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A *Drosophila* Calcium Channel α1 Subunit Gene Maps to a Genetic Locus Associated with Behavioral and Visual Defects

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We have cloned cDNAs that encode a complete open reading frame for a calcium channel α1 subunit from *Drosophila melanogaster*. The deduced 1851 amino acid protein belongs to the superfamily of voltage-gated sodium and calcium channels. Phylogenetic analysis shows that the sequence of this subunit is relatively distant from sodium channel α subunits and most similar to genes encoding the A, B, and E isoforms of calcium channel α1 subunits. To indicate its similarity to this subfamily of vertebrate isoforms, we name this protein Dmca1A, for *Drosophila melanogaster* calcium channel α1 subunit, type A. Northern blot analysis detected a single 10.5 kb transcript class that is regulated developmentally, with expression peaks in the first larval instar, midpupal, and late pupal stages. In late-stage embryos, Dmca1A is expressed preferentially in the nervous system. Variant transcripts are generated by alternative splicing. In addition, single nucleotide variations between cDNAs and genomic sequence are consistent with RNA editing. Dmca1A maps to a chromosomal region implicated in, and is the likely candidate for, the gene involved in the generation of behavioral, physiological, and lethal phenotypes of the *cacophony, nightblind-A*, and *lethal(1)L13* mutants.

Key words: cDNA sequence; RNA editing; alternative splicing; phenylalkylamine binding site; chromosome aberrations; vital gene

Calcium channels are involved in functions including membrane excitability, synaptic transmission, regulated secretion, and cell differentiation. They conduct currents with heterogeneous conductances, kinetics, and pharmacological sensitivities (Hille, 1992). Calcium channels are hetero-oligomeric assemblies of α1, α2, δ, β, and γ subunits (Campbell et al., 1988; Catterall et al., 1988; Ahlijanian et al., 1990; McEnergy et al., 1991; Witcher et al., 1993; Leveque et al., 1994); the α1 subunit forms the calcium-conducting pore of the channel. Channel diversity is generated by multiple genes, alternative splicing of transcripts from a given gene, and perhaps by combinatorial assembly of variant isoforms of the subunits (reviewed in Hofmann et al., 1994; Stea et al., 1995).

There is evidence for calcium channel diversity in *Drosophila melanogaster*. Both high- and low-affinity binding of phenylalkylamines were identified in *Drosophila* head extracts (Pauron et al., 1987; Greenberg et al., 1989). In cultured *Drosophila* embryonic neurons and myocytes, cell-attached patch-clamp studies have identified currents with variable properties, including inactivating and noninactivating barium currents with differential sensitivity to purified *Hololena* spider toxin (HoTX) (Leung et al., 1989; Leung and Byerly, 1991). Reconstitution of *Drosophila* head membrane extracts into artificial bilayers revealed calcium conductances with eight distinct conductance levels; some classes were sensitive to dihydropyridines and others to phenylalkylamines (Pelzer et al., 1989). Gielow et al. (1995) distinguished whole-cell calcium currents in *Drosophila* larval body wall muscles with differential sensitivity to dihydropyridines and amiloride.

Genetic studies of calcium channels are beginning to define the functional significance of various α1 subunits. An α1 subunit (Dmca1D) similar to L-type vertebrate channels has been cloned from *Drosophila melanogaster* (Zheng et al., 1995). Mutations in the Dmca1D gene cause embryonic lethality (D. F. Eberl and L. M. Hall, unpublished observations). A partial α1 cDNA sequence from the *Caenorhabditis elegans unc-2* locus has similarity to vertebrate non-L-type channels, and mutations in this gene disrupt physiological adaptation to dopamine and serotonin (Schafer and Kenyon, 1995). A single-base deletion mutation leading to a frame shift in a skeletal muscle α1 subunit gene has been found in the *muscular dysgenesis* mutant mouse (Chaudhari, 1992).

We have been analyzing a genetic locus defined by the courtship song mutant *cacophony*, the visually defective *nightblind-A* mutants, and by *lethal(1)L13* variants. We report here the identification and molecular analysis of a calcium channel α1 subunit. This is only the fourth such subunit cloned from invertebrates (see above; see also, Grabner et al., 1994). The Dmca1A transcript spans deletion and inversion breakpoints associated with, and is therefore likely a product of the gene responsible for, these genetic variants. In addition to providing further evidence pertaining to invertebrate calcium-channel diversity, this new α1 subunit gene may permit genetically based studies of α1 subunit variation and its connection to these behavioral- and visual system-specific phenotypes.

MATERIALS AND METHODS

cDNA cloning. As part of an analysis of transcripts from the cytogenetic region 11A1–2, known to encode several genes of interest, clone pNB53 was isolated from a 12–24 hr embryonic cDNA library (Brown and

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Kafatos, 1988). It was subcloned into pBS$^{(+)}$ (Stratagene, La Jolla, CA) after a complete *Not*I and partial *Hin*dIII digestion to generate the clone cSK53. Subclones generated by restriction digestions and by digestion into 200–500 nucleotide fragments with DNAaseI in the presence of manganese (Sambrook et al., 1989) were ligated into pBluescript IISK$^{(+)}$ for sequencing. Additional sequence was obtained from cSK53 with insert-specific primers.

A *Cla*I fragment of cSK53 corresponding to the region encoding amino acids 603–749 in Dmca1A in Figure 2 was used to probe 1.2×10^6 pfu of a λ *gt*11 *Drosophila* head cDNA library (Itoh et al., 1986), resulting in the acquisition of 11 clones. One of them, c31, extended the sequence in the 5′ direction but still was missing the 5′ end of the open reading frame. Two probes, corresponding to the regions encoding amino acids 259–637 and 608–1076 in Dmca1A (Fig. 2), were generated by PCR from clones c31 and cSK53, respectively; these were used together to screen 1.2×10^6 pfu from a λ-zapII *Drosophila* head cDNA library (DiAntonio et al., 1993) to isolate the 5′ clones cS14a and cS25a, as well as 43 additional partial cDNAs; the latter included cS26a and cS29b. These were excised into pBluescript IIKS$^{(+)}$ and sequenced with vector- and insert-specific primers.

Nineteen of 45 cDNAs isolated from the original screen of the λ-zap library could not be subcloned into pBluescript IIKS$^{(+)}$. Suspecting that some of these might represent 3′ cDNAs, we screened excised filamentous phage supernatants by PCR, using a 5′ insert-specific primer corresponding to the region encoding amino acids 1139–1145 and T3 or T7 vector-specific primers (cf. Chiang et al., 1994) to detect the inclusion and size of potential 3′ clones. Clones cS9a and cS11 were found by this analysis to extend 3′ to the existing cDNAs. We were unable to propagate cDNAs containing the 3′ ends of the open reading frame in plasmid vectors, so they were sequenced directly from PCR products.

Clone c3p1 (which extended 520 bases past the 3′ end of cS9a) was obtained in a screen of an additional 2.5×10^5 pfu from the λ-zap library probed with a PCR product generated with cS9a as template; that probe corresponds to the region encoding amino acids 1556–1802 in Dmca1A. Clone c3p1 was sequenced as described for cS9a and cS11 above.

Preparation of probes and sequencing templates. Probes for cDNA screens were labeled with ^{32}P-dCTP by random priming by either the Random Primer DNA Labeling System (BRL, Grand Island, NY) or Prime-IT II (Stratagene). PCR products were purified for labeling or sequencing either directly with the QIAquickSpin PCR Purification Kit (Qiagen, Chatsworth, CA) or after gel purification with the QIAEXII Gel Purification Kit. All sequencing used double-stranded templates prepared either with the Qiagen Plasmid Kit or by alkaline lysis and LiCl precipitation (Sambrook et al., 1989). Most sequencing was done on an ABI 373A sequencer using vector- or gene-specific primers with the PRISM DyeDeoxy Terminator Cycle Sequencing Kit [Applied Biosystems (ABI), Foster City, CA] and/or using labeled T3 or T7 primers with the *Taq* Dye Primer Cycle Sequencing kit (ABI). DNA was sequenced at least twice in each direction, except as noted in Results. When sequencing was conducted from PCR products, sequence was derived from at least two independent reactions. Sequence analysis and contig assembly were done by the GCG package of programs (Genetics Computer Group, 1991). Database searches were performed by the BLAST network service at National Center for Biotechnology Information.

