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Calmodulin binding to *Drosophila* NinaC required for termination of phototransduction

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The *ninaC* locus encodes two unconventional myosins, p132 and p174, consisting of fused protein kinase and myosin head domains expressed in *Drosophila* photoreceptor cells. NinaC are the major calmodulin-binding proteins in the retina and the NinaC–calmodulin interaction is required for the normal subcellular localization of calmodulin as well as for normal phototransduction. In the current report, we present evidence for two calmodulin-binding sites in NinaC, C1 and C2, which have different *in vitro* binding properties. C1 was found to be common to both p132 and p174 while C2 was unique to p174. To address the requirements for calmodulin binding at each site *in vivo*, we generated transgenic flies expressing *ninaC* genes deleted for either C1 or C2. We found that the spatial localization of calmodulin depended on binding to both C1 and C2. Furthermore, mutation of either site resulted in a defective photoresponse. A prolonged depolarization afterpotential (PDA) was elicited at lower light intensities than necessary to produce a PDA in wild-type flies. These results suggest that calmodulin binding to both C1 and C2 is required *in vivo* for termination of phototransduction.

Keywords: calmodulin/*Drosophila*/kinase/myosin/phototransduction

Introduction

The *Drosophila* NinaC proteins represent the most divergent members of the rapidly growing myosin superfamily (Goodson and Spudich, 1993; reviewed in Mooseker, 1993). The *ninaC* gene encodes two photoreceptor-cell specific proteins, p132 and p174, which consist of a myosin head domain linked at the N-terminus to a protein kinase catalytic domain (Montell and Rubin, 1988). Despite <30% identity between NinaC and known myosins, NinaC has retained at least some myosin-like properties including association with an actin-based cytoskeletal structure in the microvillar rhabdomeres and interaction with calmodulin (Porter *et al.*, 1992, 1993).

The two NinaC proteins have different spatial localizations and roles in the photoreceptor cells. p174 appears to function in phototransduction, maintenance of the rhabdomeral microvillar structure during illumination and for the localization of calmodulin in the rhabdomeres (Porter *et al.*, 1992, 1993). p132, which is restricted to the sub-rhabdomeral cell bodies, is required for calmodulin distribution in the cell bodies but not for phototransduction or rhabdomere maintenance (Porter *et al.*, 1992, 1993). Deletion analysis of p174 has revealed that the protein kinase, myosin head and C-terminal tail regions represent distinct functional domains: the protein kinase domain is required for phototransduction; the myosin head region also functions in phototransduction but is required in addition for rhabdomere maintenance and proper subcellular localization of p174; the C-terminal tail of p174 contains a rhabdomere localization signal; and the myosin head–tail junction is required for calmodulin binding (Porter *et al.*, 1992, 1993; Porter and Montell, 1993).

Many unconventional myosins, which are structurally distinct from conventional muscle-type myosins or myosin IIs, have been shown to bind multiple calmodulin molecules (reviewed in Cheney and Mooseker, 1992; Titus, 1993); however, the functional significance of the unconventional myosin–calmodulin interactions *in vivo* has not been well described. Yeast Myo2, a member of the myosin V class, appears to be required for polarized growth in yeast and has been shown to co-localize with calmodulin at the sites of cell growth (Johnston *et al.*, 1991; Brockerhoff *et al.*, 1994). These results raise the possibility that the calmodulin bound to the Myo2 unconventional myosin might regulate polarized cell growth, although this hypothesis remains to be tested. Calmodulin is also associated with some myosin Is, a class of unconventional myosins with a single heavy chain; however, the functions of these interactions *in vivo* have not been described (reviewed in Cheney and Mooseker, 1992; Hammer, 1994).

The importance of the calmodulin interaction for *in vivo* function of the NinaC unconventional myosin has recently been demonstrated. NinaC are the major calmodulin-binding proteins in *Drosophila* photoreceptor cells and proper localization of calmodulin in photoreceptor cells depends on both p132 and p174 (Porter *et al.*, 1993). The NinaC–calmodulin association is critical for the photoresponse since deletion of the calmodulin-binding domain results in defective phototransduction. However, deletion of the NinaC calmodulin-binding domain does not result in the light-dependent retinal degeneration typical of null *ninaC* alleles (Matsumoto *et al.*, 1987; Montell and Rubin, 1988; Porter *et al.*, 1993).

In this paper we demonstrated that the NinaC calmodulin-binding domain contained two Ca^{2+}-dependent binding sites. One was common to both NinaC proteins and the other was specific to p174. Both calmodulin-binding sites were required for normal calmodulin distribution *in vivo* and for phototransduction. Most importantly, we found that the electrophysiological phenotype resulting from mutation of the calmodulin-binding sites indicated that

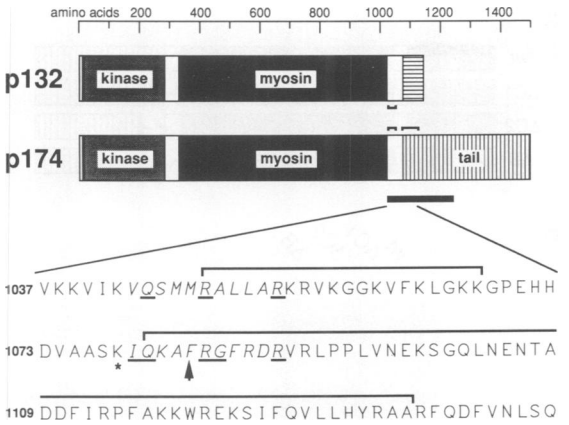

Fig. 1. The NinaC calmodulin-binding domain. The position of the previously described NinaC calmodulin-binding domain, amino acids 1037–1253, is depicted by the bold line beneath p174 (Porter *et al.*, 1993). The amino acid sequence of a portion of this domain, 1037–1144, is displayed using standard single letter designations. Residue 1082, which begins the unique region of p174, is indicated by the vertical arrow. The core region of the two IQ motifs in p174, amino acids 1043–1053 and 1079–1089, are indicated in italics and the residues which match the IQ core consensus sequence, IQxxxRGxxxR, are underlined. The asterisk under lysine 1078 shows the position of the nonsense mutation in the *ninaC*^{P225} allele. The sequences deleted in P[*ninaC*$^{\Delta C1}$] and P[*ninaC*$^{\Delta C2}$] transgenic flies, residues 1048–1067 and 1080–1134 respectively, are designated by the brackets above and below the p174 and p132 block diagrams and by the brackets above the amino acid sequence.

the p174–calmodulin interaction had a role in the termination of the photoresponse.

