Drosophila Calmodulin Mutants With Specific Defects in the Musculature or in the Nervous System

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ABSTRACT
We have studied lethal mutations in the single calmodulin gene (Cam) of Drosophila to gain insight into the in vivo functions of this important calcium sensor. As a result of maternal calmodulin (CaM) in the mature egg, lethality is delayed until the postembryonic stages. Prior to death in the first larval instar, Cam nulls show a striking behavioral abnormality (spontaneous backward movement) whereas a mutation, CamV91G, that results in a single amino acid change (V91G) produces a very different phenotype: short indented pupal cases and pupal death with head eversion defects. We show here that the null behavioral phenotype originates in the nervous system and involves a CaM function that requires calcium binding to all four sites of the protein. Further, backward movement can be induced in hypomorphic mutants by exposure to high light levels. In contrast, the V91G mutation specifically affects the musculature and causes abnormal calcium release in response to depolarization of the muscles. Genetic interaction studies suggest that failed regulation of the muscle calcium release channel, the ryanodine receptor, is the major defect underlying the CamV91G phenotype.

The small calcium sensor protein calmodulin (CaM) is one of the major mediators of the complex interactions that underlie calcium regulation (see Van Eldik and Watterson 1998 for review). CaM is present in all eukaryotic cells and is highly conserved across evolutionary orders. Two pairs of EF-hand-type calcium-binding sites are present in each of its two globular domains and conformational changes produced by calcium binding dramatically alter the target interaction properties of the protein. CaM thus converts changes in calcium levels into changes in target activity.

The large array of CaM targets identified to date includes protein kinases, the ubiquitous protein phosphatase calcineurin, adenylyl cyclase, cyclic nucleotide phosphodiesterase, and cytoskeletal proteins such as spectrin and adducin. More recently, calcium channels and calcium-regulated channels have proved to be CaM targets (reviewed in Saimi and Kung 2002). These include ryanodine receptor (RyR) channels and L-type voltage-gated dihydropyridine receptor (DHPR) channels of muscle tissues.

In vitro experimentation is elucidating the molecular detail of CaM interactions with individual targets. However, given that CaM plays a central role in coordinating calcium responses, there are aspects of function that can be revealed only by in vivo genetic studies. Genetic studies to date in unicellular eukaryotes demonstrate this point. In Paramecium, for example, Kung and coworkers uncovered striking effects of CaM mutations on avoidance responses, which entail plasma membrane depolarization and a bout of backward swimming (Saimi and Kung 1987). Surprisingly, mutations in the N- or C-terminal globular domains of CaM produce opposing effects. Mutations in the C-terminal domain show spontaneous or exaggerated backward swimming due to loss of a restorative, repolarizing, Ca2+-dependent K+ current. In contrast, mutations in the N-terminal domain give curtailed avoidance responses due to loss of a depolarizing Ca2+-dependent Na+ current (Kink et al. 1990). These studies first demonstrated a functional partitioning inherent in the domain structure of CaM (Van Houten et al. 1977; Ling et al. 1994).

More recent work with Saccharomyces cerevisiae has also provided insights that could come only from in vivo studies. Thus, genetic rescue experiments with versions of CaM defective in calcium binding (Geiser et al. 1991) provided the first evidence that Ca2+-free CaM (apo-CaM) performs important functions in the cell. Further, systematic mutation of the individual phenylalanines of S. cerevisiae CaM (Ohya and Botstein 1994) led to identification of four intragenic complementation groups and the realization that different regions of CaM are critical for different targets and different intracellular processes (reviewed in Cyert 2001).

We have used a genetic approach to address CaM function in the complex multicellular organism, Drosophila melanogaster. In contrast to other genetically tractable animals and plants, Drosophila contains only one
gene (Cam) encoding CaM (DOYLE et al. 1990), thus eliminating potential problems with functional substitution. We have generated the following classes of Drosophila Cam mutations: (i) RNA nulls that produce no zygotic CaM protein (HEIMAN et al. 1996), (ii) hypomorphs that produce decreased levels of CaM (SCOTT et al. 1997), and (iii) point mutations that alter individual amino acids within the protein (NELSON et al. 1997).

None of these Cam mutations affect embryonic development, reflecting our discovery that maternal CaM persists until immediately before hatching (HEIMAN et al. 1996; B. F. ANDRUSS and K. BECKINGHAM, unpublished results). No zygotic protein is produced until late in embryogenesis despite early zygotic transcription of Cam in neural lineages (KOVALICK and BECKINGHAM 1992). As a consequence, even RNA nulls hatch as morphologically normal first instar larvae (HEIMAN et al. 1996). Distinct phenotypes are detected among the Cam mutants, however. Although Cam nulls die in first instar, they show striking behavioral abnormalities. These include subsequent backward movement, reminiscent of Paramecium CaM mutants, and increased head swinging (HEIMAN et al. 1996). None of five point mutations affecting the CaM protein coding sequence (NELSON et al. 1997) show these defects, but several show distinctive alternative phenotypes. For example, Cam\(^{52}\), which encodes a CaM altered in calcium-binding site 1, permits survival of some morphologically normal adults but, uniquely, produces enhanced excitability of the larval neuromuscular junction (ARREDDONDO et al. 1998).

Only one of the point mutations isolated, Cam\(^{7}\), which encodes the mutant CaM V91G, produces a morphological phenotype. Cam\(^{7}\) animals form aberrant pupal cases with indentations at the larval segment boundaries, giving them a “Michelin man” appearance. The mutants all die as pupae, sometimes with “inside-out” heads buried in the thorax (NELSON et al. 1997). We have investigated the underlying defects in both Cam null and Cam\(^{7}\) animals. We have demonstrated the neural origin of the backward movement of the null and the influence of light on this phenotype. In contrast, most of the Cam\(^{7}\) phenotype is muscle specific and associated with altered-muscle calcium fluxes. Genetic interaction experiments suggest that the ryanodine receptor is one of the major muscle targets affected by this mutation.

