Genetic and Developmental Characterization of Dmca1D, a Calcium Channel $\alpha_1$ Subunit Gene in Drosophila melanogaster

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ABSTRACT

To begin unraveling the functional significance of calcium channel diversity, we identified mutations in Dmca1D, a Drosophila calcium channel $\alpha_1$ subunit cDNA that we recently cloned. These mutations constitute the l(2)35Fa lethal locus, which we rename Dmca1D. A severe allele, Dmca1D$^{110}$ truncates the channel after the IV-S4 transmembrane domain. These mutants die as late embryos because they lack vigorous hatching movements. In the weaker allele, Dmca1D$^{466}$, a cysteine in transmembrane domain I-S1 is changed to tyrosine. Dmca1D$^{466}$ embryos hatch but pharate adults have difficulty eclosing. Those that do eclose have difficulty in fluid-filling of the wings. These studies show that this member of the calcium channel $\alpha_1$ subunit gene family plays a nonredundant, vital role in larvae and adults.

Molecular diversity in voltage-gated calcium channels has been revealed by pharmacological, electrophysiological, and gene cloning studies in both vertebrates (Catterall 1988, 1995; Bean 1989; Hesch 1990; Tsien et al. 1991; Snutch and Reiner 1992; Hofmann et al. 1994) and invertebrates (Greenberg et al. 1989; Pelzer et al. 1989; Leung and Byerly 1991; Hille 1992; Skeer et al. 1992; Zheng et al. 1995; Smith et al. 1996). The well-studied vertebrate skeletal muscle L-type calcium channel is composed of five subunits $\alpha_1$, $\alpha_2$, $\beta$, $\gamma$, and $\delta$ with the $\alpha_1$ subunit forming the ion selectivity pore through the membrane. Channel diversity arises from multiple genes encoding each calcium channel subunit (Per ez-Reyes et al. 1990; Snutch et al. 1990; Hofmann et al. 1994; Catterall 1995), from alternative splicing (Per ez-Reyes et al. 1990; Hui et al. 1991; Snutch et al. 1991; Hofmann et al. 1994; Dunlap et al. 1995), from RNA editing (Peixoto et al. 1997), from posttranslational modification (Nunoki et al. 1989; De Jongh et al. 1989) and from combinatorial association of different $\alpha_1$ subunits with subtypes of non-$\alpha_1$ subunits (Wei et al. 1991).

We have recently cloned and sequenced Dmca1D, a cDNA encoding a calcium channel $\alpha_1$ subunit from Drosophila (Zheng et al. 1995). Dmca1D is most similar to the rat brain type D calcium channel $\alpha_1$ subunit (Snutch et al. 1990) and is most strongly expressed in the developing larval and adult nervous systems (Zheng et al. 1995). Binding studies of calcium channel blockers to Drosophila head membrane extracts (Greenberg et al. 1989) as well as electrophysiological recordings from reconstituted Drosophila brain membranes (Pelzer et al. 1989), from cultured Drosophila embryonic neurons (Leung and Byerly 1991), and from Drosophila larval muscle (Gielow et al. 1995) all provide evidence that in Drosophila, as in other organisms, multiple types of calcium channels are formed. Indeed, we have shown (D. Ren, H. Xu, D. F. Eberl, M. Chopra, and L. M. Hall, unpublished results) that calcium channel currents mediated by Dmca1D are sensitive to dihydropyridines (DHP), while another recently identified Drosophila calcium channel, Dmca1A, is structurally more similar to DHP-insensitive calcium channels (Smith et al. 1996; Peixoto et al. 1997).

The physiological significance of this diversity is unknown. Identification of mutations in individual calcium channel subunit genes is one approach to define the functional roles of each type of calcium channel. Here we show that the l(2)35Fa complementation group (Ashburner et al. 1990) represents the structural gene for the Dmca1D calcium channel $\alpha_1$ subunit. Therefore, we name this locus Dmca1D (Drosophila melanogaster calcium channel $\alpha_1$ subunit DHP-sensitive). We describe the developmental effects of mutations in Dmca1D on the organism and integrate these findings with our electrophysiological studies on this channel (D. Ren, H. Xu, D. F. Eberl, M. Chopra, and L. M. Hall, unpublished results).