RNA preparation and Northern blots. Samples from different developmental stages were grown, collected, and synchronized at 25°C, as described by Ashburner and Thompson (1978). Preparation of poly(A$^+$) mRNA, Northern blots, and hybridization conditions were as described in Zheng et al. (1995). Transcript abundances were quantitated with an UltroScan scanning laser densitometer with GelScan XL software, version 2.1 (Pharmacia, Piscataway, NJ).

In situ *hybridization to embryo whole mounts.* Whole-mount *in situ* hybridization to *Drosophila* embryos (stage 16) was done as described by Tautz and Pfeifle (1989). A single-stranded 245-base digoxigenin-labeled cDNA probe (corresponding to the region encoding amino acids 970–1052 in Dmca1A in Fig. 2) was prepared and applied as described by Zheng et al. (1995).

Southern blotting. The deletion *Df(1)HF368*, inversion *In(1)N66*, and balancer chromosome *In(1)FM7,B* carried in flies that were the DNA source for this experiment are described in Goralski (1985), Kulkarni and Hall (1987), and Lindsley and Zimm (1992). One breakpoint of *In(1)N66* is in cytogenetic region 11A2; this rearrangement fails to complement the phenotypes of *cacophony, nightblind-A*, and *l(1)L13* mutations (Kulkarni and Hall, 1987; Homyk and Pye, 1989). *Df(1)HF368* also is broken in 11A2 and fails to complement these mutations; this deletion removes a portion of the chromosome toward the centromere from 11A2. Preparation of DNA and probe, restriction digestions, blotting, and hybridization were performed as described in Sambrook et al. (1989). Five micrograms of genomic DNA were electrophoresed in each lane. The template for making probe was prepared by digesting the genomic phage clone 320 (cf. Goralski, 1985) with *Eco*RI, followed by electrophoretic purification of the insert. This genomic clone is homologous to portions of cDNA clones c31, cS14a, cS25a, and cS11 (see Fig. 1). The ultimate autoradiograph was scanned with a ScanJet IIc scanner and DeskScan II software (Hewlett-Packard). The scanned image was filtered to reduce mid-densities (thus reducing background), and the figure was printed from the scanned image by a commercial pictrography service (Pageworks, Cambridge, MA).

RESULTS

Isolation of cDNAs encoding a new calcium channel α1 subunit

The cDNA encoding the calcium channel α1 subunit reported here was isolated during the analysis of a region of the X chromosome known to contain the gene *cacophony* (*cac*) and the interacting genetic variants *nightblind-A* (*nbA*) and *lethal(1)L13* (also known as *l(1)11Aa*; Lindsley and Zimm, 1992). The *cac* locus was mapped cytogenetically using inversions and deletions to the X chromosomal region 11A2 (Kulkarni and Hall, 1987). Genomic phage clones 320 and 0371 (Goralski, 1985) were derived from a chromosome walk through the flanking *gastrulation defective* (*gd*) locus (which is ~10 kb from the left-hand, centromere-proximal end of the putative *cac*-locus, as depicted in Fig. 1). Clones 320 and 0371 recognized restriction-fragment-length polymorphisms (RFLPs) associated with breakpoints in this region [Goralski (1985); also see Fig. 7, below]. Northern blots of adult RNA probed with fragments of clone 320 recognized two transcripts: 0.8 kb (Goralski, 1985) and >10 kb (our preliminary data, not shown; also see Fig. 1 legend and Fig. 4, below). To clone cDNAs encoding these transcripts, we probed a 12–24 hr embryonic cDNA library (Brown and Kafatos, 1988) with genomic clone 0371 and isolated the cDNA clone cSK53 (Fig. 1). *In situ* hybridization of cSK53 to salivary gland chromosomes from third-instar larvae mapped this cDNA to the distal portion of region 11A, consistent with the origin of the original genomic probes (data not shown). Northern blotting showed that the cSK53 cDNA corresponds to a subset of the large mRNA transcribed from this X chromosomal region (see below, Fig. 4). The genomic interval that gives rise to the coding (plus untranslated) RNA indicated on the top line of Figure 1 is approximately eight times longer than the amount of sequence so depicted (see Fig. 1 legend).

Sequence analysis showed that cSK53 contains an open reading frame (ORF) encoding a fragment similar to a voltage-sensitive calcium channel α1 subunit (Fig. 2). Several rounds of cDNA isolation from *Drosophila* head cDNA libraries, starting with a probe derived from cSK53 and continuing in later rounds with probes from newly isolated cDNAs, isolated a total of 57 cDNAs. A subset of these was chosen for further analysis based on length and overlap with other cDNAs (Fig. 1). A 6522 nucleotide cDNA contig—assembled from the overlapping cDNAs cS14a, cS9a, and c3p1—contains a single large ORF of 5553 nucleotides, which encodes a voltage-sensitive calcium channel α1 subunit (Fig. 2). An AUG at nucleotide positions 553–555 is the only in-frame methionine codon between five upstream in-frame stop codons and sequences coding for the first transmembrane domain (IS1). The sequence flanking this methionine codon (UAGA AUG) shows two of four matches to the *Drosophila* translation initiation consensus sequence (C/A AA A/C AUG) (Cavener, 1987). It includes the highly conserved A at the −3 position, and the G at −2 is the second most frequently used nucleotide at this position.

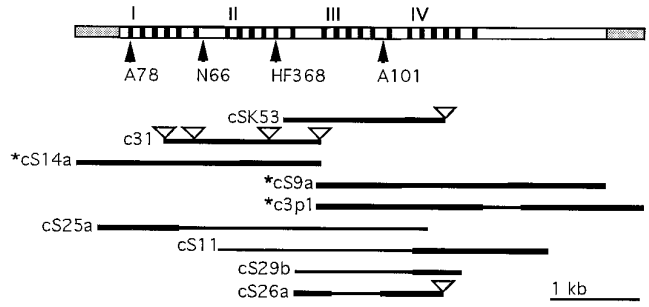

Figure 1. Overlapping cDNAs used to deduce the full-length ORF of Dmca1A. The diagram at the *top* of this figure shows the organization of the Dmca1A calcium channel α1 subunit ORF (in *white*), untranslated regions (UTRs, in *gray*), and the approximate locations of transmembrane domains (in *black*). *Roman numerals* indicate the beginning of each of the four repeat domains. Below this diagram, *bold black lines* show cloned cDNAs for which sequence was determined at least twice in each direction. *Narrow black lines* indicate regions determined by partial sequence analysis. *Triangles* show the location of unspliced introns in the indicated cDNA, as determined by comparison with genomic sequence and other cDNAs. Sequences used to assemble the full-length Dmca1A sequence shown in Figure 2 are marked with an *asterisk*. *Arrowheads* indicate the location of exons that we have mapped to genomic restriction fragments; these, in turn, were shown previously and contemporaneously by RFLP detection (Goralski, 1985; Fig. 7 of the current study) to contain an array of four 11A2 region breakpoints, associated with the following chromosome rearrangements (indicated in abbreviated form in the figure): *In(1)A78, In(1)N66*, and *In(1)A101* are chromosomal inversions that fail to complement *l(1)L13, cacophony*, and *nightblind-A* mutants (Kulkarni and Hall, 1987; Homyk and Pye, 1989). *Df(1)HF368* is a chromosomal deletion that also fails to complement these mutations (see papers just cited) and removes sequences 5′ to the indicated breakpoint (Goralski, 1985); the sequences removed are centromere-proximal to the *HF368* breakpoint (i.e., the 5′ end of the coding sequences implied by the *top line* is shown to the *left*, as usual, which makes the centromere end of the X chromosome to the *left* of this image; the *gd* gene is located centromere-proximal to, i.e., leftward of, the transcription unit shown). Phage clone 320 (see Materials and Methods) spans a genomic interval that encodes a portion of the channel running from approximately IS5 to IIS5. Clone 320 hybridizes to genomic restriction fragments containing the *In(1)N66* and *Df(1)HF368* breakpoints (Fig. 7, below); the total genomic distance between the chromosomal breakpoints flanking the two (more central lesions) just indicated is ~15 kb (Goralski, 1985); owing to the fact that this gene is especially intron-rich in genomic intervals to the *left* of the A*78* breakpoint and to the *right* of the A*101* one, the coding material and UTR shown (*top line*) arise from a ~50 kb genomic interval (Peixoto, Smith, Hall, Hall, unpublished observations). Clone 320 also was determined by Northern blotting to hybridize to an 0.8 kb transcript (Goralski, 1985). Sequence analysis of an 0.8 kb cDNA hybridizing to this clone reveals short regions of identity to the Dmca1A transcript (Smith and Hall, unpublished observations). This implies that the 0.8 kb transcript, which is the only other transcript detected in this region (also see Fig. 4, below), might be an aberrant form of the Dmca1A transcript.