**MATERIALS AND METHODS**

**Mutations:** The following mutations were used: Cam\(^{339}\), recessive RNA null mutation (HEIMAN et al. 1996); Cam\(^{2}\), recessive ethyl methanesulfonate mutation (NELSON et al. 1997); Cam\(^{52}\), recessive hypomorph, generated by excision of a P element in 5’ flanking DNA (SCOTT et al. 1997); Cam\(^{909}\), recessive hypomorph generated by a P insertion 60 bp 5’ of the transcription start site (HARVEY et al. 1998); Ryr\(^{1}\), recessive mutation of the ryanodine receptor gene (RYR; SULLIVAN et al. 2000); Df/2R;H3E1, deficiency with breakpoints at 44D1-4 and 44F12 (Bloomington Stock Center); Cam\(^{ID}\), embryonic lethal Cam\(^{ID}\) mutation (EBERL et al. 1998); Cam\(^{ID}\), hypomorphic Cam\(^{ID}\) mutation (EBERL et al. 1998; REN et al. 1998); and cn\(^{\ast}\), cinnabar (Bloomington Stock Center).

**Gal4 lines:** We used the following Gal4 lines: 24B-Gal4, P[GawB]hao\(^{A8}\), an insertion into held out wings that expresses Gal4 in muscle (BRAND and PERRIMON 1993); elav-Gal4, Gal4 expressed under the elav promoter in neurons at all developmental stages (YAO and WHITE 1994); and sco-Gal4, Gal4 expressed under the control of the scabrous promoter in the central nervous system and the peripheral nervous system before embryonic stage 14 (MLODZIK et al. 1990).

**UAS and other lines:** UAS-Cam, UAS-B12Q, UAS-B34Q, UAS-B123Q and UAS-V91G constructs were generated in the UAS vector of BRAND and PERRIMON (1993) and expressed the following CaMs, respectively: wild-type CaM, CaM with Ca\(^{2+}\)-binding sites 1 and 2 disabled, CaM with binding sites 3 and 4 disabled, CaM with all four binding sites disabled, and finally, V91G mutant CaM (WANG et al. 2002). Stocks carrying UAS-aequorin, UAS-lacZ, and UAS-green fluorescent protein (GFP) are described in ROSAY et al. (1997), BRAND and PERRIMON (1993), and BRAND (1995), respectively. A stock carrying lacZ under the control of the myosin heavy chain promoter (MHC-lacZ) was obtained from Sanford Bernstein (HESS et al. 1989).

**Stocks and crosses:** All Cam mutations were kept in y; Cam\(^{+/+}\) flies so that Cam mutant combinations could be selected from crosses of such stocks. The required larvae were identified by y’ mouth hooks. To examine the Cam\(^{2}\) hemizygous phenotype, the following cross was used: y; Cam\(^{+/+}\) × y; Cam\(^{+/+}\) (Cam\(^{+/+}\) × y Cam\(^{+/+}\)). For the Cam null phenotype, homozygous Cam\(^{339}\) larvae were examined.

To express UAS constructs with a chromosome 3 Gal4 driver in the Cam\(^{1}\) or Cam\(^{339}\) backgrounds, stocks for the following cross were generated: y; Cam\(^{1DAR66}\) × y; Cam\(^{1DAR66}\) (Cam\(^{+/+}\) × y Cam\(^{+/+}\); Cam\(^{+/+}\) Cam\(^{+/+}\)). For the Cam null phenotype, homozygous Cam\(^{339}\) larvae were examined.

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Ryr, Cam\(^{ID}\), and cinnabar (cn) mutations were recombined onto the Cam\(^{1}\) chromosome. Putative recombinant chromosomes were tested by backcrossing to confirm the presence of both mutations. For Ryr\(^{+}\), which gave an unexpected phenotype (see results), the Cam\(^{1}\) mutation was reisolated from one recombinant chromosome to confirm its presence on the double-mutant chromosomes.

**Larval/pupal studies:** First instar larvae were collected, sorted by mouth hook color, and transferred to food vials (30 per vial). Larvae were kept at 25° in a 12-/12-hr light-dark cycle. Pupal lengths and widths were measured under a dissecting microscope. Statistical analyses were performed using Statview software (Abacus Concepts, Berkeley, CA).

**Behavioral studies:** Larval locomotion assay: Larvae were placed on moist 1% agarose plates and left for a 1-min adjustment period. The number of body-wall contractions (BWC) in a 1-min interval was recorded for each animal. A total of 8000–10,000 BWC was used for strong light, and 400–500 BWC was used for low light.

Adult behavioral tests: Flies used for behavioral tests were collected immediately after eclosion and aged individually in vials for 3 days before testing. Climb, flight, and vortexing tests described previously (NELSON et al. 1997) were used with little modification.

For mating assays, male flies were placed individually in vials with two virgin Oregon-R females and observed for 1 hr. Time spent performing various elements of the mating ritual was recorded.