MATERIALS AND METHODS

Genetic strains: All Drosophila melanogaster mutations and chromosomal aberrations in the 35E-F region were obtained...
from the laboratory of M. Ashburner and from the Bloomington Drosophila Stock Center. The y w; Sb P(2-3)99AB/TM6 stock used for transformation was obtained from N. Perrimon. The X7, X10, and AR66 alleles of I(2)35Fa (= Dmca1D) were induced on a common background chromosome, b pr cn wx bw (Ashburner et al. 1990). A fourth mutation induced on this background, AR146, mutates a nearby complementation group, I(2)35Fd. Because we did not have the original b pr cn wx bw stock, we used the I(2)35Fdh14 chromosome, which should be unaltered in the Dmca1D gene, to represent the "wild-type" background for sequencing. Heterozygous Dmca1D region background DNA was obtained from I(2)35Fdh14/ Df(2L)el18 flies because Df(2L)el18 deletes Dmca1D but not I(2)35Fd (Figure 1A; Ashburner et al. 1990).

Isolation of genomic cosmid clones: The PB1 and PB2 probes (Figure 1B) were labeled with 32P-DCTP (Megaprime random primer labeling kit; Amersham, Arlington Heights, IL) and used for high stringency screening of about 16,800 colonies from the iso-1 Drosophila genomic cosmid library (J. Tamkun, University of California, Santa Cruz). PB1 is a 678-bp PCR fragment [coordinates 6885-7562 of Zeng et al. (1995)] from the 3' end of the cDNA while PB2 is a 665-bp PCR fragment [coordinates 960-1624 of Zeng et al. (1995)] from the 5' region.

Transformation: P-element-mediated transformation was carried out as described by Spradling (1986). Ca01 cosmid DNA was injected into y w; Sb P(2-3)99AB/TM6 embryos and transformants were recognized by expression of the white+ marker gene present in the cosmid vector.

Determination of lethal phase: Eggs were collected for 4 hr on yeast apple juice agar plates and 200 eggs from each cross were counted and transferred to a fresh plate. After 30-36 hr, the larvae that hatched were transferred to standard food vials at a density of 25 per vial. Unhatched eggs were dechorionated with a 2-min treatment of 50% commercial bleach, rinsed, and covered in halocarbon oil for inspection under a compound microscope using brightfield and Nomarski optics.

Northern analysis: Northern blots were prepared and analyzed by standard methods (Sambrook et al. 1989). Poly(A+) RNA was prepared by the guanidinium isothiocyanate-CsCl gradient method (Chirgwin et al. 1979) followed by one passage through an oligo(dt)-cellulose column. Poly(A+) RNA (20 μg/lane) was electrophoresed on a 0.8% agarose gel containing 6.3% formaldehyde in 1× MOPS buffer. Following capillary blotting (Nytran membrane; Schleicher & Schuell, Keene, NH) and UV crosslinking, the membrane was prehybridized for 4 hr at 42° in 50% deionized formamide, 5× SSPE, 5× Denhardt's, 0.5% sodium dodecyl sulfate (SDS), 0.01% denatured salmon sperm DNA, and then hybridized with 32P-labeled probe (2×106 cpm/ml) for 16 hr at 42°. Following high stringency washing, the blot was exposed to X-ray film for 7-21 days at -70°.

Mutation detection: For confirmation of the X10 mutant change originally detected as a Taq restriction site change, PCR was done with three different primer combinations: AmpliTaq (Perkin Elmer, Norwalk, CT); Pfu (Stratagene, La Jolla, CA); HotTub (Amersham), and 0.1 μM of each primer M13SH 14A1B [coordinates 5159-5181 of Zeng et al. (1995)] and SP6SH18A1A [coordinates 6414-6163 of Zeng et al. (1995)]. Thermal cycling conditions were: 35 cycles of 1 min at 95°, 1 min at 50°, and 90 sec at 72°, followed by 10 min at 72° and cooling to 4°. PCR products were extracted from a 1% agarose gel with GeneClean glass powder (Bio101, La Jolla, CA), digested with Taq (GIBCO BRL, Grand Island, NY) for 1 hr at 65°, and analyzed on 2.5% agarose gel.