Thus, we infer this AUG to be the translation start site. Although we have no evidence to suggest alternate initiation methionines, we cannot rule out the possibility of alternative 5′ exons in transcripts not represented in this analysis.

The first in-frame stop codon is a TAG at nucleotide position 6106. It is followed by two additional in-frame stop codons within the next 50 nucleotides. The 3′ untranslated region of this contig is 416 nucleotides in length. There is no polyadenylation signal or polyadenylated tract in this contig, suggesting that the 3′ untranslated region is incomplete. This suggestion is consistent with the difference between the length of the transcript (10.5 kb) and the assembled contig (6.5 kb).

Structure of the calcium channel α1 subunit protein

The ORF of 5553 nucleotides encodes a protein of 1851 amino acids, with a calculated molecular weight of 212,155. The protein has the canonical structure of voltage-gated calcium channel α1 and sodium channel α subunits, with four internal repeats (I–IV), each containing six presumed membrane-spanning hydrophobic domains (S1–S6). Transmembrane segments S4 of each internal repeat contain positively charged amino acids every third or fourth amino acid, consistent with the postulated role of these segments in sensing and responding to transmembrane voltage changes. In addition, the conserved domains for short segments 1 and 2 (ss1, ss2) in the loop between transmembrane domains S5 and S6 of each repeat are conserved in this protein.

Comparison of both the overall protein and of these conserved domains reveals a strikingly greater similarity to calcium channel α1 subunits than to sodium channel α subunits (see below, Fig. 6). A conserved glutamate present in ss2 in the loop between transmembrane domains S5 and S6 of each repeat is involved in ion selectivity (Kim et al., 1993; Tang et al., 1993a; Yang et al., 1993). Sodium channels contain this glutamate residue only in repeats I and II, whereas calcium channels have this glutamate in all four repeats (Heinemann et al., 1992). Changing the appropriate residue to glutamate in repeats III and IV of a sodium channel converts the ion selectivity of a sodium channel to that of a calcium channel (Heinemann et al., 1992). Conversely, changing the identity of these glutamate residues alters the ion selectivity and conductance of calcium channels (Mikala et al., 1993; Tang et al., 1993a; Yang et al., 1993; Ellinor et al., 1995). The glutamate residues relevant to ion selectivity are conserved in all four ss2 domains of the Dmca1A protein, consistent with identification of this protein as a calcium channel α1 subunit.

Possible phenylalkylamine binding site in Dmca1A

On the basis of immunoprecipitation of phenylalkylamine-labeled proteolytic peptide fragments, Striessnig et al. (1990) proposed that a fragment of the rabbit skeletal muscle α1 subunit, including transmembrane domain IVS6 and the adjacent intra- and extracellular sequences, functions as a binding site for the phenylalkylamine calcium channel blockers. Combined with previous work suggesting that phenylalkylamines block calcium channels intracellularly, this evidence identified the intracellular portion of the IVS6 transmembrane domain and the adjacent intracellular amino acids as a binding site for phenylalkylamines. Figure 2 shows the proposed phenylalkylamine binding fragment sequence defined by Striessnig et al. (1990) aligned above the Dmca1A sequence. A 17 amino acid sequence bracketing the intracellular junction of the IVS6 transmembrane domain is conserved completely between these two proteins. This conserved region is flanked on the N-terminal side by two conservative amino acid changes (isoleucine to leucine and isoleucine to methionine), preceded by two more identical amino acids (FL). The region extends to within two amino acids of the C-terminal end of the rabbit proteolytic fragment, where there is a nonconservative tryptophan-to-serine change in the Dmca1A sequence, followed by a conserved serine.

Dihydropyridine binding sites are poorly conserved in Dmca1A

Proteolytic fragments containing the IIIS6 and IVS6 transmembrane domains and regions immediately adjacent to them have been shown to bind dihydropyridines (Nakayama et al., 1991; Striessnig et al., 1991). Dihydropyridine sensitivity of an L-type

channel is abolished when a portion of the polypeptide overlapping the extracellular end of IVS6 is replaced with non-L-type sequence (Tang et al., 1993b). Conversely, dihydropyridine sensitivity can be conferred upon a non-L-type channel by replacing the IIIS5–S6 and IVS5–S6 regions with sequences from L-type (carp or rabbit) skeletal muscle subunits (Grabner et al., 1996). Because dihydropyridines bind to the channel from the outside (Bangalore et al., 1994), the portions of these fragments that begin in the extracellular domain and enter into the transmembrane segments from the outside are, most likely, involved in dihydropyridine binding.

The sequences from a dihydropyridine-sensitive carp skeletal muscle α1 subunit that confer dihydropyridine sensitivity to chimeric channels (see above) are shown in Figure 2, aligned below Dmca1A in the IIIS5–S6 and IVS5–S6 regions. Certain amino acids in the dihydropyridine-sensitive carp skeletal muscle subunit (underlined in the figure) were identified by Grabner et al. (1996) as potentially relevant to dihydropyridine sensitivity. In the region of IIIS5–S6 and IVS5–S6, there are 102 such amino acids. Of these, Dmca1A is identical to dihydropyridine-sensitive channels at only 18 sites. By comparing all known dihydropyridine-sensitive and -resistant channels, Grabner et al. (1996) also identified 23 positions within these regions where the amino acid was different between dihydropyridine-resistant and -sensitive vertebrate α1 subunit, but 100% identical within the resistant and sensitive subgroups. Of these 23 amino acids, Dmca1A was identical to the dihydropyridine-resistant channels at 20 sites and showed identity to the sensitive channels at only three sites. The lack of correspondence between Dmca1A and dihydropyridine-sensitive channels at these positions suggests that the *Drosophila* Dmca1A α1 subunit may be insensitive to dihydropyridines.

Calcium binding EF-hand motif in Dmca1A

Calcium and sodium channels often contain in their C-terminal intracellular regions an EF-hand motif, which forms a structure of two α helices flanking a calcium-binding loop (Babitch, 1990). This motif has been correlated functionally with Ca^{2+}-sensitive inactivation of calcium channels (deLeon et al., 1995). A potential EF-hand in Dmca1A, immediately C terminal to transmembrane domain IVS6, is underlined in Figure 2. In the Tufty–Kretsinger test (Tufty and Kretsinger, 1975) the Dmca1A sequence has 12 matches of 16 for residues important for calcium binding. Allowing conservative substitutions increases this match to 14 of 16 positions.