**Larval body-wall muscle staining:** Third instar larvae were im-
mobilized and their muscles relaxed with ether vapor. Pinned larvae were dissected in Ca\(^2+\)-free saline (130 mM NaCl, 5 mM KCl, 36 mM sucrose, 4 mM MgCl\(_2\), 0.5 mM EGTA, 5 mM HEPES, pH 7.3) to minimize contraction. Larvae were cut along the dorsal midline and internal organs were removed. The cleaned body walls were fixed in PBS (137 mM NaCl, 2.68 mM KCl, 10.14 mM Na\(_2\)HPO\(_4\), 1.76 mM KH\(_2\)PO\(_4\), pH 7.5) containing 4% paraformaldehyde for 30 min and the muscles were stained with rhodamine-phalloidin (10 units/ml; Molecular Probes, Eugene, OR) for 1 hr in PBX (PBS + 0.15% Triton X-100) with gentle rotation. After several rinses in PBX, the muscles were inspected by fluorescence microscopy. K-contracture and aequorin luminescence recording: Third instar larvae were relaxed on ice, pinned on glass plates using magnetic needles, and opened along the dorsal midline in bathing and show normal forward locomotion with no spontaneous backward movement. But they are sluggish, with a BWC rate about one-third that of controls. The most striking problem occurs at pupariation when a severe morphological phenotype arises. As described previously (Nelson et al. 1997; Wang et al. 2002), pupal cases with deep indentations at the larval segment boundaries are produced (Figure 1A, b–d). Control larvae decrease their body length by one-third at pupariation, whereas we determined that, on average, Cam\(^7\) larvae decrease in

Aequorin luminescence was collected using Black Night software and processed by Excel. To compensate for variation in expression of aequorin in individual larvae, a baseline was calculated for each animal by averaging the signal obtained during a 30-sec period before adding the high-K solution and used to normalize the signal after depolarization.

**RESULTS**

**Developmental and behavioral defects of the Cam\(^7\) mutant** The Cam\(^7\) phenotype was studied in the hemizygous condition. Cam\(^7\) larvae are morphologically normal and show normal forward locomotion with no spontaneous backward movement. But they are sluggish, with a BWC rate about one-third that of controls. The most striking problem occurs at pupariation when a severe morphological phenotype arises. As described previously (Nelson et al. 1997; Wang et al. 2002), pupal cases with deep indentations at the larval segment boundaries are produced (Figure 1A, b–d). Control larvae decrease their body length by one-third at pupariation, whereas we determined that, on average, Cam\(^7\) larvae decrease in

**Figure 1.**—Aberrant pupal cases and pharate adult head structures in Cam\(^7\) mutants. (A) The Cam\(^7\) mutation leads to the formation of “Michelin man” pupal cases (b–d), which are shorter than wild type (a) and have highly indented rings around the body. Cam\(^7\) pharate adults display three types of head structures: well-formed (Cam\(^7\)-W, b); malformed (Cam\(^7\)-M, c), and “inside-out” within the thorax as a result of failed head eversion (Cam\(^7\)-H, d). The adult head is formed by eversion of the head sac into an anterior gas bubble (a). The presence of a gas bubble is variable in Cam\(^7\) and does not correlate with the degree of failure of head eversion. (B) Correlation of pupal length:width ratio with the degree of head abnormality in Cam\(^7\) pharate adults. The more severe head defects in Cam\(^7\) animals are associated with shorter pupal cases. Cam\(^7\)-W, Cam\(^7\)-M, and Cam\(^7\)-H as in A. n, number of animals measured. P values for the length:width ratios: between control and Cam\(^7\)-W, \(P < 0.0001 (**\)); between Cam\(^7\)-W and Cam\(^7\)-M or Cam\(^7\)-H, \(P < 0.0001 (**\)); between Cam\(^7\)-M and Cam\(^7\)-H, \(P = 0.07 (-\)). Genotypes: control, yw; Cam\(^7\), yw; Cam\(^7\).
length by 50%. Measurements of length:width ratios for control and Cam7 pupal cases (Figure 1B) confirmed that only the long axis of the body is abnormally compressed.

One possible explanation is that the longitudinal body-wall muscles of Cam7 animals are hypercontracted during pupariation. Consistent with this possibility, wandering third instar Cam7 larvae grow progressively incapable of relaxing at the end of each body-wall contraction and show increasing stiffness during locomotion. Examination of third instar body-wall muscles revealed that, despite artificial relaxation with ether, all mutant animals had groups of longitudinal muscles with a “bunched” appearance (Figure 2c) that showed structural disorganization and misaligned myofibrils (Figure 2d). These abnormalities suggest muscle degeneration, possibly as a result of hypercontraction.

Cam7 mutant pupae never eclose, but most develop into pharate adults with head defects. The adult head is formed from a head sac that is everted from the thorax into an anterior gas bubble ~10 hr after pupariation. Examination revealed three categories of heads among Cam7 pharate adults, each approximately equally represented (Table 2): (i) normal heads with no obvious defects (W class; Figure 1A, b), (ii) malformed heads (M class) that are partially everted (Figure 1A, c), and (iii) “inside-out” heads (H class; Figure 1A, d). Inside-out heads arise when head eversion fails completely and development proceeds in a noneverted head sac buried in the thorax.

Head eversion entails strong contractions by residual larval abdominal muscles. From videorecordings we determined that in all Cam7 mutant larvae, these contractions were weak and poorly synchronized or absent and often failed to give complete head eversion. Thus, failure of the residual larval muscles probably represents the major cause of the Cam7 head eversion defects. We found a correlation in the Cam7 pupae between length:width ratios and the three classes of pupal heads (Figure 1B). Thus, Cam7 pharate adults with normal heads have longer pupal cases than those with malformed or noneverted heads. This correlation suggests that both parameters reflect the degree of severity of the Cam7 defects.

It could be argued that the Cam7 mutant V91G CaM is less stable than wild-type CaM and that the phenotype simply reflects low levels of CaM. However, examination of the Cam352 mutant, which has severely decreased levels of CaM (Scott et al. 1997), discounted this possibility. Like Cam7, Cam352 produces larval sluggishness and 100% pupal lethality. However, the Cam352 pupal cases are normal and the animals die as pharate adults with no head defects.