DNA sequencing: To sequence the AR66 allele, double-stranded sequencing was performed on an Applied Biosystems Sequencer Model 373A (Applied Biosystems, Foster City, CA), using the dideoxy chain terminal method either with fluorescent dye-tagged primers (M13 or SP6) according to instructions supplied with the Taq Dye Primer Cycle Sequencing kit (Applied Biosystems, Inc.) or with fluorescent dye-tagged terminators. Purified PCR products from the X10 mutant heterozygote were digested with Accl and XbaI, subcloned into pBluescriptKS-I (Stratagene), and colonies with different Taq restriction digestion patterns were sequenced in the same way. The AR66 mutant change detected by automated sequencing was confirmed by sequencing two PCR products, from different primer pairs that flank the change, with the CircumVent Cycle Sequencing kit (New England Biolabs, Beverly, MA) using the PCR primers and incorporation of 35S-dATP.

Antibody staining of mutant embryos: Embryos homozygous for recessive lethal alleles (X10, X7, and AR66) of Dmca1D were analyzed with the following antibodies: Mab22C10 (provided by the laboratory of S. Benzer, CalTech, Pasadena, CA), MabBP102 and Mab1D4 (provided by the laboratory of C. Goodman, University of California, Berkeley), and anti-HRP (Cappell, Organon Teknika Corp., Durham, NC) and anti-cut (provided by L. Jan and Y. Jan, University of California, San Francisco). Embryos from heterozygous mutant/Cyo, wg1111 parents were collected overnight and prepared for staining by dechorionating in 2.5% sodium hypochlorite (50% bleach) for 5 min and rinsing with 0.1% Triton X-100. Embryos then were permeabilized and fixed for 5 min in a mixture of 2.5% glutaraldehyde (Sigma Chemical, St. Louis, MO) and heptane (1:1). Vitelline membranes of embryos in the heptane layer were removed by adding 1–2 volumes of methanol and vortexing for 10 sec on moderate speed. Embryos were rehydrated in PTween (phosphate-buffered saline containing 0.1% Tween) for approximately 30 min. All embryos then were reacted with primary antibodies (Mabs 22C10, BP102, and 1D4 diluted 1:10, anti-HRP diluted 1:1000 and anti-cut diluted 1:1000) in PTween for 3 hr at room temperature. After washing in PTween, embryos were incubated in biotinylated horse anti-mouse or goat anti-rabbit secondary antibodies (Vector Laboratories, Burlingame, CA) for 3 hr at room temperature. Secondary antibodies were detected using a Vectastain Elite kit (Vector Labs, Burlingame, CA) followed by a solution of 0.3% H2O2 (diaminobenzidine), 0.08% NiCl and 0.01% H2O2 in PTween. Embryos were mounted in 70% glycerol and homozygous mutant embryos of all stages were distinguished from sibling embryos by a failure to express β-galactosidase from an enhancer trap insertion in the wg gene on the Cyo, wg1111 balancer chromosome.

RESULTS

Genetic and cytological mapping of Dmca1D: Genetic and cytological mapping of Dmca1D: To analyze the consequences of genetically disrupting the α1 subunit of Dmca1D, we used deletion mapping to determine whether any existing mutants corresponded to Dmca1D. We first used in situ hybridization to wild-type salivary gland polytene chromosomes with biotinylated probes from Dmca1D to determine the approximate map position and found that it hybridized to 35E3-F3 on the left arm of chromosome 2 (Zeng et al. 1995). Next, we extended this in situ hybridization analysis to deletions and other chromosomal aberrations to compare this map position with the extensive array of mutations that were previously mapped to this area (Ashburner et al. 1990). This analysis revealed a single complementation
Figure 1.—Identification of Dmca1D mutant complementation group. (A) Genetic map of the Dmca1D region. Genes shown in the left column have been ordered by numerous deletions (Ashburner et al. 1990; Alphey et al. 1992), a subset of which are shown in the right column. The extent of each deletion is indicated by a vertical line, while the breakpoints are indicated by horizontal lines. Vertical lines connected to only one horizontal line represent deletions that extend beyond the limits of this figure. Dmca1D co-maps with the complementation group, l(2)35Fa (arrow), which we identify as the Dmca1D locus. (B) Genomic cosmid clone Ca01 that rescues Dmca1D mutations. The Ca01 cosmid used for P element-mediated transformation rescue of l(2)35Fa (Dmca1D) was isolated from a Drosophila genomic library using probes PB1 and PB2. Exons (black rectangles) in the Dmca1D cDNA clone are shown above the genomic clone. Arrows above the exon map indicate the proposed start and end of the ORF (Zheng et al. 1995). The positions of the white eye marker gene (w+) and the flanking P element ends (arrow heads) in the transforming construct are shown. C1NB6.7, C1B17, and C1BN14 are subclones of Ca01 used for probing Northens shown in C. Restriction enzyme sites are: B = BamHI; K = KpnI; N = NotI; Q = XbaI; S = SalI; SI = SstI; W = NheI; Y = SpeI. The numbers within the long rectangle indicate the length in kilobases from the initial NotI site. (C) Embryonic Northern blots. mRNA from early (3–12 hr) or late (13–21 hr) embryos grown at 25°C was used for replicate blots probed with the three subclones of Ca01 shown in B. A single transcript size was detected in late embryos (arrows).