Potential sites of post-translational modification

There are several sites of possible post-translational modification of the Dmca1A protein. A single extracellular site matching the consensus sequence [N]-[~P]-[S/T]-[~P] for N-linked glycosylation (cf. Hubbard and Ivatt, 1981) was found at N865 near the N terminus of the IIIS4 transmembrane domain. Nine intracellular consensus sites for cAMP-dependent protein kinase phosphorylation [R/K]-[X]-[X]-[S/T] were found (cf. Krebs and Beavo, 1979): in the N terminus at T31; in the I/II loop at S386 and S392; and in the C terminus at S1348, S1519, S1559, S1616, S1650, and S1836. Fifteen intracellular sites matching the consensus PKC phosphorylation site [S/T]-[X]-[R/K] were found (cf. Woodgett et al., 1986): in the I/II loop at T437; in the IIS4–S5 loop at S552 and S563; in the IIIS4–S5 loop at S900; in the III/IV loop at T1083; in the IVS4–S5 loop at T1209 and S1220; and in the C terminus at T1370, S1432, S1493, S1496, S1559, S1683, S1748, and T1820. The clustering of 13 potential phosphorylation sites in the C terminus suggests that this region may be involved in phosphorylation-dependent modification of calcium channel function.

Of particular interest because of their possible functional significance are five conserved PKC sites that are found in the S4–S5 loops of all non-L-type channels. These sites are not found in any L-type channels and thus may mediate a property that distinguishes these channels functionally. In Dmca1A these sites are S552, S563, S900, T1209, and S1220. Their proximity to the voltage-sensing S4 transmembrane domain is intriguing. There is, in addition, a cAMP-dependent protein kinase phosphorylation site conserved in the segment between IVS6 and the EF-hand in all calcium channels sequenced to date. This site is likely to modulate a function common to all calcium channels.

Alternative exons are different in the region of the β subunit binding site

Calcium channel β subunits interact with α1 subunits to stimulate peak current amplitude, to increase the rate of activation, and to modify the voltage dependence of activation and inactivation in *Drosophila* (D. Ren, M. Chopra, L. M. Hall, unpublished observations) as well as in other species (Lacerda et al., 1991; Varadi et al., 1991; Neely et al., 1993). Pragnell et al. (1994) identified a conserved 18 amino acid sequence (QQ-E–L-GY–WI—E) in the I–II cytoplasmic linker that binds β subunits. Mutations in this conserved domain inhibit β subunit binding. Analysis of the cDNA clones summarized in Figure 1 shows that alternative splicing in the region encoding this I/II linker in Dmca1A generates α1 subunits with major differences in the β subunit binding domain.

The ORF of clone c31 is interrupted by four unspliced introns (Fig. 1). The introns are bounded by consensus splice-site sequences (cf. Mount et al., 1992) and contain no regions of similarity to overlapping cDNAs. Identity as introns was confirmed by comparison with genomic sequences (A. A. Peixoto, L. A. Smith, L. M. Hall, J. C. Hall, unpublished observations). In addition, in the region immediately downstream of the IS6 transmembrane domain, the c31 ORF diverges from that of cS14a for 116 nucleotides, encoding a 38 amino acid sequence beginning at amino acid 315. Each of these divergent sequences is of the same length and is in frame with the Dmca1A ORF. The two divergent sequences (alternative cassettes) are encoded in separate (albeit nearby) genomic regions (Peixoto, Smith, Hall, Hall, unpublished observations), where they each are flanked by consensus splice-site sequences (cf. Mount et al., 1992). In Figure 2, the c31-encoded amino acid sequence is shown aligned above Dmca1A, which contains the sequence encoded by cS14a. Comparison of these divergent transcripts with representative vertebrate sequences (Table 1) shows that the pattern of similarity to these sequences differs between the exons. The c31-encoded exon is more similar to vertebrate α1 subunits in this region (58–84% identity) than is the cS14a sequence (37–47% identity). The c31 form is most similar to the non-L-type isoforms A, B, and E in this region.

The c31 exon contains the first 17 amino acids of the conserved β subunit binding domain. The final conserved glutamate (E) is encoded by the first codon of the downstream exon. Interestingly, the c31-encoded exon has 100% conservation of the nine amino acids required for β subunit binding, whereas the cS14a-encoded exon has only a 4/9 match, with the tyrosine (Y), tryptophan (W), isoleucine (I), and terminal glutamate (E) being conserved. If the cS14a exon is incorporated into a functional α1 subunit, this subunit might not bind β subunits or may be involved in differential interactions with β isoforms.

Transcript diversity in the IVS3–S4 extracellular region

As summarized in Figure 3, sequencing of six cDNAs from different libraries revealed substantial heterogeneity in the IVS3–S4 loop.

