstrates that ω -CgTx also interacts with the

DHP-sensitive $\alpha_{1D}\alpha_{2b}\beta_2$ channel. Reversible block by ω -CgTx of L-type (Aosaki and Kasai, 1989), T-type (McCleskey et al., 1987), and a subclass of N-type (Plummer et al., 1989) Ca^{2+} channels has been reported. Furthermore, Ca^{2+} -dependent ATP release from elasmobranch electroplax synaptosomes is blocked reversibly by ω -CgTx with micromolar affinity (Yeager et al., 1987). It thus appears that variable affinity for ω -CgTx may be shared by several types of voltage-dependent Ca^{2+} channels. A weak block such as we have demonstrated for the $\alpha_{1D}\alpha_{2b}\beta_2$ L-type channel may account for the conflicting results reported in the literature concerning the ability of ω -CgTx to block neuronal L-type channels (McCleskey et al., 1987; Suzuki and Yoshioka, 1987; Aosaki and Kasai, 1989; Plummer et al., 1989).

Conclusion

The function of DHP-sensitive Ca^{2+} channels in skeletal and cardiac muscle has been extensively studied (Hess, 1990). In contrast, the role of the neuronal L-type Ca^{2+} channel is poorly understood (Miller, 1987). L-type Ca^{2+} channels may mediate the release of neurotransmitters from some types of neurons (Holz et al., 1988). However, functional analysis is difficult due to the mixed population of voltage-dependent Ca^{2+} channel subtypes in continuous cell lines as well as cells in primary tissues. For example, L-type Ca^{2+} channels contribute a minor fraction of the I_{Ba} detectable in the cell bodies of IMR32 cells (Carbone et al., 1990; unpublished data), PC12 cells (Plummer et al., 1989), certain sensory neurons (Aosaki and Kasai, 1989), and sympathetic neurons (Plummer et al., 1989; Jones and Jacobs, 1990). In contrast, L-type channels contribute substantial I_{Ba} in some populations of sensory neurons (Scroggs and Fox, 1991) and certain CNS neurons (Mogul and Fox, 1991; Regan et al., 1991).

Our characterization of a novel human neuronal voltage-dependent Ca^{2+} channel firmly establishes the existence of multiple subtypes of DHP-sensitive L-type Ca^{2+} channels. Furthermore, this human neuronal Ca^{2+} channel appears to have functional and pharmacological properties distinct from any other recombinant Ca^{2+} channel expressed to date. Together with the evidence for differentially processed mRNAs encoding three subunits of voltage-dependent Ca^{2+} channels, these results indicate that the molecular diversity of this ion channel class is much greater than previously proposed by traditional biophysical and pharmacological studies.

Experimental Procedures

Nomenclature

The following nomenclature is used for the α_1 gene family and the differentially processed α_2 and β transcripts. The structurally distinct human neuronal α_1 gene product described here is designated α_{1D} in accordance with its 96.3% deduced amino acid sequence identity to the rat brain class D sequence (Snutch et al., 1990). The α_2 gene product expressed in skeletal muscle is designated α_{2a} ; the differentially processed α_2 transcript expressed in neuronal tissues is designated α_{2b} ; the aorta α_2 tran-

script is designated α_{2c} . The β gene product expressed in skeletal muscle is designated β_1 ; the β transcript expressed in neuronal tissues is differentially processed to produce β_2 and β_3 transcripts. An additional β transcript expressed in aorta is designated β_4 .

cDNA Libraries

Recombinant cDNA libraries were prepared, and individual cDNA clones were characterized essentially as previously described by Ellis et al. (1988). Unless otherwise noted, the nucleotide numbers in the text refer to cDNA coding sequence. For the isolation of human neuronal α_{1D} subunit cDNAs, RNA was isolated from the human neuroblastoma IMR32 cell line (ATCC #CCL127), which had been grown in 1.0 mM dibutyryl cAMP for 10 days. Four different cDNA libraries were constructed into the phage vector λ gt11: oligo(dT)-primed double-stranded cDNA, 1–3 kb size fractionated by agarose gel electrophoresis; oligo(dT)-primed double-stranded cDNA, 3–9 kb size fractionated; random-primed double-stranded cDNA, >1.8 kb; and specifically primed (nucleotides 2417–2446 of α_{1D}) double-stranded cDNA, >1.5 kb. Human neuronal α_{2b} subunit cDNAs were isolated from a human basal ganglia cDNA library (ATCC #37433) and a human brain stem cDNA library (ATCC #37432). Human brain β_2 and β_3 subunit cDNAs were isolated from a human hippocampus cDNA library constructed in the λ phage vector λ ZAPII (Stratagene, La Jolla, CA, #936205).