Dmca1D Ca2+ Channel Mutations

Rescue of the l(2)35Fa lethality with a genomic cosmid clone: To determine whether the l(2)35Fa gene encodes Dmca1D, we used P-element-mediated transformation to test whether Dmca1D could rescue the lethal phenotype. We isolated a genomic cosmid clone, Ca01, that carries the entire Dmca1D coding region (19 kb) plus 6.63 kb of the 5' upstream region (Figure 1B). Ca01 was injected into embryos that endogenously express the P-element transposase (Robertson et al. 1988). One resulting fly (Ca01.88) transmitted an integrated copy of the cosmid clone. Two derivatives (Ca01.88C4 and Ca01.88C7) were stabilized in the following generations during removal of the transposase from the genotype. To test whether Ca01 rescues l(2)35Fa mutations, flies carrying the transforming cosmid in a mutant background were constructed. The transforming cosmid-bearing chromosome was crossed into a strain carrying the deletion Df(2L)RA5, which deletes a region including the l(2)35Fa gene (Figure 1A). Thus, flies heterozygous for the deletion and a mutant allele of l(2)35Fa will live only if the transformed Ca01 cosmid clone provides the function missing in the mutant. As shown in Table 1, both stable insertions (Ca01.88C4 and Ca01.88C7) were fully able to rescue the lethality caused by each of the three mutant alleles of l(2)35Fa because we recovered w+ Cy+ flies in the
The wild-type Taq eliminates this l(2)35Fa

1vors (w Cy

The appearance of a new, larger band (849 bp) in type expected of the balancer homozygotes. The third been mutated. This lost Taq l(2)35FaX10

AR66

ble 1). The transformation results indicate that the gene changes, mostly silent, in Ca01 cosmid clone. be polymorphisms because they are present in all the

Table 1

<table>
<thead>
<tr>
<th>Cosmid insert</th>
<th>l(2)35Fa allele</th>
<th>Cy</th>
<th>Cy⁺</th>
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<td>Ca01.88C4</td>
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<td>AR66</td>
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<tr>
<td></td>
<td>AR66</td>
<td>198</td>
<td>214</td>
</tr>
</tbody>
</table>

Ratio expected with a fully penetrant lethal allele: 2 2 1 0

2 Crosses were: w;Df(2L)RA5/CyO; P{Ca01, w⁺}/+ crossed to w;l(2)35FaCyO; +/+ , where P{Ca01} is either Ca01.88C4 or Ca01.88C7 and the l(2)35Fa allele is indicated in the table.

expected Mendelian frequencies. Some mutant survivors (w Cy⁺ flies) are produced by the leaky allele, l(2)35Fa666, even in the absence of rescue by Ca01 (Table 1). The transformation results indicate that the gene product(s) that rescues l(2)35Fa is encoded within the Ca01 cosmid clone.

Embryonic transcripts encoded by the Ca01 cosmid clone: To determine how many different candidate embryonic transcripts were encoded by the rescuing Ca01 cosmid clone, three subclones (C1NB6.7, C1B17, and C1BN14) shown in Figure 1B were used to probe replicate wild-type embryonic Northern blots. Only one size class of message, 9.5 kb, was seen with each probe (Figure 1C). This is the size expected for Dmca1D (Zheng et al. 1995). Thus, a band the size of the Dmca1D message is the only one detected from the Ca01 genomic region. Further analysis (see Materials and Methods) confirmed this result.