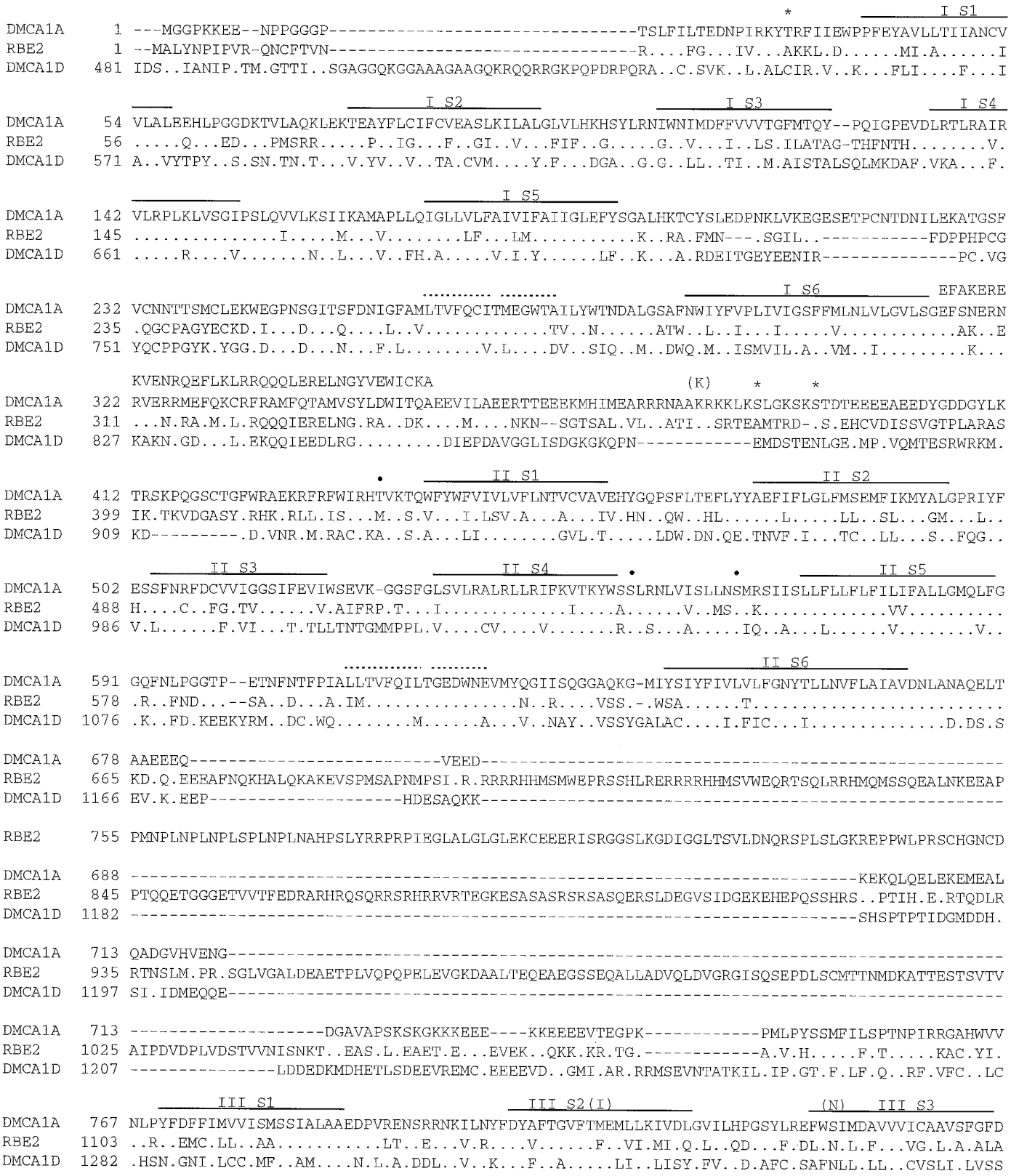

Figure 2. Comparison of the deduced amino acid sequence of Dmca1A with rat brain E (rbE2) and *Drosophila* Dmca1D sequences. Sequences were aligned with ClustalW software (gap penalty, 20; gap extension, 0.05); the first 480 amino acids of Dmca1D align 5′ to the included sequences and were omitted. Identical amino acids are indicated by *dots* and gaps, by *dashes*. Putative transmembrane domains are indicated by a *single line over* the Dmca1A sequence. The short segment 1 and 2 regions in each repeat are indicated by *dotted lines above* the Dmca1A sequence. The sequence of the alternative exon detected in clone c31 is aligned *above* Dmca1A, just downstream of IS6. The amino acids encoded by the nine bases representing the largest variant at the IVS3–S4 variable region are *double-underlined*. Amino acid sequence from carp skeletal muscle (CARPSk) identified as containing dihydropyridine binding sites (Grabner et al., 1996) is included within *brackets* and aligned *below* the IIIS5–S6 and IVS5–S6 domains in (*Figure legend continues*)

←

Dmca1A. A proposed phenylalkylamine binding sequence (Striessnig et al., 1990) is aligned *above* the IVS6 region of Dmca1A. A calcium-binding EF-hand structure downstream of IVS6 is *single-underlined* in Dmca1A. The potential N-glycosylation site at the N terminus of IIIS4 is marked with Ψ. cAMP kinase sites are marked with *; PKC sites with ●. Sites of potential RNA editing are indicated by aligning the unedited codon identity in *parentheses above* the Dmca1A sequence. The GenBank accession number for Dmca1A is U55776.

Table 1. Comparison of alternative exons encoding the β subunit binding domain

	rbA1	rbB1	rbE2	rbC1	a1D	RSk
cS14a	15	16	15	18	18	14
	0.39	0.42	0.39	0.47	0.47	0.37
c31	32	32	30	22	23	22
	0.84	0.84	0.79	0.58	0.61	0.58

Alternative exons encoded by cDNAs cS14a and c31 were aligned with the mammalian calcium channel subunit sequences named in the top row. The top number in each cell represents the number of amino acid identities at 38 positions; the bottom number is the proportion of the 38 residues that are identical between a given *Drosophila* subsequence (second and third rows) and the mammalian ones. Abbreviations for the mammalian α1 channel subtypes are as indicated in Figure 6.

Some of this sequence diversity may arise from incomplete splicing, because the sequence downstream of the common region in cS26a and cSK53 contains no large ORF and begins with 5/6 or 6/6 matches to *Drosophila* 5′ consensus splice-site sequences (Mount et al., 1992). Relative to cS26a, cSK53 contains six additional in-frame nucleotides before the start of the presumed unspliced exon.

Additional heterogeneity in the length of the cDNAs changes the number of amino acids in the IVS3–S4 loop from 9 to 10 or 12. Clone cS9a is the shortest (encoding the 9 amino acid IVS3–S4 loop). Clone c3p1 is slightly longer, containing an in-frame insertion of three nucleotides that are not present in cS9a but are found in both cS11 and cS26a. The latter two clones contain identical in-frame insertions of nine nucleotides; these have identical sequence to the six nucleotides in cSK53 plus the three in c3p1. The nine nucleotides found within cS11 and cS26a encode the amino acids HDD. This variable HDD segment is included as amino acids 1181–1183 in Dmca1A (Fig. 2).

Possible post-transcriptional modifications of the Dmca1A transcript

Additional single nucleotide differences were detected at seven positions in the cDNAs (Table 2). In each case, the differences are between guanosine and adenosine nucleotides. Each difference, except the one at nucleotide 1691, causes an amino acid change. The positions and amino acid differences involving these A- versus G-containing codons are presented in Table 2 and are shown in context in Figure 2. We examined the corresponding genomic sequence at these seven positions (Peixoto, Smith, Hall, Hall, unpublished observations); in all cases, the relevant nucleotide in the genomic sequence was an adenosine.

Alteration of adenosines in genomic DNA to guanosines in cDNA is thought to reflect RNA editing by deamination of adenosine to inosine (reviewed by Bass, 1993). A double-stranded RNA-specific deaminase activity has been reported in *Drosophila* (reported in Bass, 1993). Although a few of these changes may be attributable to genetic polymorphism, the observed changes are uniformly consistent with this mechanism of RNA editing. It seems likely that a majority of these adenosine-to-guanosine differences are caused by a deamination mechanism similar to that observed in vertebrates.

cDNA	Variable region sequence
cS26a	TGGATGGAATTCGGGgtaattatcagttg
cSK53	TGGATGGAATTCGGGCACGATgtaagtacgaac
c3p1	TGGATGGAATTCGGG.....GATTCGAACTCAATCAACGTC
cS29b	TGGATGGAATTCGGGCACGATGATTCGAGCTCAATCAACGTC
cS11	TGGATGGAATTCGGGCACGATGATTCGAGCTCAATCAACGTC
cS9a	TGGATGGAATTCGGG.....TCGAGCTCAATCAACGTC
Amino Acid	W M E F G H D D S S S I N V (N)

Figure 3. Alignment of cDNA sequences at the variable region encoding the IVS3–S4 loop. Sequences matching 5′ splice-site consensus sequences are *double-underlined*, and the sequence from inferred introns is in *lower case*. These intron junctions begin large unspliced introns (see Fig. 1) 3′ to the variable region in these cDNAs. *Underlined* A and G nucleotides in the completely spliced cDNAs correspond to an A in genomic sequence and likely reflect RNA editing at this position. The conceptually translated protein sequence, determined by the predominant G nucleotide at the edited position, is aligned *underneath*; use of the A nucleotide at this position changes this codon identity from Ser (S) to Asn (N). Terminal residues of the IVS3 and IVS4 transmembrane domains are *solid-underlined*, and the variable HDD amino acid sequence is marked with a *dotted underline*.

Temporal pattern of expression of Dmca1A

We used quantitative Northern blotting to determine the developmental profile of expression of the Dmca1A calcium channel α1 subunit. As shown in Figure 4*A*, the probe used in these studies recognized a single major mRNA species of 10.5 kb at each stage tested. To correct for apparent differences in expression because of variations in RNA recovery, we reprobed the blot in Figure 4*A* with a cDNA encoding a widely expressed ribosomal protein (rp49) (O'Connell and Rosbash, 1984), and we determined the relative expression of Dmca1A and rp49 by scanning densitometry, as summarized in Figure 4*B*.

There are three peaks of expression during development. The first peak begins to rise in mid-to-late embryo stages (Fig. 4*B, lanes 5–7*) and reaches a peak during the first larval instar (Fig. 4*B, lanes 8–9*). Expression then declines over the remaining larval instars but begins to rise again after pupariation. There is a second peak in midpupal stages and a final peak in late pupae just before adult eclosion.

Spatial pattern of expression of Dmca1A

To determine where the message for the Dmca1A calcium channel α1 subunit is expressed, we used a digoxigenin-labeled antisense DNA probe on relatively late-stage embryos (equivalent to *lanes 5* or *6* in Fig. 4). As shown in Figure 5, this α1 subunit RNA is expressed widely in the embryonic nervous system. Intense, dark staining is seen in the dorsal cerebral hemispheres as well as throughout the ventral nerve cord. In addition, as shown in Figure 5*B*, bilaterally symmetric, lightly stained nerves can be seen extending anteriorly from the CNS toward the region of the antennomaxillary complex at the extreme anterior end of the animal.