Results

Mapping the NinaC calmodulin–agarose binding site

Both NinaC proteins bind to calmodulin in solution, using calmodulin–agarose; however, only p174 binds calmodulin immobilized on a nitrocellulose filter (overlay assay) (Porter *et al.*, 1993). One interpretation of these results is that p132 and p174 have distinct calmodulin-binding sites with different *in vitro* binding properties. Alternatively, there may be one or more sites common to both NinaC proteins, mediating the calmodulin–agarose binding, and at least one additional site unique to p174 required for binding in an overlay assay.

To address whether the site required for binding to calmodulin–agarose was located in a segment common to p132 and p174, we tested whether any of the existing *ninaC* alleles expressed a truncated version of the NinaC proteins containing only the common portion of NinaC. We found that *ninaC*^{P225}, previously shown to express a single NinaC isoform of ~120 kDa (Matsumoto *et al.*, 1987; Montell and Rubin, 1988), contained a nonsense mutation at amino acid 1078 (see Materials and methods; calculated molecular weight of 125 kDa). This mutation eliminated the C-terminal 54 and 440 amino acids specific to p132 and p174 respectively, as well as the last four residues shared by both NinaC proteins (residues 1078–1081; Figures 1 and 2A).

To determine whether the 125 kDa isoform expressed in *ninaC*^{P225} was capable of binding to calmodulin–agarose, we incubated head extracts with the affinity

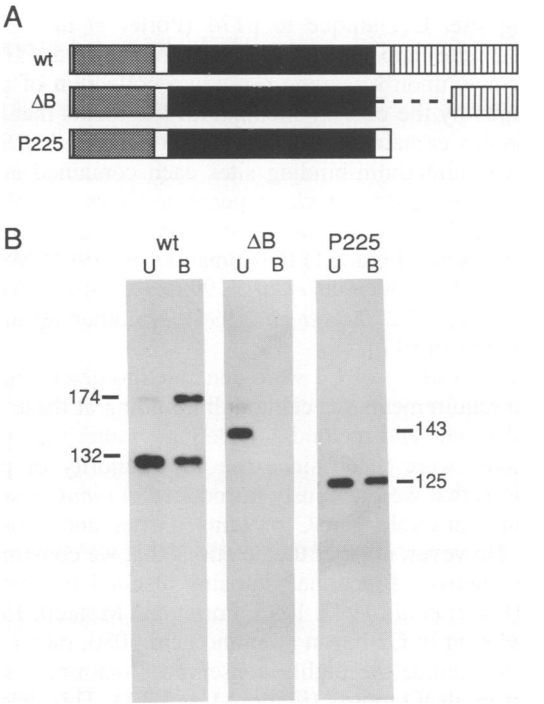

Fig. 2. Binding of the truncated NinaC protein expressed in *ninaC*^{P225} to calmodulin–agarose. (**A**) Domain structure of p174 expressed in wild-type (wt) flies and the truncated versions of NinaC expressed in P[*ninaC*$^{\Delta B}$] (ΔB) and *ninaC*^{P225} (P225) flies. The protein kinase, myosin head and tail domains are represented by the stippled, black and vertically lined boxes respectively. The broken horizontal line connecting the myosin head and tail domains in ΔB indicates the region deleted in this protein. (**B**) NinaC binding to calmodulin–agarose. Head extracts from wild-type (wt), P[*ninaC*$^{\Delta B}$] (ΔB) and *ninaC*^{P225} (P225) flies were incubated with calmodulin–agarose. The unbound (U) and bound (B) fractions were fractionated by SDS–PAGE (6% gel), transferred to nitrocellulose and probed with NinaC specific antibodies. The sizes of the wild-type and truncated proteins are indicated in kilodaltons.

resin. The bound and unbound fractions were transferred to nitrocellulose and the NinaC proteins present in each pool were detected with anti-NinaC antibodies. As previously described, the majority of p174 and nearly half of p132 extracted from wild-type heads bound to calmodulin–agarose (Figure 2B) (Porter *et al.*, 1993) but not to agarose alone (data not shown). Furthermore, the truncated version of p174 expressed in the transgenic flies, P[*ninaC*$^{\Delta B}$] (143 kDa), in which residues 1037–1253 were deleted (Figure 2A; Porter *et al.*, 1993), remained in the unbound fraction (a deleted form of p132 was not detected since the mutation in P[*ninaC*$^{\Delta B}$] prevented synthesis of the mRNA encoding p132; Figure 2B). Of primary significance in this experiment, we found that the proportion of the 125 kDa isoform expressed in *ninaC*^{P225} that bound to calmodulin–agarose was similar to p132 expressed in wild-type (Figure 2B). Thus, the region common to p132 and p174, residues 1037–1077, was required for binding to calmodulin–agarose. This site, which was located in the myosin head–tail junction, will be referred to as C1.

Generation of transgenic lines containing mutated NinaC calmodulin-binding sites

The observation that p174, but not p132, binds calmodulin in an overlay assay, indicates that there is a calmodulin-

binding site, C2, unique to p174 (Porter *et al.*, 1993). The C2 site appears to map to amino acids 1087–1128 since, upon screening a random collection of p174 fragments by the overlay method, all fragments that bind calmodulin contain these residues (Porter *et al.*, 1993). The two calmodulin-binding sites each contained an IQ motif, a sequence which appears to have a role in calmodulin binding in other proteins including unconventional myosins (Figure 1) (Chapman *et al.*, 1991; Mercer *et al.*, 1991; Espreafico *et al.*, 1992; Swanljung-Collins and Collins, 1992; Xie *et al.*, 1994). No other IQ motifs were found in p174.

Deletions of C1 or C2 were generated to determine the *in vivo* requirements for calmodulin binding at these sites (see Materials and methods). Deletions, rather than point mutations, were made since the vast majority of point mutations that we previously introduced in *ninaC* resulted in highly unstable NinaC proteins (Porter and Montell, 1993). However, none of the deletions that we constructed which removed functional domains resulted in unstable p174 (Porter *et al.*, 1992, 1993; Porter and Montell, 1993). The deletion in C2 began at amino acid 1080, rather than 1087, to include the highly conserved glutamine residue present in all IQ motifs (Figures 1 and 3A). This deletion would be predicted to prevent synthesis of p132; however, this would not complicate the phenotypic analyses since p132 is not required for the light response or to prevent retinal degeneration. The deletion in C1 ended at amino acid 1067, instead of 1077, to reduce the possibility that this deletion would also affect the C2 site (Figures 1 and 3A). The transgenic flies, P[$ninaC^{\Delta C1}$] and P[$ninaC^{\Delta C2}$], expressed the altered NinaC proteins, p132ΔC1, p174ΔC1 and p174ΔC2, at wild-type levels (Figure 3B).