Isolation of Recombinant cDNAs Encoding Different Ca^{2+}

Channel Subunits

α_{1D} Subunit

Approximately 1×10^6 recombinants of the 1–3 kb library were screened with the rabbit skeletal muscle α_1 subunit cDNA (Ellis et al., 1988). Clone $\lambda\alpha 1.36$ (nucleotides 2347–3771 of α_{1D}) was isolated and characterized, and the insert was used to screen the 3–9 kb library. Clone $\lambda\alpha 1.80$ (nucleotides 1573–5958) was isolated and characterized, and the 3' portion of the insert was used to screen the random-primed library from which clone $\lambda\alpha 1.163$ (nucleotides 4690–7125) was isolated. The 5' portion of $\alpha 1.80$ was subsequently used to screen the random-primed library, resulting in the isolation of clone $\lambda\alpha 1.144$ (nucleotides –510 to 1921). The 5' portion of $\alpha 1.80$ was then used to screen the specifically primed library from which clone $\lambda\alpha 1.136$ (nucleotides 1117–2478) was isolated.

α_{2b} Subunit

Human genomic α_2 clones were isolated to use as α_2 -specific probes of human neuronal cDNA libraries. A rabbit skeletal muscle α_{2a} cDNA fragment, clone SkMCaCH α 2.2, comprising nucleotides 43–272 (Ellis et al., 1988), was used to identify and clone two α_2 -specific, human genomic EcoRI fragments, HGCaCH α 2.20 (3.5 kb) and HGCaCH α 2.29 (3.0 kb). Restriction mapping and DNA sequencing revealed that HGCaCH α 2.20 contains an 82 bp exon (nucleotides 96–177 of the human α_{2b} coding sequence) and that HGCaCH α 2.29 contains 105 bp of an exon (nucleotides 178–282 of the coding sequence). These restriction fragments were used to screen the human basal ganglia cDNA library. HBCaCH α 2.1 was isolated (nucleotides –6 to 1129) and used to screen the human brain stem cDNA library. Two clones were isolated, HBCaCH α 2.5 (nucleotides –34 to 1128) and HBCaCH α 2.8 (nucleotides 680–1528 followed by 1600 nucleotides of intervening sequence). HBCaCH α 2.8 was used to rescreen the brain stem library and to isolate HBCaCH α 2.11 (nucleotides 845–3566).

β_2 and β_3 Subunits

A rabbit skeletal muscle β_1 subunit cDNA fragment (Ellis et al., 1988; Ruth et al., 1989) was used to screen the human hippocampus cDNA library. Two clones, $\lambda\beta 1$ and $\lambda\beta 4$, were isolated that appear to encode alternative splice products of the human β subunit transcript expressed in the brain, β_2 and β_3 , respectively. $\lambda\beta 1$ begins at nucleotides 69 and extends 107 nucleotides beyond the translation stop codon, encoding 1367 nucleotides of coding sequence. $\lambda\beta 1$ also contains a 448 nucleotide intron between nucleotides 1146 and 1147 of the coding sequence. $\lambda\beta 4$ begins at nucleotide 246 of the coding sequence and diverges from β_2 at nucleotide 1333 as described in the Results. $\lambda\beta 1$ was used to

rescreen the hippocampus cDNA library from which clone $\lambda\beta 1.18$ was isolated, characterized, and determined to encode nucleotides 1–325 of the β_2 coding sequence.

PCR Analysis

PCR analyses were performed essentially as described by Innis et al. (1990). IMR32 cell cytoplasmic RNA was prepared as described by Ausubel et al. (1988). For the analysis of the series of 5' methionine codons in the α_{1D} cDNA, four oligonucleotide primers were synthesized (numbered in the 5' to 3' orientation): (1) nucleotides –39 to –18, beginning 39 nucleotides 5' of the first methionine codon; (2) nucleotides 58–81; (3) nucleotides 164–187; and (4) nucleotides 314–291. The oligonucleotide pairs (1, 4), (2, 4), and (3, 4) were used to prime PCR assays of cytoplasmic RNA and human genomic DNA. PCR amplification of human genomic DNA and IMR32 cytoplasmic RNA with oligonucleotide pairs (2, 4) and (3, 4) gave the predicted size product (260 and 150 bp, respectively). The cytoplasmic RNA assayed with the pair (1, 4) gave the predicted size product (350 bp); a PCR product of genomic DNA primed with the pair (1, 4) was not detected. The lack of a PCR product primed with pair (1, 4) on genomic DNA suggested the possible presence of an intron between oligonucleotides 1 and 2 and indicated that the positive results with the RNAs could not be due to genomic DNA contamination of the RNA preparations. The cytoplasmic RNA PCR product of the (1, 4) oligonucleotide pair was cloned and sequenced.