**Rescue of the Cam7 lethality and pupariation defects by expression of exogenous wild-type CaM in the musculature:** All aspects of the Cam7 phenotype suggest that muscle function, or neural control of muscle function,
is specifically affected by the mutation. To address these possibilities, we expressed wild-type CaM in either the muscles or the nervous system using the Gal4-UAS system (Brand and Perrimon 1993). For muscle-specific expression we used the 24B-Gal4 driver (Brand and Perrimon 1993) and for neurons we expressed Gal4 under the control of promoters from (i) elav (elav-Gal4; Yao and White 1994) or (ii) scabrous (sea-Gal4; Mlodzik et al. 1990).

Expressing wild-type CaM with the 24B-Gal4 driver rescues the lethality and pupariation defects caused by the Cam′ mutation (Table 1; see also Wang et al. 2002). The rescued Cam′ larvae form pupal cases that are morphologically wild type and have more normal length:width ratios. Almost all of the rescued pupae eclose as adults and no aberrant pharate head structures are found. In contrast, expressing wild-type CaM specifically in the nervous system with either neural driver failed to rescue the lethality and the pupariation defects. Therefore, these aspects of the Cam′ phenotype specifically originate in the muscles, not in the nervous system (Table 1). However, the sluggish larval locomotion was incompletely rescued by either muscle or neural expression of CaM (Figure 3), suggesting a more generalized origin. Both Cam null and Cam′ mutant larvae also show larval sluggishness.

Interestingly, Cam′ mutant animals rescued to adulthood by expression of wild-type CaM in the muscles (rescued adults) were behaviorally abnormal. They failed to climb after being gently knocked to the bottom of a vial and showed poor flight and a reduced ability to right themselves after brief vortexing. In addition, rescued males, but not females, showed defective mating behavior. Initial stages of male courtship were normal, but, whereas wild-type males could penetrate females successfully after a few attempts, the rescued males could not achieve junction despite repeated mounting. For the Drosophila male to bring his genitalia into the correct position for intromission, he must bend his abdomen into a strongly curved position. The rescued males could not bend their abdomens sufficiently to achieve penetration.

The aberrant behaviors in rescued Cam′ adults could arise from two sources: (i) defects present in nonmuscle tissues in adults or, given that the adult expression pattern of 24B-Gal4 is uncharacterized, (ii) inadequate Gal4 expression from the 24B driver in adult muscles. This latter possibility seemed relevant to the male mating problem because the male-specific abdominal Muscles of Lawrence are required for abdominal bending during copulation. We compared the expression of the 24B-Gal4 line (using UAS-GFP and UAS-lacZ) to that of an MHC-lacZ construct in male abdominal muscles. MHC-lacZ gave strong expression in both the somatic and male-specific muscles. However, 24B-Gal4-induced expression was weak in the abdominal dorsal muscles and was not detectable in the Muscles of Lawrence. In contrast, 24B-Gal4 gave strong expression in adult leg muscles (data not shown). Therefore, the abnormal male mating behavior probably reflects inadequate expression in the required muscles.

**Partial rescue of the Cam′ phenotype by a ryanodine receptor mutation:** The aspects of the Cam′ phenotype that are unique to this Cam mutation arise specifically in the musculature and are (i) the permanent hypercontraction of the longitudinal body muscles at pupariation and (ii) the subsequent failure of the residual body-wall muscles during head eversion. This combination of defects is unprecedented in Drosophila. Failed head eversion due to abdominal muscle defects is also seen for mutations associated with the ecdysone pulses that orchestrate the puparial and pupal molts (Hadorn and Gloor 1943; Chadfield and Sparrow 1985; Fletcher et al. 1995; Hewes et al. 2000). In these mutants, muscle failure probably reflects premature death of the larval muscles (Fletcher et al. 1995; Hewes et al. 2000). However, none of these mutants show the hypercontraction at pupariation seen in Cam′ animals.

Although we could find no other Drosophila mutations producing hypercontraction at pupariation, we discovered a strikingly similar pharmacological “pheno-copy” in the related Dipteran Sarcophaga bullata. Injection of ryanodine immediately prior to pupariation (Zdarek and Fraenkel 1972; Zdarek et al. 1979) pro-

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**TABLE 1**

Rescue of Cam′ lethality by muscle-specific expression of wild-type CaM

<table>
<thead>
<tr>
<th>Genotype</th>
<th>No. of first instar larvae collected</th>
<th>Larvae that pupariate (%)</th>
<th>Larvae that eventually eclosed as adults (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cam′</td>
<td>87</td>
<td>97.7</td>
<td>97.7</td>
</tr>
<tr>
<td>Cam′</td>
<td>90</td>
<td>78.9</td>
<td>0</td>
</tr>
<tr>
<td>Cam′ + muscle Cam′</td>
<td>60</td>
<td>91.7</td>
<td>88.3</td>
</tr>
<tr>
<td>Cam′ + neural(e) Cam′</td>
<td>60</td>
<td>85.0</td>
<td>0</td>
</tr>
<tr>
<td>Cam′ + neural(s) Cam′</td>
<td>60</td>
<td>83.3</td>
<td>0</td>
</tr>
</tbody>
</table>

Cam′, y w; Cam′/+; Cam′, y w; Cam′/Cam′; 24B-Gal4+, Cam′ + muscle Cam′, y w; Cam′/Cam′; 24B-Gal4/UAS-CaM, Cam′ + neural(e) Cam′, y w; Cam′/Cam′ elav-Gal4/Cam′; +/UAS-CaM. Cam′ + neural(s) Cam′, y w; Cam′ Cam′/H11001, sea-Gal4/Cam′; +/UAS-CaM.
produced the same shortened, highly indented pupal cases type muscles, which include the Drosophila larval body- onto the Cam7 doses, the channel is locked into a subconductance state smaller alleviation of the

The excitatory depolarization of the plasma membrane and the RyR on the sarcoplasmic reticulum. Controls were performed to investigate the unex-

cium-release channel of muscles. The action of ryano- expression of wild-type CaM (see above). In marked

failing to eclose showed no head defects (Table 2).