C1 and C2 mutations alter calmodulin binding

To determine the effects of deleting C1 and C2 on calmodulin binding, we tested p174ΔC1 and p174ΔC2 in overlay and calmodulin–agarose assays. Retinal extracts were fractionated by SDS–PAGE, transferred to nitrocellulose and probed with [^{125}I]calmodulin. The results showed that p174ΔC1 bound calmodulin in a gel overlay; however, binding to p174ΔC2 was eliminated (Figure 4A). The opposite result was obtained in the calmodulin–agarose assay. We found that the majority of p132ΔC1 and p174ΔC1 failed to bind calmodulin–agarose while most of p174ΔC2 bound the affinity resin (Figure 4B). p174ΔB does not bind calmodulin–agarose or calmodulin in the overlay assay since it is missing both C1 and C2 (Figure 4A and B) (Porter *et al.*, 1993). Thus, *in vitro* binding of calmodulin to C1 occurs only in solution and to C2 primarily in a gel overlay.

Stoichiometry of calmodulin binding at the C1 and C2 sites

We estimated the stoichiometry of calmodulin binding to NinaC by co-immunoprecipitating NinaC with calmodulin. Wild-type head extracts were incubated with p174-specific antibodies and subsequently with protein-A beads. p174 and any associated proteins were then pelleted by centrifugation and fractionated by SDS–PAGE. Proteins in the high molecular weight range, which included p174, were detected by staining with Coomassie Blue (Figure 5A) and the low molecular weight proteins were transferred

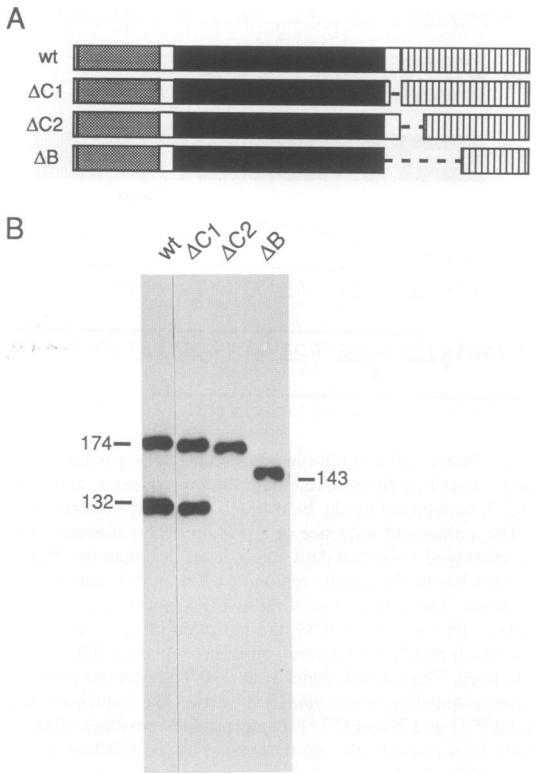

Fig. 3. NinaC protein expression levels in calmodulin-binding domain deletion mutants. (**A**) Domain structure of NinaC p174 expressed in wild-type (wt), P[$ninaC^{\Delta C1}$] (ΔC1), P[$ninaC^{\Delta C2}$] (ΔC2), and P[$ninaC^{\Delta B}$] (ΔB) flies. (**B**) Protein blot showing expression levels of the wild-type and truncated NinaC proteins. Head extracts from wild-type (wt), P[$ninaC^{\Delta C1}$] (ΔC1), P[$ninaC^{\Delta C2}$] (ΔC2), and P[$ninaC^{\Delta B}$] (ΔB) flies were fractionated by SDS–PAGE (6% gel), transferred to nitrocellulose and probed with the NinaC-specific antibodies αZB551 (Montell and Rubin, 1988). The sizes of the NinaC proteins expressed in wild-type and P[$ninaC^{\Delta B}$] are indicated in kilodaltons. The mutations which created the deletions in the p174 isoforms expressed in P[$ninaC^{\Delta C2}$] and P[$ninaC^{\Delta B}$] transgenic flies prevented synthesis of p132.

to a PVDF membrane and probed with a calmodulin antibody (Figure 5B). Quantification of p174 and calmodulin in the immunoprecipitates indicated a p174 to calmodulin ratio of 1:1.1 (see Materials and methods). p174ΔC1 and p174ΔC2 also co-immunoprecipitated with calmodulin, but at ratios of 1:0.75 and 1:0.63 respectively (Figure 5). p174ΔB, the p174 derivative missing 217 amino acids encompassing both C1 and C2, did not appear to have any associated calmodulin (Figure 5B).

Ca^{2+} dependence of NinaC–calmodulin binding in solution

The effect of varying the Ca^{2+} concentration on calmodulin binding to NinaC during the immunoprecipations was investigated. We found that NinaC and calmodulin co-precipitated in the range ~0.5–100 μM Ca^{2+} but did not appear to interact strongly in the absence of Ca^{2+} (Figure 6A and B).

The requirement for Ca^{2+} was also examined using the calmodulin–agarose solution binding assay. Proteins from a wild-type head extract were incubated with calmodulin–agarose in 10 μM Ca^{2+}, 160 mM KCl. Those proteins which bound to the affinity resin and subsequently eluted with 5 mM EGTA, 160 mM KCl and then with