Construction of Full-Length cDNAs

α_{1D} Subunit

pVDCIII(A) was constructed using $\alpha 1.144$ (nucleotides –184 to 1222), $\alpha 1.136$ (nucleotides 1222–2157), $\alpha 1.80$ (nucleotides 2157–4784), and $\alpha 1.163$ (nucleotides 4784–7125). PCR analysis of the α_{1D} transcript revealed that $\alpha 1.80$ contained a 148 nucleotide deletion (nucleotides 2474–2621). To correct this deletion, PCR was performed on IMR32 RNA, and the Accl-BgIII fragment (nucleotides 2254–3380) was isolated and used to replace the $\alpha 1.80$ fragment.

α_{2b} Subunit

pHBCaCH α 2.5(A) was constructed using HBCaCH α 2.5 (nucleotides –34 to 1027) and HBCaCH α 2.11 (nucleotides 1027–3566).

β_2 Subunit

To construct pHBCaCH β_2 .RBS(A), the 448 nucleotide intron of $\lambda\beta 1$ first was deleted via site-directed mutagenesis (Sambrook et al., 1989). $\lambda\beta 1$ was subcloned into M13mp19. The mutagenic oligonucleotide was the sense strand of β_2 encoding nucleotides 1128–1165. The final construct was designated p $\beta 1$ (–). pHBCaCH β_2 .RBS(A) then was constructed using $\lambda\beta 1.18$ (nucleotides 1–282) and p $\beta 1$ (–) (nucleotides 282–1547). The 5' untranslated sequence in $\lambda\beta 1.18$ was replaced with an efficient ribosomal-binding site so that the sequence reads 5'-GAATT (EcoRI) ACCACC (ribosomal-binding site) ATG (start codon) ... –3'. Each α_{1D} , α_{2b} , and β_2 full-length construct was subcloned into pcDNA1 (Invitrogen, San Diego, CA).

Expression Studies in *Xenopus* Oocytes

In vitro transcripts of human neuronal α_{1D} , α_{2b} , and β_2 subunit cDNAs were synthesized according to the instructions of the mCAP mRNA Capping Kit (Stratagene, La Jolla, CA, #200350). Each plasmid first was linearized by restriction digestion: pVDCIII(A) with Xhol, pHBCaCH α 2.5(A) with Xhol, and pHBCaCH β_2 .RBS(A) with EcoRV. T7 RNA polymerase was used to transcribe each cDNA. *Xenopus laevis* oocytes were dissociated and defolliculated by collagenase treatment and maintained in 100 mM NaCl, 2 mM KCl, 1.8 mM CaCl₂, 1 mM MgCl₂, 5 mM HEPES (pH 7.6), 20 μ g/ml ampicillin, and 25 μ g/ml streptomycin at 19°C–25°C for 2–5 days after injection and prior to recording. Oocytes were injected with 6 ng of each in vitro synthesized mRNA species per cell in a volume of 50 nl and were assayed by the two-electrode voltage-clamp method (Dascal, 1987) using the pClamp (Axon Instruments) software package in conjunction with a Labmaster 125 kHz data acquisition interface (Scientific Solutions) to generate voltage commands and to acquire and analyze data. Current signals were digitized at 1–5 kHz and filtered appropriately. I_{B_2}

was recorded in a solution intended to minimize currents carried through K^+ , Cl^- , or Na^+ channels (Snutch et al., 1990): 40 mM BaCl₂, 36 mM tetraethylammonium chloride, 2 mM KCl, 5 mM 4-aminopyridine, 0.15 mM niflumic acid, 5 mM HEPES (pH 7.6). Currents were leak subtracted by the P/n method provided in pClamp, where n was -4 or -6. Drugs were applied directly into the 60 μ l bath while the perfusion pump was turned off. Bay K 8644 and nifedipine were prepared fresh from stock solutions (in dimethyl sulfoxide) and diluted into the bath solution. The dimethyl sulfoxide concentration of the final drug solutions in contact with the cell never exceeded 0.1%. Control experiments showed that 0.1% dimethyl sulfoxide had no effect on membrane currents. ω -CgTx was prepared in a 15 mM BaCl₂ bath solution plus 0.1% cytochrome C (Sigma) (Feldman et al., 1987) to serve as a carrier protein. Control experiments showed that cytochrome C had no effect on currents. Before and during ω -CgTx application, test pulses were recorded at 20 s intervals from the holding potential (-90 mV or -50 mV) to the peak I_{Ba} (-10 mV to 10 mV). To reduce the inhibition of ω -CgTx binding by divalent cations (McCleskey et al., 1987), recordings were made in 15 mM BaCl₂, 73.5 mM tetraethylammonium chloride, and the remaining ingredients identical to the 40 mM Ba²⁺ recording solution.

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GenBank Accession Numbers

The nucleotide sequences of the human α_{1D} , α_{2B} , and β_2 cDNAs will appear in the EMBL, GenBank, and DDBJ nucleotide sequence data bases under the accession numbers M76558 (α_{1D}), M76559 (α_{2B}), and M76560 (β_2).