Identification of a premature stop codon in the X10 allele: To determine whether the three l(2)35Fa alleles have alterations in the Dmca1D coding sequence, we used heteroduplex analysis with mutation detection enhancement (MDE) gels (Keen et al. 1991) to identify single base substitutions in heterozygote DNA. Heteroduplex analysis of genomic DNA including over 4 kb of the 7.5-kb open reading frame revealed an alteration in one allele, l(2)35FaX10, that suggested a TaqI site had been mutated. This lost TaqI site was confirmed by restriction enzyme digestion of a PCR fragment showing the appearance of a new, larger band (849 bp) in l(2)35FaX10 heterozygotes but not in wild type (Figure 2A) and not in heterozygotes for the other mutant alleles (data not shown). Therefore, the X10 allele of l(2)35Fa eliminates this TaqI site.

Subsequent genomic DNA sequencing showed that the wild-type TaqI site TCGA is mutated to TTGA (Figure 2B) in the X10 allele, thereby changing an arginine CGA codon [R1800 of Zheng et al. (1995)] to a TAG stop codon. This change is in the cytoplasmic loop just following the IV-S4 transmembrane domain in the deduced Dmca1D protein (Figure 2E). This stop codon would produce a truncated protein that is missing the last two transmembrane domains (IV-S5 and -S6) and the cytoplasmic carboxy tail, which contains an EF hand motif, thought to bind Ca2⁺ (Babitch 1990; de Leon et al. 1995), part of the DHP-binding domain (Catterall and Striessnig 1992; Grabner et al. 1996), and the phenylalkylamine binding domain (Striessnig et al. 1990). Loss of these regions would produce a nonfunctional channel. Indeed, while deletions that remove 70% of the cytoplasmic carboxy terminus of a cardiac calcium channel α1 subunit lead to increased ionic currents, more extensive deletions abolish detectable current (Wei et al. 1994).

Identification of a missense mutation in the AR66 allele: To investigate the ability to isolate homozygous mutant DNA from the surviving AR66 homozygotes allowed us to test for the mutant change(s) by direct sequencing. Sequencing of most of the coding region with the exception of some of the larger introns revealed several changes, mostly silent, in AR66 relative to the Dmca1D cDNA. All except one of these changes were found to be polymorphisms because they are present in all the mutant alleles and the wild-type chromosome from which they were generated (see Materials and Methods). The exceptional change, present only in AR66 and not in X7, X10, or in the wild type (Figure 2C), mutates a cysteine TGT codon [C629 of Zheng et al. (1995)] to a tyrosine TAT codon (Figure 2D). This cysteine is within the I-S1 transmembrane domain, closer to the extracellular side, of the deduced Dmca1D protein (Figure 2E). We have examined the functional significance of this residue in a separate study (D. Ren, H. Xu, D. F. Eberl, M. Chopra, and L. M. Hall, unpublished results) and find that the I-S1 segment is involved in determining the rate of channel activation and peak current.

Dmca1D mutant phenotype: To investigate the lethality of l(2)35Fa alleles in more detail, we collected eggs from each mutant stock carrying the CyO, wg111 balancer (see Materials and Methods) and followed them through development (Table 2). For the severe alleles, X7 and X10, there are three classes of offspring in a roughly Mendelian ratio of 1:1:1. About half, the heterozygotes, live to adulthood. A quarter die as embryos with the characteristic wingless (wg) phenotype of the balancer homozygotes. The third class, representing the l(2)35Fa homozygotes, die uniformly at the late embryonic stage as pharate larvae with no gross morphological abnormalities. Nevertheless, the tracheae of X7 and X10 mutant embryos generally do not become gas-filled as they do in normal embryos. Gas-filling of tracheae may be associated with motor...
exertion or may be under control of the nervous system (see Manning and Krasnow 1993). The mutant embryos do move, but the movements are very weak, slow localized twitches, usually at the posterior end. Some peristaltic motion is visible in the gut, which is also very slow. Occasionally the embryos bend their heads, but movement of the cephalopharyngeal apparatus has not been seen. Pumping of the heart, which appears to require L-type calcium channel function (Gu and Singh 1995), is not seen in these mutant embryos. Certainly the vigorous writhing movements and extension of the mouthparts required for hatching are absent.