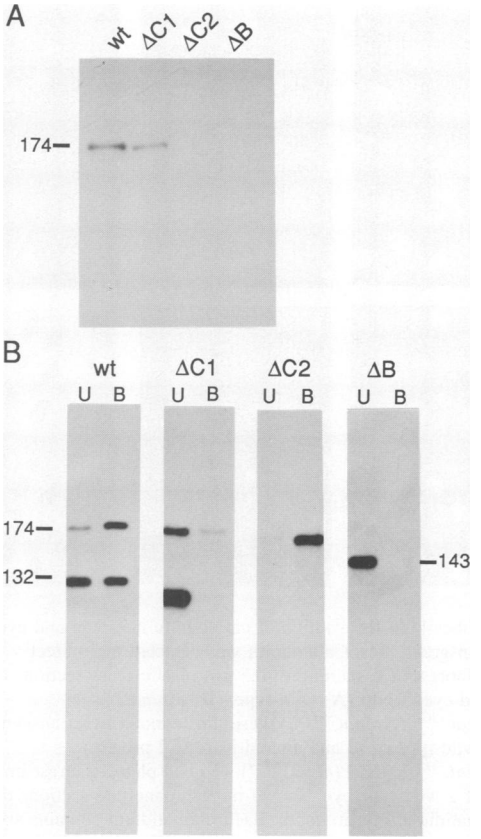

Fig. 4. Calmodulin binding to NinaC derivatives expressed in deletion mutants. (**A**) Calmodulin overlay assay. Retinal extracts from wild-type (wt), P[*ninaC*$^{\Delta C1}$] (ΔC1), P[*ninaC*$^{\Delta C2}$] (ΔC2), and P[*ninaC*$^{\Delta B}$] (ΔB) flies were fractionated by SDS–PAGE (6% gel), transferred to nitrocellulose and probed with [^{125}I]calmodulin in the presence of 0.1 mM $CaCl_2$. (**B**) Binding to calmodulin–agarose. Head extracts from the indicated fly stocks were incubated with calmodulin–agarose in the presence of EGTA/$CaCl_2$ (5 mM each; ~10 μM free Ca^{2+}). The beads and the supernatants were then combined with 2× SDS sample buffer. The unbound (U) and bound (B) fractions were fractionated by SDS–PAGE (6% gel), transferred to nitrocellulose and probed with the NinaC specific antibodies αZB551.

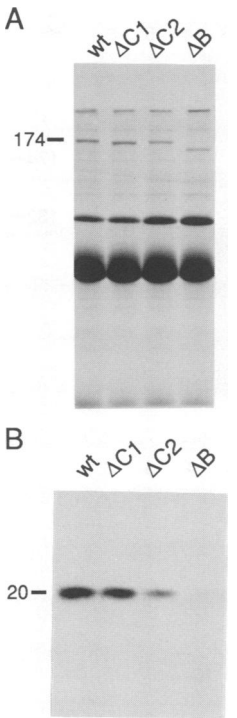

Fig. 5. Co-immunoprecipitation of NinaC and calmodulin from wild-type and mutant head extracts. Immunoprecipitations were performed as described in Materials and methods in a buffer containing 5 mM EGTA and 5 mM $CaCl_2$ (~10 mM Ca^{2+}). (**A**) Coomassie stained gel of the immunoprecipitated p174 and derivatives from wild-type (wt), P[*ninaC*$^{\Delta C1}$] (ΔC1), P[*ninaC*$^{\Delta C2}$] (ΔC2), and P[*ninaC*$^{\Delta B}$] (ΔB) flies. Head extracts were prepared under non-denaturing conditions and incubated with p174-specific rabbit antisera (Porter *et al.*, 1992). The p174 derivatives and associated proteins were then pelleted with protein-A beads and fractionated by SDS–PAGE (12% gel). (**B**) Calmodulin present in NinaC immunoprecipitation complexes. Proteins in the NinaC immunoprecipitation complex (described in A) with apparent molecular weights <40 kDa were transferred to a PVDF membrane and probed with a calmodulin-specific antibody. Panels (A) and (B) were obtained from the top and bottom portions respectively of the same SDS–polyacrylamide gel.

5 mM EGTA, 500 mM KCl were examined on a Coomassie-stained gel (Figure 6C). The results indicated that both NinaC proteins required EGTA and high salt to elute efficiently from calmodulin–agarose. NinaC could bind calmodulin–agarose in the absence of Ca^{2+} in 160 mM KCl (data not shown) but eluted during the high salt washes (500 mM KCl) used to eliminate non-specific binding. Thus, solution binding to calmodulin–agarose via the C1 site required Ca^{2+} in 500 mM KCl; although, some Ca^{2+}-independent binding was detected in 160 mM KCl.

Calmodulin distribution requires both NinaC calmodulin-binding sites

Deletion of both NinaC calmodulin-binding sites disrupts the normal distribution of calmodulin in the photoreceptor cells (Porter *et al.*, 1993). To determine whether deletion of just C1 or C2 affected the subcellular localization of calmodulin, we stained cross-sections of adult compound eyes with calmodulin antibodies and visualized the results by indirect immunofluorescence. Wild-type photoreceptors showed strong calmodulin staining in the rhabdomeres and diffuse staining in the cell bodies as previously described (Porter *et al.*, 1993) (Figure 7A; see Figure 7F for phase contrast image of two ommatidia). In P[*ninaC*$^{\Delta C1}$] transgenic flies, calmodulin staining was detected almost exclusively in the rhabdomeres (Figure 7B) and the intensity of this rhabdomeric staining was less than in wild-type. In P[*ninaC*$^{\Delta C2}$] (expressing wild-type p132), the relative intensity of staining in the rhabdomeres was reduced compared with wild-type (Figure 7C). For comparison, the calmodulin distribution in previously analyzed transgenic flies lacking either the cell body specific p132 (P[*ninaC*$^{\Delta 132}$]) or rhabdomere-specific p174 (P[*ninaC*$^{\Delta 174}$]) are shown (Figure 7D and E). Calmodulin is primarily restricted to the rhabdomeres in P[*ninaC*$^{\Delta 132}$] (Figure 7D) and to the sub-rhabdomeral cell bodies in P[*ninaC*$^{\Delta 174}$] (Figure 7E). The alterations in calmodulin localization in P[*ninaC*$^{\Delta C1}$] and P[*ninaC*$^{\Delta C2}$] were not due to changes in p174 localization or retinal degeneration since the p174ΔC1 and p174ΔC2 proteins were rhabdomere-specific and neither transgenic line showed any retinal degeneration (data not shown).