Interestingly, some of the eclosed Ryr16 Cam7 adults showed failed wing expansion as seen for weak Ca-α1D mutations. All of the eclosed adults performed poorly in the climb test, like the Cam7 adults rescued by musclespecifically with the ryanodine receptor, the major cal-

The Ryr16 mutation is not a null (SULLIVAN et al. 2000), so we also used deletion Df(2R) H3E1, which completely removes Ryr. The presence of one copy of the Ryr16 mutation produced a striking rescue of the Cam7 pupal and lethal phenotypes (Figure 4 and Table 2). The pupae (termed here Ryr16 Cam7 pupae) were smooth and indentation free in the anterior with only mild ridges in the posterior (Figure 4A). Their length:width ratios were actually higher than those of wild type (Figure 4B). While none of the Cam7 pupae eclosed, >40% of the Ryr16 Cam7 animals emerged as adults, and those failing to eclose showed no head defects (Table 2).

Controls were performed to investigate the unexpected difference in behavior between Ryr16 and the H3E1 deletion. Since Ryr is on the second chromosome as is Cam, the Ryr16 and H3E1 mutations were recombined onto the Cam1 chromosome (see MATERIALS AND METHODS). Thus, an alternative explanation for this difference could be that the Cam1 phenotype is partially due to a second mutation on the Cam1 chromosome that was recombined away in generating the Ryr16 Cam1 chromosome but not the H3E1 Cam1 chromosome. To address this possibility, we examined five different recombinant Ryr16 Cam1 chromosomes and four H3E1 Cam1 chromosomes and found that all the recombinant chromosomes of the same type behaved identically. Thus, random differences in the chromosome regions ex-
changed during recombination did not affect either phenotype. As a further test, an unrelated mutation, cn, which affects eye color, was recombined onto the Cam^7 chromosome. cn is located close to Ryr on chromosome 2 and a similar region of the Cam^7 chromosome must be exchanged to introduce cn onto the Cam^7 chromosome. Four recombinant cn Cam^7 chromosomes were examined and none showed any effects on the Cam^7 mutation (Table 2). As a final control, we demonstrated that chromosomes carrying the Cam^7 mutation alone and displaying the original Cam^7 phenotype could be recovered from one of the Ryr^16 Cam^7 chromosomes. The difference in alleviation of the Cam^7 phenotype shown by Ryr^16 and H3E1 thus seems to reflect a real difference in the effects of the two mutations.

To examine the effects of reduced DHPR channel function, we used two Ca-α1D mutations, the strong mutation X7, which behaves as a null in larvae, and the weaker allele, AR66, containing a point mutation (Eberl et al. 1998). The heterozygous Ca-α1D^X7 mutation produced a small but significant increase in the length:width ratio of the pupal cases (Figure 4B) but had no obvious effect on the pupal case indentations. This mutation also enabled a very small fraction of the Cam^7 pupae to eclose as weak, uncoordinated adults with failed wing expansion (Table 2). The weaker Ca-α1D^AR66 mutation increased the length:width ratio of the Cam^7 pupal cases (Figure 4B) but none of these pupae eclosed (Table 2).

Effects of the Cam^7 mutation on larval body-wall calcium fluxes in response to K contracture: To determine whether calcium fluxes are altered in Cam^7 muscles, we used aequorin to monitor calcium release upon muscle depolarization. Aequorin, when bound to its cofactor coelenterazine, luminesces in response to calcium binding. UAS-aequorin under Gal4 control has been used in

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**Figure 4.**—Rescue of the Cam^7 pupal case phenotype by introduction of one copy of an Ryr or Ca-α1D mutation. (A) Rescue of the Cam^7 pupal case indentations by introduction of one copy of the Ryr^16 mutation. (a) Control, yw; + / Cam^339. (b) Cam^7 pupal case, yw; Cam^7/Cam^339. (c) Ryr^16 Cam^7 pupal case, yw; Ryr^16 Cam^7/Cam^339. Note that only mild indentations are present in the posterior half of Ryr^16 Cam^7 pupal cases. (B) Effects of mutations on pupal length:width ratios. Genotypes are as follows: Cam^7, yw; Cam^339/Cam^7. Ryr^16 Cam^7, yw; Cam^339/Ryr^16 Cam^7. cn Cam^7, yw; Cam^339/cn Cam^7; Ryr^16 Cam^7, yw; Cam^339/Ryr^16 Cam^7. Ca-α1D^X7 Cam^7, yw; Cam^339/Ca-α1D^X7 Cam^7. Ca-α1D^AR66 Cam^7, yw; Cam^339/Ca-α1D^AR66 Cam^7. Control, yw; Cam^339/+.

**P < 0.0001; *P < 0.01; – not significant; n, number of animals measured.**
Drosophila to examine calcium fluxes in the adult brain (Rosay et al. 2001) and Malpighian tubules (Rosay et al. 1997). We expressed aequorin in the muscles of Cam7 and control larvae and induced calcium release in preparations of body-wall muscles with high external \([K^+]\) (K contracture; Hodgkin and Horowicz 1960).