Conversely, flies homozygous for the weak allele, AR66, are all able to hatch as larvae, indicating that this allele retains partial function. In the uncrowded conditions used in this experiment 48% of the AR66 homozygotes are able to eclose as adults (Table 2), although their development is delayed by 1–2 days at 25°C relative to their siblings (data not shown). The wings of these homozygotes are usually unexpanded and many of the flies are found stuck in the food. The remaining 52% (Table 2) develop completely and usually manage to open the puparium but fail to eclose, so they die as pharate adults. No obvious differences could be detected in larval movements or heart rate between AR66 homozygotes and heterozygotes.

To gain more insight into the nature of the hypomorphic AR66 allele, we crossed it to the two strong alleles and to Df(2L)RA5, a deficiency for the region (Figure 1A). In all three cases (Table 2), the hatching and pupation frequencies are as high as from the AR66 stock, indicating that the trans-heterozygous larvae are able to hatch and pupate. As with AR66 homozygotes, there appears to be a major threshold during eclosion. Those that manage to eclose are usually found stuck in the using three different polymerases (only AmpliTaq is shown). TaqI digestion produces an 849-bp fragment in mutant DNA that is cut into 531- and 318-bp pieces in wild type. (B) Premature stop codon in the X10 allele. In wild-type DNA, the TaqI site is present but in the X10 mutant allele, this site is destroyed by a C to T transition. This changes an Arg to a stop codon. (C) Missense mutation in the AR66 allele. A sequencing gel using the CircumVent cycle sequencing kit (New England Biolabs) shows a substitution of A in the AR66 allele for G in the wild-type background. The X7 and X10 alleles are unaltered (not shown). Because these sequence reactions are for the complementary strand, the gel image was rotated 180° to portray the coding strand. (D) Amino acid substitution in the AR66 allele. A cysteine residue in the wild-type protein is substituted with a tyrosine residue in the AR66 mutant protein. (E) Location of the X10 and AR66 mutant changes in the α1 subunit. The α1 subunit transmembrane configuration is shown diagramatically. The amino and carboxyl termini are cytoplasmic. There are four repeats (I, II, III, IV) each of which is composed of six transmembrane domains (S1-S6) (Catterall 1988). Positions of the truncation in the X10 allele and amino acid substitution in the AR66 allele are indicated (black squares).
### TABLE 2

<table>
<thead>
<tr>
<th>Cross</th>
<th>Eggs Total</th>
<th>Eggs Fertilized</th>
<th>Eggs wg&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Eggs Late&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Eggs Hatched</th>
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<th>Pupae</th>
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<td>126 (52)&lt;sup&gt;d&lt;/sup&gt;</td>
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<tr>
<td>AR66 × X10 × CyOen11</td>
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<td>148</td>
<td>148 (92)&lt;sup&gt;d&lt;/sup&gt;</td>
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<sup>a</sup> The dead embryos with a wingless (wg) phenotype are homozygous for the CyO, wg<sup>en11</sup> chromosome in which the wingless gene has been disrupted by an enhancer trap transposon insert.

<sup>b</sup> These dead embryos develop to a late stage, and appear structurally normal at a gross level. They are homozygous for the mutant allele indicated.

<sup>c</sup> These surviving flies are all Cy and are genotypically l(2)35Fa/CyO, wg<sup>en11</sup>.

<sup>d</sup> These adults included both Cy and Cy<sup>1</sup> flies in a 2:1 ratio. The Cy<sup>1</sup> flies represent the critical class (mutant homozygotes) and include both flies that eclosed (these usually had unextended wings and were stuck in the food) and flies that did not fully eclose but died as pharate adults. In parentheses is the percent of Cy<sup>1</sup> flies that died as pharate adults.

food with unexpanded wings; those that fail usually open their puparia but die before being able to crawl out.

AR66/Df(2L)RA5 flies appear to be indistinguishable from AR66 homozygotes (Table 2) in that about half of these flies die as pharate adults. AR66/X7 flies, however, are almost all able to escape the puparium while very few AR66/X10 flies do so (Table 2). These results were reproduced in a second independent experiment (data not shown). This may reflect differences in the nature of the X10 and X7 alleles.