Evolutionary relationship of Dmca1A to other calcium channel α1 subunits

To examine the relationship between Dmca1A and other α1 subunits, we generated a phylogenetic tree containing the invertebrate channels and representative members of each of the six classes of vertebrate channels (Fig. 6). The structure of this tree is consistent with those reported previously for the relationship of the α1 subunits (Grabner et al., 1994; Stea et al., 1995). The Dmca1A sequence branches at the most ancestral node of the non-L-type channels, indicating that it is less similar to any of the vertebrate class A, B, or E subunit sequences than they are to each other and implying that the diversification of the vertebrate non-L-type lineage occurred after the evolutionary divergence of vertebrate and invertebrate lineages.

A partial α1 subunit sequence from the *unc-2* gene of *C. elegans* was reported recently (Schafer and Kenyon, 1995). Inclusion of the extant sequence in a similar analysis indicates that the *C. elegans* protein occupies the same branch of the tree as Dmca1A

Table 2. Positions and codon identity of possible RNA editing sites

	1691	2997	3069	3269	3361	3597	4106
cSK53		ATA	AAT	AAC	AGT	ATG	
c31	AAG						
cS14a	AAA	ATA					
cS9a		ATG	AGT	AGC	GGT	GTG	AGC
c3p1			AAT	AGC	AGT	ATG	AAC
genome	AAA	ATA	AAT	AAC	AGT	ATG	AAC
	380	815	839	906	937	1016	1185
	K>K	I>M	N>S	N>S	S>G	M>V	N>S

The position of the variable nucleotide in the assembled contig is indicated at the top of the table. Codon sequences of the cDNAs and genomic sequence are shown with the relevant adenosine or guanosine indicated in **bold** font. Aligned at the bottom of a given column are the amino acid position number from Figure 2 and the amino acids encoded by unedited and edited codons, respectively.

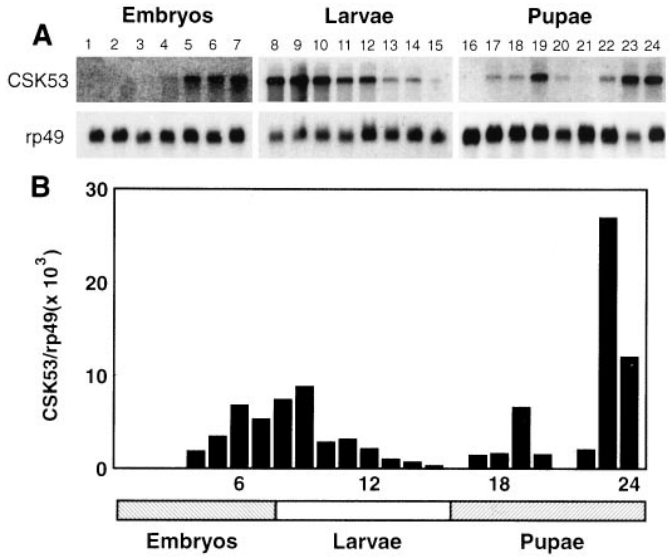

Figure 4. Developmental profile of the Dmca1A calcium channel α1 subunit mRNA expression. *A*, Each lane contains ~10 μg of poly(A^+) RNA. Blots were probed first with a 1 kb *Eco*RI fragment of Dmca1A cDNA and later reprobed with a ribosomal protein (rp49) cDNA probe (O'Connell and Rosbash, 1984). Exposure time for the Dmca1A autoradiographs was 21 d for the embryonic and pupal blots and 7 d for the larval blot. Exposure times for the rp49-probed blots were 18 hr for embryos and pupae and 6 hr for larvae. The collection times in hours postoviposition for animals in the embryonic (*lanes 1–7*) and larval (*lanes 8–15*) stages were *lane 1*, 0–3 hr; *lane 2*, 3–6 hr; *lane 3*, 6–9 hr; *lane 4*, 9–12 hr; *lane 5*, 12–15 hr; *lane 6*, 15–18 hr; *lane 7*, 18–21 hr; *lane 8*, 21–36 hr; *lane 9*, 36–48 hr; *lane 10*, 48–60 hr; *lane 11*, 60–72 hr; *lane 12*, 72–84 hr; *lane 13*, 84–96 hr; *lane 14*, 96–108 hr; *lane 15*, 108–120 hr. The collection times for pupal stages (*lanes 16–24*) in hours postpuparium formation were *lane 16*, 0–12 hr; *lane 17*, 12–24 hr; *lane 18*, 24–36 hr; *lane 19*, 36–48 hr; *lane 20*, 48–60 hr; *lane 21*, 60–72 hr; *lane 22*, 72–84 hr; *lane 23*, 85–96 hr; *lane 24*, 96–108 hr. *B*, The autoradiographs were quantitated by scanning densitometry. Dmca1A/rp49 ratios were calculated from the densities of the Dmca1A and rp49 signals for each lane after correction for exposure times.

(data not shown). Dmca1A and Unc-2 could represent orthologous channels in these two species or could be representative of separate invertebrate α1 subunits that diverged after the evolutionary separation of vertebrate and invertebrate lineages.

The structure of the L-type branch of the tree is also consistent with that reported for the relationship of the L-type vertebrate channel classes and the Mdla1 subunit cloned from housefly larva (Grabner et al., 1994); the Mdla1 subunit is on a branch arising at the most ancestral node of this clade. It has been reported previously that, when compared with known vertebrate sequences, the Dmca1D α1 subunit is most similar to class D subunits (Zheng et al., 1995). In this analysis, we find additionally that Mdla1 and Dmca1D are more similar to each other than to any of the L-type vertebrate α1 subunits. Also, Mdla1 and Dmca1D are less similar to the vertebrate C, D, and Sk subunits than the latter three are to each other and occupy a branch at the most ancestral node on the L-type side of the tree.

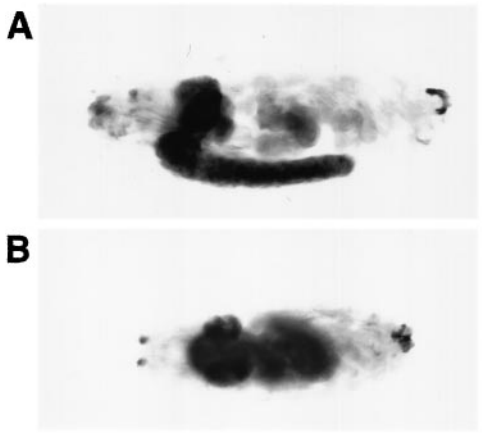

Figure 5. Expression of Dmca1A mRNA in the embryonic nervous system. A single-stranded antisense DNA probe labeled with digoxigenin was hybridized to whole-mounted stage 16 embryos. The *darkly stained* areas represent regions of RNA expression. *A*, Side view in which anterior is to the *left* and dorsal is *up*. *B*, View of the dorsal surface in which anterior is to the *left*.

Dmca1A maps to chromosomal breakpoints that define a locus containing the interacting genetic variants *cacophony, nightblind-A*, and *l(1)L13*

Goralski (1985) molecularly mapped chromosomal breakpoints that were later found to damage or remove functions associated with the *cac, nbA*, and *l(1)L13* variants (Kulkarni and Hall, 1987; Homyk and Pye, 1989). We have confirmed the original RFLP data (Goralski, 1985) for a subset of the relevant chromosome aberrations by Southern blotting that compared the banding patterns from inversion- and deletion-bearing flies with those of control flies devoid of chromosomal lesions near the *cac*/*nbA*/*l(1)L13* locus (Fig. 7). We also have mapped portions of the Dmca1A cDNA to this genomic region (Peixoto, Smith, Hall, Hall, unpublished observations; also see legend to Fig. 1). Combining the findings from the current Fig. 7 (Goralski, 1985; Peixoto, Smith, Hall, Hall, unpublished data), we infer that the *l(1)L13*-minus (hence *cac*- and *nbA*-minus) lesions are almost certainly within the Dmca1A locus. In particular (and as is summarized in Fig. 1), the deletion *Df(1)HF368* has a breakpoint within 2 kb of transmembrane domain IIS5 and removes sequences 5′ to this; the inversion *In(1)A78* has a breakpoint within 4 kb of the putative transcription initiation site and the first transmembrane domain IS1; *In(1)N66* has a breakpoint within 2 kb of the alternatively spliced exons in the I–II loop; and *In(1)A101* has a breakpoint within 2 kb of the IIIss1–ss2 domain.