Preliminary experiments were performed to identify a method of increasing \([K^+]\) that produced synchronized depolarization of all muscle groups in the larval body wall. By rapidly introducing a large volume of high \([K^+]\) solution, luminescence transients could be produced that had a single peak and a time frame comparable to those recorded in single fiber studies (Taylor et al. 1982). To allow for the variation in the expression of aequorin in individual animals, depolarization-induced luminescence was corrected for each animal to its individual baseline luminescence level. Thus these experiments did not address resting calcium levels in control or Cam7 animals. Figure 5A shows three superimposed luminescence transients for control larvae; these transients increased rapidly to a maximum and returned smoothly and sharply to baseline within 10 sec. Figure 5B shows transients for three Cam7 larvae; the rise in luminescence was not as rapid or as great and, most strikingly, the decline to baseline was slow and interrupted by secondary peaks. In these larvae, a complete return to baseline is not achieved even 50 sec after initial depolarization. These altered features of the Cam7 luminescence transients suggest three abnormalities in the Cam7 muscles: (i) loss of synchrony of calcium release in response to depolarization, (ii) lower overall calcium release, and (iii) failure of the mechanisms that inhibit calcium release quickly after depolarization.

Rescue of the backward movement phenotype in Cam null animals by neuronal expression of wild-type CaM:

The phenotype of the Cam null mutation is very different from that of the Cam7 point mutation. As discussed above, Cam null animals die in the first instar showing behavioral abnormalities, the most pronounced of which is spontaneous backward locomotion. The one phenotypic characteristic shared with Cam7 animals is sluggishness. We investigated the tissue of origin of these two Cam null phenotypes with the same Gal4 drivers used for the Cam7 experiments.

Expression of wild-type CaM in the musculature did not rescue the backward movements or overall sluggishness of Cam nulls. In contrast, the backward movement was efficiently rescued by neural expression of CaM with a concomitant increase in forward movement (Figure 6). The larvae were still sluggish, however, with a BWC rate of \(~40\%\) that of controls. Significantly, the sea-Gal4 driver, which gives neural expression only between embryonic stages 6–14 (Mlodzik et al. 1990), produced no rescuing effect (Figure 6). As discussed earlier, all embryonic CaM is maternally derived, and zygotic protein appears not to be required until the perihatching period. The findings for the sea-Gal4 driver are consistent with this hypothesis and support the idea that the backward movement of Cam nulls is caused by neuronal defects after hatching and not by developmental problems.

Although the N- and C-terminal globular domains of CaM are similar, there is evidence that the two domains have discrete functions in target regulation (see Introduction). We recently discovered that muscle-specific expression of a mutant CaM with the two C-terminal Ca2+-binding sites inactivated (termed B34Q) exacerbates the Cam7 phenotype, producing hypercontraction and larval death (Wang et al. 2002). In contrast, a mutant CaM with the two N-terminal Ca2+-binding sites
inactivated (termed B12Q) produces an opposite effect; the Cam⁰ pupariation phenotype is “overrescued” and unusually long, smooth pupal cases are produced. A CaM with all four Ca²⁺-binding sites inactivated (B1234Q) has little effect on the Cam⁰ phenotype. As a further approach to comparing the affected targets in Cam⁰ and Cam null animals, we examined the ability of these binding site mutants to rescue the backward movement of Cam null larvae when expressed in the nervous system. Interestingly, none of them could rescue the backward movement (Figure 7). This result indicates that different CaM target(s) are compromised in the Cam null mutant as compared to the Cam⁰ mutant and that the Cam null target(s) require fully calcium-saturated CaM for normal regulation. The Cam⁰-encoded CaM (V91G CaM) rescues the Cam null defects as efficiently as wild-type CaM when expressed in the nervous system, providing further proof that different targets are affected by the Cam null and Cam⁰ mutations.

**Light-induced spontaneous backward movements in hypomorphic Cam mutants:** In addition to backward movements, Cam null larvae show increased head swinging (Heiman et al. 1996). Recently, the Campos laboratory (Busto et al. 1999) established that head swinging represents the first component of an aversion response to light. We therefore investigated the role of light in backward movement by Cam mutant larvae. As shown in Figure 8, backward movement in Cam null larvae is only slightly increased by high light levels. High light intensity could not induce backward movement in any of the single amino acid mutations of Cam, including Cam⁰ (Figure 8), or in wild-type larvae. However, for two hypomorphic mutants, Cam⁰² and Cam⁰⁰⁰⁰, backward movements are significantly increased by high light in-

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**Figure 5.**—Aequorin luminescence transients in control and Cam⁰ larval body-wall muscles. Aequorin transients produced by rapid high [K⁺]-induced depolarization are shown. Transients for three control larvae (A) and three Cam⁰ larvae (B) are shown superimposed on one another. Arrow indicates the time of high [K⁺] solution application.
tensity. Hemizygous Cam"null" larvae contain less than one-tenth the amount of CaM found in wild-type larvae (Scott et al. 1997). The Cam"null" mutation results from a P element insertion upstream of the Cam transcription start site (Harvie et al. 1998). It has a similar, although milder, phenotype to Cam"null" and presumably also produces decreased levels of wild-type CaM.

**DISCUSSION**

**The Cam"null" phenotype:** The goal of these studies was to gain insight into the in vivo functions of CaM by examining the differing phenotypes of Drosophila Cam null and Cam"null" (V91G) mutations. Our studies demonstrate that the Cam"null" lethal phenotypes arise specifically from defects in the larval musculature, since muscle, but not neural, expression of wild-type CaM rescues Cam"null" animals to adulthood. Larval sluggishness is not rescued by muscle-specific expression of CaM, however, indicating some mutant effects in other tissues. The poor coordination shown by Cam"null" adults rescued with muscle-specific CaM expression may also reflect Cam"null" defects in nonmuscle tissues. However, the failed mating behavior seen in rescued males correlates well with the poor expression achieved in the Muscles of Lawrence using the 24B driver.