To determine whether genetic disruption of this calcium channel subunit compromises nervous system formation, various antibodies were used to examine the developing nervous systems of mutant embryos (Figures 3 and 4). Mab22C10 stains subsets of neurons and axons in the central nervous system (CNS) and peripheral nervous system (PNS) (Fujita et al. 1982) (Figure 3, A–D); MabBP102 labels CNS axons (Seeger et al. 1993) (Figure 3, E and F); Mab1D4 was generated against the cytoplasmic domain of transmembrane forms of fasciclin II and recognizes subsets of neurons and axons in the CNS (Seeger et al. 1993) (Figure 4, A and B) and motorneuron growth cones and axons in the PNS (Van Vactor et al. 1993) (Figure 4, C and D). In addition to the antibodies shown in the figures, we have also used anti-HRP (data not shown), which stains neuronal membranes (Jan and Jan 1982), and anti-cut (data not shown), which labels nuclei of many CNS cells and all external sensory organ cells in the PNS (Blochlinger et al. 1990). With one exception, in mutant embryos of the three l(2)35Fa alleles tested, no abnormalities were detected with any of these antibodies, indicating that at this level of analysis the mutant embryonic nervous systems appear morphologically normal.

The exception is that, in the CNS of a few (about 20%) homozygous AR66 embryos, neurons in the longitudinal tracts appeared to stall at some of the commissures, forming nodular growths (Figure 4B) rather than the normal smooth longitudinal tracts seen in the strong X10 mutant (Figure 4A) and in wild-type embryos (Seeger et al. 1993) (not shown). In a similar fraction of the AR66 homozygous embryos, the motorneurons in the SNb branch of the developing PNS also showed stalling (Figure 4D) at stage 17 when the SNb has normally already formed the three characteristic muscle attachments (Figure 4C) into the target muscles (Van Vactor et al. 1993). The observations that these defects are low in frequency and that they do not appear in X10, the strong allele tested, suggest that they may be caused by homozygosity of another lesion on the AR66 chromosome unrelated to the Dmca1D locus. Alternatively, they could be generated by the altered channel properties caused by the AR66 missense mutation (D. Ren, H. Xu, D. F. Eberl, M. Chopra, and L. M. Hall, unpublished results).

We have also tested for maternal effects that might
be associated with these lethal mutations. Many lethal mutations with apparently late effects have much earlier requirements if the normal gene products provided maternally to the egg are eliminated (Perrimon et al. 1984). We tested for maternal effects by generating heterozygous mutant ovaries in heterozygous mothers using the “FLP-DFS technique” (Chou and Perrimon 1996). We have found no evidence for maternal effects associated with the three l(2)35Fa alleles tested (data not shown).

**DISCUSSION**

The l(2)35Fa gene (Dmca1D) encodes the Dmca1D channel: We have presented several lines of evidence indicating that l(2)35Fa is the structural gene encoding the calcium channel α1 subunit Dmca1D. First, it is the only candidate complementation group that co-maps with Dmca1D, even after extensive mutagenesis screens in a number of laboratories (Ashburner et al. 1990). Second, we rescued l(2)35Fa with a genomic cosmid that encodes the calcium channel α1 subunit and demonstrated that the α1 subunit transcript is the only one detectable in embryos by probes from this cosmid. Third, we have identified within the calcium channel open reading frame a premature stop codon in the X10 mutant allele and a missense mutation in the AR66 allele of l(2)35Fa.

Additional electrophysiological studies on the AR66 mutation (D. Ren, H. Xu, D. F. Eberl, M. Chopra, and L. M. Hall, unpublished results) demonstrated reduced DHPR-sensitive calcium channel current density with slower activation kinetics in third instar larval muscles. In addition, the embryonic lethality of l(2)35Fa is consistent with our earlier observation that flies fed with
the calcium channel blocker verapamil show dose-dependent lethality (Hall et al. 1994). Those flies that do survive at moderate doses of verapamil show delayed development, consistent with delayed development of AR66 mutant flies. Furthermore, late embryonic lethality is consistent with the peak of $\alpha_1$ subunit mRNA expression detected with Dmca1D probes in late embryos (Zheng et al. 1995). Taken together, these results constitute overwhelming evidence that l(2)35Fa encodes Dmca1D. Based on these results and arguments, we propose to rename the l(2)35Fa locus Dmca1D.