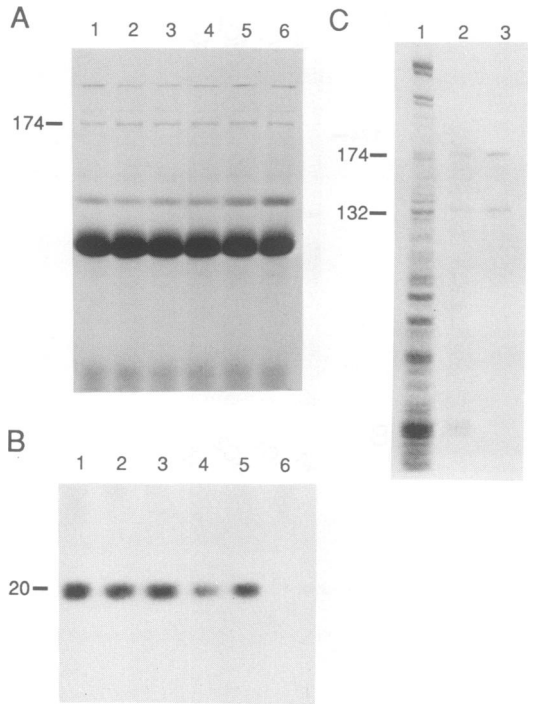

Fig. 6. Ca^{2+} dependence of p174–calmodulin binding. (**A**) Wild-type head extracts were prepared in different Ca^{2+}/EGTA buffers and the p174 protein complexes were immunoprecipitated with a p174 specific antiserum. The immunoprecipitated proteins were fractionated by SDS–PAGE (12% gel) and detected by staining with Coomassie Blue. The approximate free Ca^{2+} levels used to prepare the extracts and during the immunoprecipitations were as follows: lane 1, 100 μM; lane 2, 10 μM; lane 3, 5 μM; lane 4, 1 μM; lane 5, 0.5 μM and lane 6, no free Ca^{2+}. The position of p174 is indicated. (**B**) Immunoprecipitation of calmodulin with p174 at different Ca^{2+} concentrations. Proteins with mobilities <40 kDa were transferred from the gel described above (A) to PVDF membrane and probed with a calmodulin-specific antibody. Panels (A) and (B) were derived from the top and bottom portions of the same SDS–polyacrylamide gel. (**C**) Binding of NinaC to calmodulin–agarose in the presence and absence of Ca^{2+}. Wild-type fly head extracts were incubated with calmodulin–agarose in 10 μM $CaCl_2$ and washed in a high salt buffer. Those proteins which did not bind and which eluted under the following conditions were fractionated by SDS–PAGE (8% gel) and detected by staining with Coomassie Blue. Lane 1, proteins which did not bind to the affinity resin; lane 2, proteins which bound and eluted with 2 mM EGTA; lane 3, proteins eluted with 2 mM EGTA/500 mM KCl subsequent to the elution with 2 mM EGTA (see lane 2). p132 and p174 are indicated.

Strong electroretinogram phenotype results from deletion of C2 but not C1

The phenotypic consequences of deleting either the C1 or C2 site on phototransduction was assessed by performing electroretinogram (ERG) recordings, a method which measures the summed responses of all retinal cells to light. A wild-type ERG is characterized by a corneal negative response to light followed by a rapid return to baseline upon cessation of the light stimulation (Figure 8A). The response of wild-type flies to a second pulse of light is similar to the first. P[$ninaC^{\Delta B}$] flies display an ERG indistinguishable from the null allele, $ninaC^{P235}$ (Figure 8A). The features of this ERG include a large corneal negative response, after a 90 s dark adaptation, followed by a slow return to baseline and a small or absent off-transient upon cessation of the initial light stimulus. The ERG obtained with P[$ninaC^{\Delta C1}$] flies was

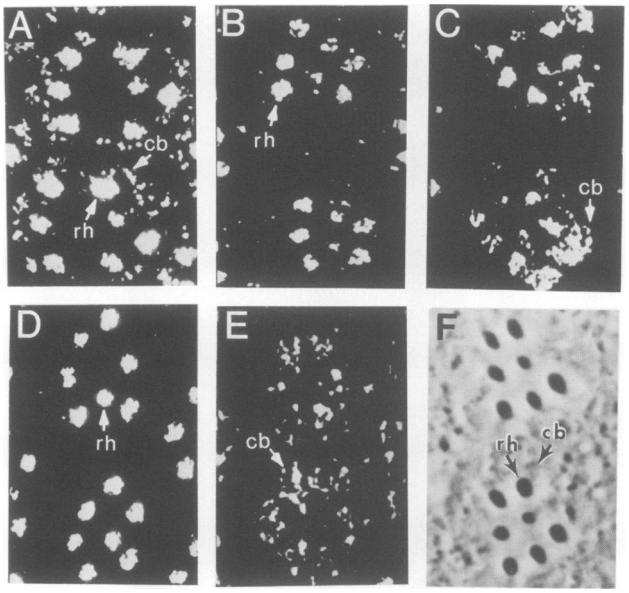

Fig. 7. Subcellular localization of calmodulin in compound eyes of *ninaC* transgenic flies. Calmodulin was detected by indirect immunofluorescence staining of 0.5 μm plastic cross-sections of compound eyes from: (**A**) wild-type; (**B**) P[$ninaC^{\Delta C1}$]; (**C**) P[$ninaC^{\Delta C2}$]/P[$ninaC^{\Delta 174}$] (these flies contain two transgenes and express wild-type p132 and altered p174ΔC2 protein); (**D**) P[$ninaC^{\Delta 132}$]; (**E**) P[$ninaC^{\Delta 174}$]. (**F**) is a phase contrast image of a section of a wild-type eye. Shown in each panel are sections through two ommatidia at 30 μm depth. Each ommatidium contains six outer photoreceptor cells, R1-6, and one central R7 cell at this depth. Several rhabdomeres and cell bodies are indicated. In (F) the rhabdomeres are seen as seven dark ovals in each ommatidium.

similar to wild-type except for a small effect on the rate of return to baseline after termination of the initial light stimulus (Figure 8A). P[$ninaC^{\Delta C2}$] flies exhibited an ERG phenotype similar to the null allele, $ninaC^{P235}$ (Figure 8A); however, the off-transients were often larger than typical of $ninaC^{P235}$.