DISCUSSION

Dmca1A participates in generation of calcium channel diversity

The sequence and deduced structure of Dmca1A places it in the superfamily of voltage-sensitive calcium and sodium channels.

A

	Dmca1A	rbA1	rbB1	rbE2	rbC1	a1D	RSkm	Mdla1	Dmca1D
Dmca1A	.000	.401	.403	.411	.513	.513	.532	.527	.532
rbA1	.211	.000	.213	.243	.514	.513	.525	.520	.521
rbB1	.225	.076	.000	.259	.515	.512	.520	.520	.519
rbE2	.244	.101	.095	.000	.527	.534	.529	.528	.525
rbC1	.388	.405	.409	.409	.000	.174	.258	.347	.355
a1D	.384	.411	.415	.424	.107	.000	.274	.344	.359
RSkm	.405	.409	.407	.401	.159	.202	.000	.273	.372
Mdla1	.407	.409	.419	.417	.244	.252	.374	.000	.182
Dmca1D	.405	.415	.417	.424	.242	.250	.264	.081	.000
Na alpha	.601	.605	.603	.605	.597	.610	.603	.599	.610

B

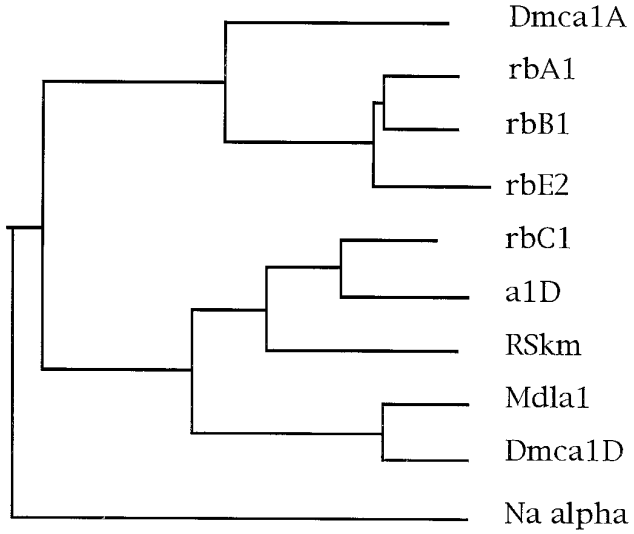

Figure 6. Phylogenetic analysis of Dmca1A and α1 subunits representative of other calcium channel subfamilies. The channels indicated are rbA1, rat brain class A; M64373 (Starr et al., 1991); rbB1, rat brain class B; M92905 (Dubel et al., 1992); rbE2, rat brain class E; M94172 (Soong et al., 1993); rbC1, rat brain class C; M67516 (Snutch et al., 1991); a1D, human class D; M76558 (Williams et al., 1992); RSkm, rat skeletal muscle; X05921 (Tanabe et al., 1987); *Mdl*a1, *Musca* larvae; Z31723 (Grabner et al., 1994); Dmca1D, *Drosophila*; U00690 (Zheng et al., 1995). Transmembrane sequences of each of these channels were concatenated into a single sequence, and the resulting "core" sequence files were aligned with the ClustalW program (default BLOSUM scoring matrix series; gap penalty, 20; gap extension, 0.5) (Thompson et al., 1994). Full-length sequences also were aligned. Small discrepancies between endpoints of reported transmembrane domains were resolved by reference to the multiple alignment in Stea et al. (1995). A sodium channel α subunit sequence (Noda et al., 1986) was included to indicate relatively greater similarity of Dmca1A to calcium channel α1 subunit sequences. *A*, Distance matrix for calcium channel α1 subunits. The distance between channel sequences, representing the minimum number of nucleotide changes necessary to the observed amino acid differences, was calculated for both the core sequences and for the full-length sequences, using a neighbor-joining algorithm as implemented in ClustalW (Thompson et al., 1994) (ignore gaps = on; multiple substitutions = off). In the *bottom left* of the matrix, distances are calculated from alignment of transmembrane core sequences. In the *top right* of the matrix, distances are calculated from alignment of full-length sequences. *B*, Phylogenetic tree for core sequence alignment. The Retree and Drawtree programs of the Phylip software package (Felsenstein, 1989) were used to display a phylogenetic tree using data from the core sequence alignment shown in the *bottom left* corner of the distance matrix. Branch lengths between subunits are proportional to divergence. A tree generated from full-length sequence alignments had identical topology to the one shown.

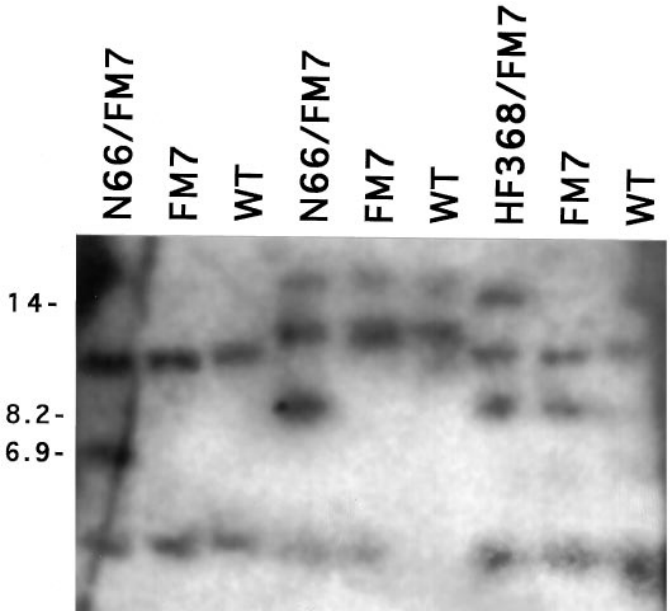

Figure 7. Southern blot detection of lethal chromosomal lesions, the breakpoints of which map to the region encoding Dmca1A. Goralski (1985) localized breakpoints of inversion- and deletion-containing chromosomes to a relatively small genomic interval within the cytogenetic region called 11A2 by RFLP detection with multiple restriction enzymes. To confirm elements of these findings, we prepared genomic DNA from *In(1)N66* and *Df(1)HF368* flies heterozygous for the *In(1)FM7* balancer chromosome, from homozygous *In(1)FM7* flies, or from a wild-type strain (*Canton-S*). DNA in *lanes 1–3* was subjected to restriction digestion with *Hin*dIII, in *lanes 4–6* with *Bam*HI and *Kpn*I, and in *lanes 7–9* with *Hin*dIII and *Kpn*I (following Goralski, 1985). The blot was probed with genomic phage clone 320 (cf. Goralski, 1985), which contains a portion of the Dmca1A ORF extending approximately from transmembrane domains IS5 to IIS5 (see Fig. 1). The novel restriction fragments present in *lanes 1, 4*, and *7* are as originally detected by Goralski (1985); the size of these fragments (in kb) is indicated in the *left* margin of the figure.

The protein sequence is more similar to calcium channel α1 subunits than to sodium channel α subunits, both overall and within conserved transmembrane and ss1–ss2 motifs. Key glutamate residues in the ss2 motifs that have been implicated in ion selectivity are present in a pattern that is conserved perfectly in calcium channels and is required for ion selectivity. Near-perfect conservation of a motif implicated in phenylalkylamine binding implies that Dmca1A is sensitive to this class of calcium channel-specific pharmacological agents. In combination, this evidence clearly establishes that Dmca1A belongs to the family of calcium channel α1 subunits. Dmca1A maps to a different chromosome from the Dmca1D gene (Zheng et al., 1995) and encodes a structurally distinct α1 subunit. Thus, in *Drosophila*, as in vertebrates, one source of calcium channel diversity involves separate genes encoding distinct α1 subunits.