Theoretically, the Cam"null" mutation could disrupt muscle function by affecting (i) muscle development and structure, (ii) the contraction mechanism itself, or (iii) excitation-contraction coupling. Our previous findings on the role of maternal CaM in embryogenesis (see Introduction) eliminate developmental defects as the underlying cause. We found structural abnormalities in the body-wall muscles of wandering Cam"null" larvae, but these probably reflect secondary muscle damage or degeneration. Some mutations to the human RyR that produce calcium leakage into the muscles lead to degenerative changes producing so-called central core disease (Lynch et al. 1999).

Mutations that disrupt the contraction mechanism per se produce pupariation defects that are the exact opposite of those produced by Cam"null"—that is, flaccid, elongated pupal cases (Bunch et al. 1998). The hypercontraction in Cam"null" thus suggests that the contraction apparatus is intact, functional, and overactivated. This recognition, and the phenocopy induced by ryanodine, indicate that excess contraction due to increased calcium levels is the primary defect in Cam"null" muscles.

We demonstrated that calcium release in the body-wall muscles just prior to pupariation is severely disrupted in Cam"null" animals. Upon membrane depolarization, low levels of asynchronous calcium release that did not return to baseline were observed. These results seem paradoxical given the excessive contraction seen in Cam"null" animals. However, an incomplete return to baseline calcium levels with each successive contraction could lead to progressive calcium accumulation in the sarcoplasm and thus to hypercontraction. Our calcium measurement technique could not address resting calcium levels in individual animals, but the progressive failure of relaxation in Cam"null" animals as they age through third instar is consonant with this possibility. Further, over the lifetime of the animal this effect would slowly deplete the calcium stores so that by late third instar,
Figure 7.—Disruption of Ca\(^{2+}\)-binding by CaM abolishes its ability to rescue backward movement in Cam nulls. The rescue of backward movement seen when wild-type CaM (WT CaM) is expressed in the nervous system is lost when binding sites 1 and 2 (B12Q) or 3 and 4 (B34Q) or all four sites (B1234Q) are inactivated. Expression of two CaMs, one with sites 3 and 4 active (B12Q) and one with sites 1 and 2 active (B34Q), cannot compensate for wild-type CaM. In contrast, neural expression of V91G CaM does rescue the behavior. Genotypes are as follows: Cam\(^{n339}\), as in Figure 6. WT CaM, as elav\(^{\dagger}\) Cam\(^{n339}\) in Figure 6. B12Q, B34Q, B1234Q, and V91G, as WT CaM except that UAS constructs expressing the appropriate mutant CaM were used. B12Q and B34Q, (yw; Cam\(^{n339}\) elav-Gal4/Cam\(^{n339}\) UAS-B12Q; UAS-B34Q/\(^{+}\)). *, **, n, and error bars as in Figure 6.

when these assays were performed, calcium available for excitation-induced release would be significantly diminished.

In contrast to the hypercontraction at pupariation, the head eversion defects seen hours later are associated with failed muscle contractions. It is possible that by this stage permanent damage has occurred to the muscles. In adult insects, the calcium leakage caused by ryanodine produces muscle rigidity followed by progressive flaccid paralysis (Jenden and Fairhurst 1969). Alternatively, by this stage, the muscle calcium stores may be completely depleted and contraction may be impossible.

Both channel types responsible for calcium flux into the muscles (RyR and DHPR) are CaM-regulated proteins (reviewed in Teng et al. 2002) and thus are good candidates to be the target(s) affected by Cam\(^{\dagger}\). CaM regulation of RyR is relatively well-characterized. Each subunit of the tetrameric channel binds a single molecule of CaM, which regulates the channel in a biphasic manner. Ca\(^{2+}\)-free CaM (apoCaM) sensitizes the channel to opening at low calcium, whereas Ca\(^{2+}\)-bound CaM (holoCaM) inhibits channel opening, thus providing feedback regulation and channel closure after activity. CaM regulation of DHPR channels is less well investigated. In mammals, the \(\alpha 1\) subunits of both skeletal- and cardiac-muscle DHPR channels bind CaM and, in the case of cardiac DHPRs, holoCaM mediates channel closure at high calcium. However, no equivalent studies have yet been reported for the skeletal \(\alpha 1\) subunit.

The loss of prompt channel closure suggested by our aequorin experiments could thus reflect failed regulation of either RyR or DHPR channels. However, several factors suggest that the RyR is the target more affected by Cam\(^{\dagger}\). First, if CaM-induced closure of the RyR channels were still intact, then failed inactivation of the DHPR channels would have to override this functional, downstream regulation to keep the RyR channels open. In contrast, failed closure of the RyR channels would allow calcium to leak into the muscles independently of DHPR function.

Second, the tissue mRNA expression patterns for the two channels also point to a major role for the RyR. Embryonic expression of RyR mRNA is primarily in the body wall and visceral musculature (Sullivan et al. 2000). Mosaic experiments with the \(Ryr^{16}\) mutation failed to reveal any roles for RyR in nonmuscle tissues during development (Sullivan et al. 2000). Thus, the rescue of the Cam\(^{\dagger}\) phenotype by muscle expression of wild-type CaM is the expected result if RyR is the major target affected. In contrast, although Dmos ID encodes the \(\alpha 1\) subunit of the L-type channel in larval muscles, the major site of mRNA expression in embryogenesis is the nervous system (Zheng et al. 1995). Thus, if this
Figure 8.—Relationship between illumination and backward movement in Cam mutants. At low light intensity, the Cam hypomorphic mutants Cam352 and Cam3909 make almost no backward movements. At high illumination, both these mutants show robust backward movement. For control and Cam7 larvae, the presence or level of backward movement is unaffected by light. All mutations and the control chromosome were studied in the y w background, hemizygous to Camn339. *, **, n, and error bars as in Figure 6. --, not statistically significant.