Deletion of C1 or C2 causes a prolonged depolarization afterpotential phenotype

Using a different electrophysiological paradigm, a defect was seen in both P[$ninaC^{\Delta C1}$] and P[$ninaC^{\Delta C2}$]. Wild-type flies show a prolonged depolarization afterpotential (PDA) upon exposure to intense blue light (Minke *et al.*, 1975; Hamdorf and Razmjoo, 1979; Hillman *et al.*, 1983). However, moderate or low intensity blue light does not elicit a PDA (Figure 8B). The PDA can be terminated by exposing flies to orange or white light (Figure 8B). We found that P[$ninaC^{\Delta B}$], P[$ninaC^{\Delta C1}$] and P[$ninaC^{\Delta C2}$] all showed PDAs under reduced blue light intensities that did not induce PDAs in wild-type or in the *ninaC* null mutant, $ninaC^{P235}$ (Figure 8B). This low light PDA phenotype in the transgenic flies did not appear to be due to increased rhodopsin levels since the amplitude of the M-potential, a predominantly second order neuronal response that reflects the levels of light-activated rhodopsin in the R1-6 photoreceptors (Minke and Selinger, 1992b), was smaller rather than larger than in wild-type (Figure 8C). Furthermore, a protein blot containing head extracts from these flies was probed with rhodopsin antibodies demonstrating a decrease in rhodopsin concentration of ~40% in P[$ninaC^{\Delta B}$] and P[$ninaC^{\Delta C2}$] and 25% in P[$ninaC^{\Delta C1}$] (data not

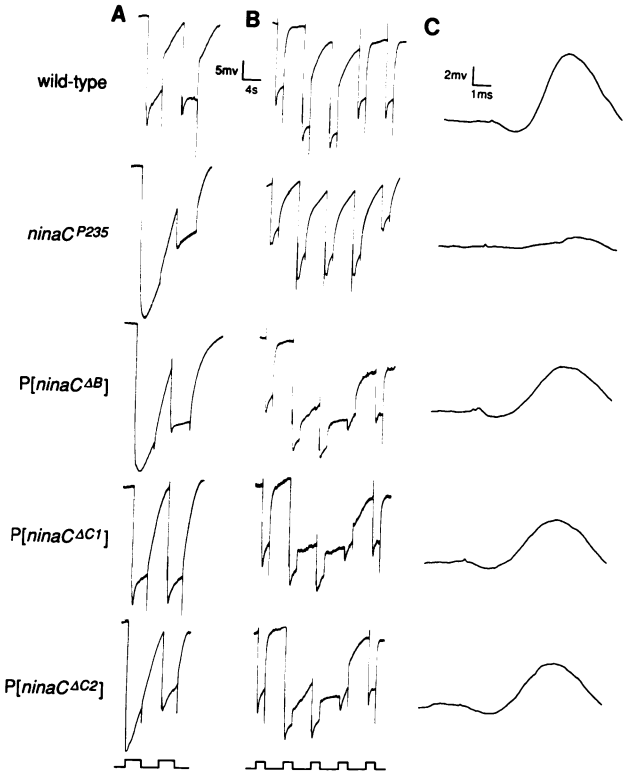

Fig. 8. Electrophysiological phenotypes of calmodulin-binding domain deletion mutants. (**A**) Two 4 s pulses of bright white light interrupted by a 5 s dark adaption. The flies were dark adapted for 90 s before exposure to the first light pulse. (**B**) Flies were crossed into a white-eyed background to remove the screening pigment and exposed to five moderate intensity light pulses of 2 s each. The first, fourth and fifth pulses were orange light and the second and third pulses were blue light. The intensity of the light was 0.09 mW. The event markers below the panels indicate the initiation and cessation of the light stimuli. A time and mV scale bar is shown between panels (A) and (B). The on-transients were frequently not detected even in the wild-type ERGs due to limitations in the sampling rate (400/s) of the analog-digital converter. (**C**) M-potentials (metarhodopsin-potentials) recorded from flies in a white-eyed background. Flies were exposed to bright blue light and then to a bright orange flash, from a photographic strobe, to photoconvert the metarhodopsin to rhodopsin. The orange flash occurred 2 ms after the traces commenced. The positive response centered ~5.5 ms after initiation of the traces is the M-potential.

shown). The lack of PDA in $ninaC^{P235}$ was presumably due to the decreased rhodopsin concentration resulting from retinal degeneration (Porter *et al.*, 1992) since the amplitude of the M-potential was very small (Figure 8C). In newly eclosed dark-reared $ninaC^{P235}$, which do not show retinal degeneration but some reduction in rhodopsin levels, a PDA was observed, but less pronounced than in P[$ninaC^{\Delta B}$], P[$ninaC^{\Delta C1}$] or P[$ninaC^{\Delta C2}$] (data not shown).

Discussion

Several unconventional myosins have been shown to bind multiple calmodulin molecules (reviewed in Cheney and Mooseker, 1992; Titus, 1993); however, with the exception of NinaC, the *in vivo* consequences of disrupting this interaction have not been described for any unconventional myosin. We have previously shown that the NinaC proteins are the major calmodulin-binding proteins in *Drosophila* photoreceptor cells (Porter *et al.*, 1993). Furthermore, the NinaC–calmodulin interaction is required for the normal spatial distribution of calmodulin and for the photoresponse. Since many other unconventional myosins bind multiple calmodulin molecules, in the current work, we addressed whether the NinaC calmodulin-binding domain also bound more than one calmodulin light chain and analyzed the phenotypic consequences of disrupting the individual binding sites.

IQ motifs are in vitro calmodulin binding sequences in p174

We found that p174 contained two calmodulin-binding sites, C1 and C2 near the myosin head–tail junction. C1 was in a region common to p174 and p132 and C2 was just C-terminal to C1 in the unique region of the p174 C-terminal tail. Both the C1 and C2 sites mapped to an IQ motif, a sequence of ~25 amino acids containing the core consensus IQxxxRGxxxR, proposed to be involved in calmodulin–light chain binding in many myosins (Mercer *et al.*, 1991; Espreafico *et al.*, 1992; Swanljung-Collins and Collins, 1992; Xie *et al.*, 1994). All myosins have one or more sequences similar to the IQ motif in a position similar to the NinaC sequences (reviewed in Cheney and Mooseker, 1992). Additional evidence that the IQ motifs were calmodulin-binding sites in p174 were that no similar motifs were present in p174, other than those mapping to C1 and C2, and no calmodulin-binding sites were identified that did not contain an IQ motif.

p174 calmodulin-binding sites display Ca^{2+} requirements distinct from other unconventional myosins

A key difference between the NinaC calmodulin-binding sites and those in most other unconventional myosins is the Ca^{2+} sensitivity of the calmodulin binding. Although most calmodulin-binding proteins require Ca^{2+} for light chain/calmodulin binding, the unconventional myosin–calmodulin interaction is typically Ca^{2+} independent. Examples include the chicken brush border myosin I (BBMI), brain myosin V and yeast Myo2p (Swanljung-Collins and Collins, 1991; Espindola *et al.*, 1992; Wolenski *et al.*, 1993; Brockerhoff *et al.*, 1994). An exception to this rule is rat myr 4 which contains both Ca^{2+}-dependent and Ca^{2+}-independent calmodulin-binding sites (Bähler *et al.*, 1994). However, binding of calmodulin to p174 was distinct from all other unconventional myosins in its requirement for Ca^{2+} for optimal binding at each calmodulin-binding site. This suggests that the functions regulated by the NinaC–calmodulin interaction (see below) are Ca^{2+} dependent.