Role of Dmca1D in embryos and adults: These studies demonstrate that Dmca1D is first required in the developing embryo and later in late pupal stages, both times when Dmca1D mRNA is expressed at peak levels (Zheng et al. 1995). There is no maternal effect and we have found no obvious structural abnormalities in the mutant embryonic CNS or PNS that we could attribute to the Dmca1D mutations. These observations, together with those of abnormal movement of the mutant embryos, suggest that the embryonic defect is primarily physiological rather than a gross disruption of nervous system development. Our finding that AR66 larvae have defects in muscle calcium channel currents is consistent with this interpretation. Thus, function of Dmca1D appears to be required for the muscle contractions (or their neuronal modulation) for the pharate larva to make proper hatching movements. Dmca1D also appears to be necessary for gas-filling of the tracheae, either actively or, as more classically thought, passively, through metabolic exertion or physical movement (see Manning and Krasnow 1993). This could be resolved by a detailed mapping of the cells which require Dmca1D function for the gas-filling process. Dmca1D may also be involved in modulating contraction in the gut and heart.

While these pharate larval movements are disrupted by the strong mutations X7 and X10, they are not detectably altered by the weak allele, AR66. Larvae expressing this mutation are able to hatch normally, and are behaviorally indistinguishable from their wild-type siblings throughout the larval period, displaying the first abnormalities as late pupae. These abnormalities are manifest as a delay in development, difficulty in eclosion and disturbed fluid-filling of the wings for proper expansion. The mechanistic significance of the AR66 mutation for the larva appears to be that the slower activation and reduced current density of the AR66 channel provides reduced larval calcium channel activity, but still enough to meet the larval requirements. However, AR66 mutant channel function clearly is not sufficient to fulfill the adult requirements of Dmca1D.

As in the embryo, it is possible to explain the pharate adult defects as either muscle or neuronal defects. First, the process of eclosion from the puparium requires...
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vigorou s muscular activity. The ptilinum, or inflatable head, of the pharate adult is cyclically inflated and deflated, allowing it to wedge into the anterior part of the puparium in order to pry it open (Laing 1935; Crossley 1978). Inflation of the ptilinum is achieved by the contraction of the supercontracting abdominal muscles (of larval origin; they degenerate within a few days of eclosion), forcing the hemolymph into the head. Deflation occurs by contraction of the ptilinal retractor muscles in the head (these also degenerate in a couple of days). Second, the process of wing expansion in the first hour after eclosion is based on pharyngeal muscle activity. Upon eclosion the fly begins to swallow air until (Smith 1924; Fraenkel 1935). This increase in gut volume greatly increases the pressure of the hemolymph, distending the entire fly and forcing a steady stream of hemolymph into the wings to inflate them (Lagueux and Perron 1973). Thus, all the phenotypes we see, from defects in embryonic muscular contractions in strong mutants, to defects in third instar larval muscle electrophysiology (D. Ren, H. Xu, D. F. Eberl, M. Chopra, and L. M. Hall, unpublished results), to defects in eclosion and wing expansion in pharate adults carrying the weaker mutation, are consistent with defects in muscle contraction, though neuronal modulation of the contractions could also be affected.

Use of heteroallelic mutant combinations revealed differences in the proportion of mutant pupae that die as pharate adults suggesting differences in the nature of the X7 and X10 alleles. One possibility is that the X10 truncated protein sequesters some of the other We gratefully acknowledge the technical assistance of Nancy Bourgeois, John Mulawka, and Daniella Scalice. John Root and Michael Ashburner were exceptionally helpful in providing mutations and chromosome rearrangements for the genetic analysis. We thank Maninder Chopra and Jeff Hall for comments on the manuscript. This work was supported in part by grants from the BioAvenir program sponsored by Rhone Poulenc, the Ministry in charge of Research and the Ministry in charge of Industry (France). It was also supported by grants to L. M. H. from National Institutes of Health (NHHL39369), and the New York Affiliate of the American Heart Association. D. F. E. was supported by a Natural Sciences and Engineering Research Council of Canada Postdoctoral Fellowship; G. F. by a Pharmaceutical Manufacturers Association Predoctoral Fellowship; and L. J. L. by an NIH Postdoctoral Fellowship.

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