DHPR channel were the major target affected by Cam7, some component of the phenotype would be predicted to be neural in origin.

Our genetic interaction experiments also indicate that RyR is an important, if not the primary, muscle target affected by Cam7. The RyR16 mutation produces a remarkable suppression of the Cam7 phenotype. Almost half of the Cam7 animals are rescued to become morphologically normal adults. The ability of RyR16 to rescue Cam7 more dramatically than an RyR null mutation probably reflects the fact that each RyR channel is a tetramer. Whereas a heterozygous deletion will result in 50% fewer wild-type channels, a heterozygous mutant protein, assuming it can be incorporated into tetramers, will represent one or more subunits in 15 of 16 of all channel tetramers. RyR16 is not dominant and thus its dramatic effect on the Cam7 phenotype suggests that (i) V91G CaM has an altered interaction with the wild-type RyR channels and (ii) when most of the channels contain at least one RyR16 type subunit, the effects of that altered interaction are alleviated. It will be of interest to determine the extent to which calcium transients are restored in the RyR16 Cam7 larvae. The protein encoded by the RyR16 allele has not been characterized, although preliminary Western blot experiments indicate that it is similar in size to the wild-type protein (R. Liou, K. M. C. Sullivan and K. Beckingham, unpublished observations). The dynamics of CaM binding to the RyR are also not yet fully understood, although there is evidence that the conversion of CaM from an activator to an inhibitor upon calcium binding involves a shift in the position of CaM on RyR (Rodney et al. 2001). Thus, more molecular work is required to explain the RyR16, Cam7 and RyR16-Cam7 interactions. We have evidence, however, that V91G CaM shows altered binding to one of the CaM-binding regions of RyR (B. Wang and K. Beckingham, unpublished observations).

In addition to the two channels discussed above, misregulation of the calcium-ATPase (SERCA) pump that sequesters calcium into the sarcoplasmic reticulum could contribute to the Cam7 phenotype. In mammals, the cardiac muscle SERCA pump is activated by phosphorylation by CaM kinase II (Toyofuku et al. 1994). Failure of this activation would lead to accumulation of calcium in the muscles. However, in Drosophila, the CaM kinase II phosphorylation site is not conserved (Shi et al. 1998). V91G CaM has an altered interaction with the wild-type RyR channels and (ii) when most of the channels contain at least one RyR16 type subunit, the effects of that altered interaction are alleviated. It will be of interest to determine the extent to which calcium transients are restored in the RyR16 Cam7 larvae. The protein encoded by the RyR16 allele has not been characterized, although preliminary Western blot experiments indicate that it is similar in size to the wild-type protein (R. Liou, K. M. C. Sullivan and K. Beckingham, unpublished observations). The dynamics of CaM binding to the RyR are also not yet fully understood, although there is evidence that the conversion of CaM from an activator to an inhibitor upon calcium binding involves a shift in the position of CaM on RyR (Rodney et al. 2001). Thus, more molecular work is required to explain the RyR16, Cam7 and RyR16-Cam7 interactions. We have evidence, however, that V91G CaM shows altered binding to one of the CaM-binding regions of RyR (B. Wang and K. Beckingham, unpublished observations).

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The Cam null phenotype: The spontaneous backward movement seen in Cam nulls clearly reflects target interaction defects that differ from those produced by Cam7. The affected target(s) is neural, as opposed to muscular, and the requirements for rescue, in terms of functional calcium-binding sites on CaM, are different from those for rescue of Cam7. As for the Cam7 mutation, however, the overall sluggishness of Cam nulls is not cleanly rescued by CaM expression in either neural or muscle tissue, reinforcing the idea that this defect reflects generalized loss of CaM.
The spontaneous backward movements of *Cam* null larvae seem compellingly similar to the enhanced backward swimming produced by C-terminal lobe CaM mutants in *Paramecium* (see Introduction). However, our experiments with versions of CaM defective in either N-terminal or C-terminal calcium binding indicate that the underlying target(s) in the two species show different regulation by CaM. Given that only C-terminal mutations produce exaggerated avoidance responses in *Paramecium*, a CaM with a functional C terminus might be expected to restore some functional interaction with a comparable target in *Drosophila*. But as shown in Figure 7, B12Q CaM is as ineffective as B34Q CaM in reducing backward movements.

Nevertheless, our findings on the influence of light on the *Cam* null phenotype suggest that, as in *Paramecium*, the defect results from hyperexcitability of the organism. Comparisons to the work of Busto et al. (1999) suggested that both the excessive head swinging and the backward movement seen in *Cam* nulls reflect components of a light-avoidance response. Although head swinging in response to light is seen in control larvae, we could not induce backward movement in wild-type animals subjected to high light levels. In contrast, reduction of CaM protein levels in hypomorphic mutants produced light-induced backward movements, and in *Cam* null animals, this response seems to be constitutively activated. It is possible that the nervous system is extremely hypersensitive in *Cam* nulls and that even mild sensory input continuously triggers avoidance responses.

In *Paramecium*, *Cam* mutant-induced hyperexcitability results from failed regulation of a Ca$^{2+}$-activated repolarizing K$^+$ channel in the plasma membrane. Although differences in CaM target regulation are indicated by our experiments, defective control of key ion channels that regulate neuronal excitability seems likely to underlie the *Cam* null defect in *Drosophila*. Further experimentation will address this hypothesis.

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