Both in vitro calmodulin-binding sites appear to be sites in vivo

Examination of the spatial localization of calmodulin in flies containing a mutated C1 or C2 site provided evidence that NinaC contained two calmodulin-binding sites *in vivo*. Calmodulin is normally concentrated in the rhabdomeres and present at lower concentrations in the sub-rhabdomeral cytoplasm. We have previously shown that the spatial localization of calmodulin is dependent on NinaC (Porter *et al.*, 1993). P[$ninaC^{\Delta 132}$] flies, which lack the sub-rhabdomeral p132 isoform, contain no detectable calmodulin in the sub-rhabdomeral cell bodies and P[$ninaC^{\Delta 174}$] flies, which are missing the rhabdomeral-

specific p174 isoform, do not concentrate calmodulin in the rhabdomeres. In the current study, we found that in P[*ninaC*$^{\Delta C1}$], nearly all of the calmodulin staining was restricted to the rhabdomeres, a localization pattern similar to that observed in the transgenic flies, P[*ninaC*$^{\Delta 132}$]. These results not only indicated that C1 was a calmodulin-binding site *in vivo*, but provided evidence that C1 was the only binding site in p132 *in vivo*. The slight decrease in intensity in the rhabdomeral staining in P[*ninaC*$^{\Delta C1}$], relative to P[*ninaC*$^{\Delta 132}$], could have been due to the absence of the C1 site in p174.

Examination of the spatial localization of calmodulin in P[*ninaC*$^{\Delta C2}$] photoreceptor cells indicated that C2 was also a binding site. In contrast to wild-type photoreceptors, in which the calmodulin is concentrated in the rhabdomeres, the calmodulin appeared to be distributed more uniformly in the rhabdomeres and sub-rhabdomeral cytoplasm in P[*ninaC*$^{\Delta C2}$]/P[*ninaC*$^{\Delta 174}$]. This spatial distribution was different from that seen in P[*ninaC*$^{\Delta 174}$] flies in which the staining in the rhabdomeres was difficult to detect. The higher level of calmodulin in P[*ninaC*$^{\Delta C2}$] rhabdomeres relative to P[*ninaC*$^{\Delta 174}$] was probably due to the intact C1 site in p174. Thus, calmodulin appeared to bind both *in vitro* p174 binding sites *in vivo* as well.

Calmodulin binding to both p174 sites required for termination of the photoresponse

We found that deletion of either C1 or C2 resulted in an electrophysiological phenotype indicating that binding to both sites was required *in vivo*. This contrasts with the recent finding that a mutation in a *Dictyostelium* myosin II that eliminates binding to the regulatory light chain, an EF hand Ca^{2+}-binding protein molecule similar to calmodulin, had minimal effect *in vivo* (Uyeda and Spudich, 1993).

The type of electrophysiological phenotype resulting from mutation of C1 or C2 suggested that calmodulin binding to p174 was required for inactivation of the phototransduction cascade. Phototransduction is initiated by photoconversion of rhodospsin (R) into a thermally stable metarhodopsin (M). In the major class of photoreceptor cells, R1-6 cells, the absorption maxima for the R- and M-states are 480 and 580 nm, respectively (Hamdorf, 1979; Hardie, 1983). In *Drosophila*, the R-state is not spontaneously regenerated; instead, a termination mechanism is required to inactivate the M-state. Since the R- and M-forms have widely different absorption maxima, a PDA can be induced in wild-type upon exposure to a bright 480 nm light stimulus. An intense 480 nm stimulus is necessary since the PDA requires conversion of a large absolute number of R-state molecules to the M-state (Hamdorf and Razmjoo, 1979; Hillman *et al.*, 1983). Exposure of the M-state to 580 nm light terminates the PDA. Mutations that reduce the total rhodopsin amount, either as a result of a defect in rhodopsin synthesis or due to retinal degeneration, interfere with the PDA (Pak, 1991; Smith *et al.*, 1991; Minke and Selinger, 1992a). Null mutations in *ninaC* induce light-dependent retinal degeneration and as a consequence cause a decreased PDA (Matsumoto *et al.*, 1987; Porter *et al.*, 1992). However, mutation of either C1 or C2 did not cause retinal degeneration and there was no reduction in the PDA. Of primary importance in the current work, we found that a PDA can be elicited in P[*ninaC*$^{\Delta C1}$] or P[*ninaC*$^{\Delta C2}$] flies at lower light intensities than that needed to induce a PDA in wild-type. This PDA phenotype was not due to increased rhodospsin levels in the transgenic flies. This suggests that there was a defect in the termination of the M-state.

The only other mutation identified that results in a PDA, under reduced light intensity, is *arrestin2* (*arr2*) (Dolph *et al.*, 1993). In wild-type, Arrestin2 (Arr2) binds to M-form rhodopsin, which is subsequently phosphorylated at multiple serine and threonine residues by rhodopsin kinase. The phosphorylated rhodopsin is then protected from dephosphorylation, as a consequence of the association with Arr2, and is no longer able to bind to the G-protein causing termination of the photoresponse (Bentrop *et al.*, 1993; Byk *et al.*, 1993). Arr2 also undergoes serine/threonine phosphorylation, apparently in a Ca^{2+}/calmodulin-dependent fashion (Yamada *et al.*, 1990; Byk *et al.*, 1993). Although the functional consequences of the Arr2 phosphorylation remain to be determined, it is possible that it plays a role in the termination of the photoresponse. It is intriguing to speculate that p174 may be a calmodulin-dependent serine/threonine kinase that phosphorylates Arr2. Consistent with this proposal, we have found that deletion of the NinaC protein-kinase domain results in a PDA phenotype indistinguishable from deletion of either the C1 or C2 calmodulin-binding sites (J.A.Porter and C.Montell, unpublished). An alternative possibility is that p174 phosphorylates metarhodopsin since a substantial proportion of the protein kinase that phosphorylates metarhodopsin is associated with the rhabdomeres (Doza *et al.*, 1992; Bentrop *et al.*, 1993).