Zheng et al. (1995) presented evidence for variant transcripts

from the Dmca1D locus. The diversity of cDNA sequences reported here demonstrates further that alternative splicing plays a role in generation of calcium channel diversity in invertebrates. The 116 nucleotide alternative exons in the I–II loop encode different amino acid sequences. These differences in a motif important for interaction with β subunits (cf. Pragnell et al., 1994) imply that isoforms encoded by these alternative exons might exhibit different affinities for β subunit interactions.

The pattern of variable nucleotide insertions at the extracellular IVS3–S4 loop is consistent with nonexclusive differential inclusion of three- and six-base exons, generating variants differing by zero, three, six, or nine bases. Differential inclusions of small exons (15 or 3 bases in length) have been reported for transcripts from the mammalian NCAM gene (Santoni et al., 1989; Reyes et al., 1991). In Dmca1A, the variable splice site is only five amino acids N terminal to the IVS4 transmembrane domain, which forms part of the voltage sensor in these channels. In addition to the splice variants in this region, a possible RNA editing site (see below) has been found between the variable splice region and the S4 voltage sensor. Thus, in this small region there is the potential for significant transcript variability. This raises the intriguing possibility that these differences might play a role in modulating the voltage dependence of calcium channels containing Dmca1A.

Direct sequence analysis of PCR products derived from RNA from whole flies indicates that each of the alternatively spliced transcripts described here is expressed at detectable levels (L. A. Smith and J. C. hall, unpublished observations). Of the two forms at the I–II loop, the c31-encoded exon, containing a perfectly conserved β subunit binding motif, is predominant. At the IVS3–S4 variable region, the shortest form is predominant. In *Drosophila*, optional or differential splicing of exons of the *para* sodium channel α subunit occurs at six known sites (Loughney et al., 1989; Thackeray and Ganetzky, 1994; O'Dowd et al., 1995), whereas the *Shaker* potassium channel gene generates multiple developmentally regulated classes of transcripts (Kamb et al., 1988; Pongs et al., 1988; Schwartz et al., 1988; Mottes and Iverson, 1995). Although the known splicing-generated transcript diversity of Dmca1A is not as extensive as for these well characterized *Drosophila* ion channels, further analysis of the new calcium channel gene and its products may reveal additional instances of alternative splicing.

The Dmca1A mRNA seems to be post-transcriptionally modified (Table 2). This is the first evidence for RNA editing of a neurobiologically relevant gene in *Drosophila* (and in any invertebrate, to our knowledge). In vertebrates, adenosine deamination is often dependent on an editing site-complementary sequence in an adjacent intron (Higuchi et al., 1993; Lomeli et al., 1994). For five of the seven potential editing sites inferred for Dmca1A, preliminary analysis of genomic sequence has detected flanking intronic complementary sequences, ranging in length from seven to nine nucleotides and, in each case, perfectly centered on the relevant adenosine residue (Peixoto, Smith, Hall, and Hall, unpublished observations). Six of the seven adenosine-to-guanosine differences result in changes of codon identity, suggesting that RNA editing may contribute to functional diversity of the Dmca1A protein. The apparent preferential localization near conserved transmembrane domains implies that these amino acid changes might be relevant to regulated functions of the Dmca1A calcium channels. The apparent lack of editing in the embryonic cDNA cSK53 suggests that editing may be stage- or tissue-specific.

Northern blot analysis of the first cloned *Drosophila* calcium channel α1 subunit detected three size classes of mRNA in heads: a major band at 9.5 kb and two minor bands at 10.2 and 12.5 kb (Zheng et al., 1995). Because the RNA encoding this subunit undergoes extensive alternative splicing, it was not possible to determine whether the minor bands were minor splice forms or represented distinct members of a calcium channel α1 subunit gene family. The results reported here suggest that the 10.2 kb band previously detected with a Dmca1D probe (Zheng et al., 1995) might encode the Dmca1A subunit. Dmca1A is expressed throughout the embryonic nervous system; the relatively head-enriched expression of the 10.2 kb message (seen with the Dmca1D probe) implies predominantly neural expression in the adult. The slight discrepancy in size (10.2 vs 10.5 kb measured in this study) could be attributable to the difficulty in accurately estimating the size of high-molecular-weight RNAs.

Comparison of pharmacological motifs of Dmca1A and Dmca1D

Conservation of a proposed phenylalkylamine binding site near the 3′ end of the IVS6 transmembrane domain suggests that Dmca1A may bind phenylalkylamines. Relatively poor conservation of amino acids in the proposed dihydropyridine binding sites suggests that this α1 subunit does not bind dihydropyridines, consistent with the phylogenetic similarity to the non-L-type channels. Because both Dmca1A and Dmca1D α1 subunits similarly are conserved in the proposed phenylalkylamine binding region, both may contribute to the phenylalkylamine binding activity found in *Drosophila* extracts (Pauron et al., 1987; Greenberg et al., 1989). On the basis of sequence analysis, it was suggested initially that Dmca1D might encode the predominant dihydropyridine-insensitive calcium channel in *Drosophila* heads (Zheng et al., 1995). However, recent electrophysiological studies show that Dmca1D encodes a dihydropyridine-sensitive current in larval muscle (D. Ren, H. Xu, G. Feng, M. Chopra, L. M. Hall, unpublished observations) consistent with its structural similarity to L-type channels. If Dmca1A is expressed in muscles, it is a candidate for encoding the amiloride-sensitive current (Gielow et al., 1995).

Dmca1A may be encoded by a gene defined by behavioral, physiological, and lethal mutations

The *cacophony, nightblind-A*, and *lethal(1)L13* mutations all map by deletion analysis to the same genetic interval (Kulkarni and Hall, 1987). Breakpoints of certain physically lesioned inversion chromosomes that fail to complement *cacophony, nightblind-A*, and *lethal(1)L13* mutations not only map genetically to the sites of these mutations (see introductory remarks) but now also have been mapped molecularly to the Dmca1A-encoding locus (Figs. 1, 7). Although chromosomal breakpoints can induce spreading effects that cause perturbation of neighboring genes not directly disrupted by the genetic lesion, the fact that all of these breakpoints disrupt the Dmca1A locus (Fig. 1) strongly suggests involvement of this calcium channel α1 subunit gene in the generation of the physiological, behavioral, and lethal phenotypes associated with the *cac, nbA*, and *l(1)L13* mutants. It follows that *cac, nbA*, and *l(1)L13* are likely to be Dmca1A mutants and that these genetic variants likely define a single gene.

Mutant alleles of *l(1)L13* cause late embryonic lethality. The first expression peak of the Dmca1A transcript begins in late embryogenesis, consistent with a requirement for Dmca1A function at this developmental stage. The diversity of the Dmca1A transcript suggests that the complicated complementation interations of *cac, nbA*, and *l(1)L13* mutations (Kulkarni and Hall, 1987) could be attributable to isoform-specific lesions. In adults, the *cac* mutation causes defects in the male courtship song (Kulkarni and Hall, 1987), and

nbA mutants exhibit increased light thresholds for optomotor and phototactic behaviors (Heisenberg and Götz, 1975) as well as defects in the shape and amplitude of the electroretinogram (Homyk and Pye, 1989). The particular song defect exhibited by *cac* males—larger than normal numbers of cycles within a given "burst" of tone—could be rationalized in terms of modified calcium channel function [cf. Hille (1992), Chapter 5]. The cellular etiology of the abnormal singing behavior is difficult to speculate on, because it could involve defects in neural or muscular physiology (or even anatomy), yet it is difficult to imagine a non-neural etiology for the abnormal ERG in *nbA* mutants. Taken together, this analysis implies involvement of the Dmca1A voltage-dependent calcium channel in visual transduction and may suggest involvement of calcium-dependent beating or bursting cells in the generation of the rhythmic wingbeat behavior underlying the generation of courtship song. Further experiments on the molecular etiologies of these three types of mutants may reveal how variation within the Dmca1A gene can cause either severe and rather global neurobiological problems or more subtle ones involving these discrete elements of behavior and physiology.

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