Possible function of NinaC–calmodulin interaction

There are several potential functions for the calmodulin binding to NinaC, none of which are mutually exclusive. As suggested above, NinaC may be a novel type of calmodulin-dependent protein kinase. Alternatively, the putative NinaC myosin activity might be regulated by calmodulin since the properties of at least some unconventional myosins, such as the magnesium-ATPase activity of BBMI, are affected by calmodulin association (Swanljung-Collins and Collins, 1991; Wolenski *et al.*, 1993). The calmodulin could also translocate from NinaC to other proteins as part of a mechanism to regulate the activities of other photoreceptor cell calmodulin-binding proteins (Porter *et al.*, 1993). The proper spatial localization of calmodulin in photoreceptor cells depends on calmodulin binding to both the NinaC C1 and C2 sites and a variety of protein kinases, channels and other calmodulin-binding proteins are present in the *Drosophila* retina (Phillips *et al.*, 1992; M.Yu, P.Wes, A.Kreuz, Y.Liu and C.Montell, unpublished). The apparent calmodulin to p174 ratio of 1.1 rather than 2 might be due to the translocation of calmodulin from some p174 sites to other proteins. If NinaC does provide calmodulin to other proteins in a regulated manner, the release of the calmodulin from NinaC does not appear to be controlled by physiological Ca^{2+} levels since NinaC binds calmodulin in Ca^{2+} concentrations in both the low and high physiological ranges (~0.6–40 μM; reviewed in Nagy, 1991).

Materials and methods

Identification of the mutation in ninaC^{P225}

Genomic DNA was prepared from wild-type and *ninaC*^{P225} flies, digested with *Bam*HI and DNA encoding the NinaC head–tail junction (corres-

ponding to p132 amino acids 1017–1135 in wild-type) was amplified by the PCR technique. The PCR products were subcloned into pBluescript KS+ and subjected to DNA sequence analyses using Sequenase (USB).

Construction of transgenic lines with calmodulin-binding site deletions

The ΔC1 and ΔC2 deletions were generated by oligonucleotide-directed mutagenesis, using single stranded pBSXX1 or pBSKK DNA, respectively, as described (Porter *et al.*, 1992). The oligonucleotides used to construct the ΔC1 and ΔC2 deletions were: CAAAGTGCAGTCCATGATGGGACCGGAGCATCATGG and GTGGCCGCGTCCAAAATACGCTTCCAGGACTTTG, respectively. The underlined nucleotides corresponded to the nucleotides flanking the deleted sequence. The mutated ΔC1 DNA fragment was subcloned into a shuttle vector, pG*ninaC*ΔXb-RI (Porter *et al.*, 1992), and then into the transformation vector pDM30 (Mismer and Rubin, 1987) and the ΔC2 mutation was subcloned directly into pDM30 all as described (Porter *et al.*, 1992). The mutated *ninaC* genes were then introduced into the germline of $ninaC^{P235}$; ry^{506} flies according to P-element mediated germline transformation (Rubin and Spradling, 1982; Spradling and Rubin, 1982). Multiple transformant lines were isolated for each construct and the lines were made homozygous for the transgene.

Calmodulin binding assays

To perform the calmodulin overlay assays, 15 retinas per line were dissected and homogenized in 2× SDS sample buffer. The proteins were then fractionated by SDS–PAGE (6% gel), transferred to nitrocellulose, blocked for 30 min at RT in blocking buffer (5% BSA in 10 mM imidazole pH 7.4, 150 mM KCl, 0.1 mM $CaCl_2$, 0.2% Tween 20), probed for 4 h at room temperature (RT) with [^{125}I]calmodulin (NEN) (~1×10^7 c.p.m./ml; 50 mCi/μg) in blocking buffer, washed three times for 15 min each in blocking buffer without BSA and the bound calmodulin was detected by exposing photographic film. All incubations were performed at RT.

Calmodulin–agarose pelleting assays were performed by equilibrating calmodulin–agarose (Sigma; 50 μl) in buffer A [10 mM imidazole pH 7.35, 10% sucrose, 5 mM $MgCl_2$, 1 mM DTT, 160 mM KCl, 5 mM EGTA, 5 mM $CaCl_2$ (~10 μM free Ca^{2+}), 10 μg/ml PMSF, 1 μg/ml leupeptin] by several washes and then incubated for 30 min with extract supernatants prepared by homogenizing fly heads (100 heads) at 4°C in a microfuge mortar and pestle (Kontes) in buffer A (400 μl) and centrifuging at 100 000 *g* for 30 min. The calmodulin–agarose beads were then pelleted at 6 000 *g* for 5 min and the supernatant was diluted 1:1 with 2× SDS sample buffer and retained for analysis by SDS–PAGE. The pelleted beads were then washed several times with buffer A plus 340 mM KCl and the bound proteins were subsequently eluted either with (i) SDS sample buffer (pellet was resuspended in a volume of buffer A equal to the supernatant volume and then an equal volume of 2× SDS sample buffer); (ii) with buffer A minus $CaCl_2$; (iii) buffer A minus $CaCl_2$ plus 340 mM KCl. Proteins eluted by treatments (ii) or (iii) were diluted 1:1 with 2× SDS sample buffer. The samples were fractionated by SDS–PAGE (6% gel) and either stained with Coomassie Blue or transferred to nitrocellulose and probed with the NINAC-specific antibodies αZB551 as described (Montell and Rubin, 1988).

Indirect immunofluorescent localization of calmodulin

Fly heads were hemisected and fixed in a 3.5% sucrose, 3% paraformaldehyde, 0.1 M phosphate buffer solution for 2–3 h at 4°C, embedded in L.R.White, sectioned, blocked, incubated with rabbit calmodulin-specific antibody (gift of C.Klee), with a fluorescent secondary antibody and washed as previously described (Porter and Montell, 1993). Fluorescent images were obtained using a MRC 600 confocal imaging system (Bio-Rad Laboratories, Richmond, CA) on a Nikon Optiphot microscope.

Electrophysiology

Electroretinogram (ERG) recordings were performed as previously described (Porter *et al.*, 1992) with the addition of a second light paradigm for measuring the PDA described below. Briefly, flies were immobilized with a mixture of equal volumes of vaseline, lanolin and paraffin and glass recording and reference electrodes filled with Ringer's solution were applied to a small drop of electrode cream (Sigma Chemical Co., St Louis, MO) placed on the eye and thorax respectively. For standard ERGs, flies were dark adapted for 90 s and then exposed to two bright light flashes of 4 s duration interrupted by a 5 s dark adaptation. The ERGs were amplified using a WPI Dam 60 differential amplifier and recorded on a Macintosh SE using a MacLab analog-to-digital convertor and the Chart/4 v3.1 program. The light source was a projector (model 765; Newport Corp., Irvine, CA) with a 100 W quartz tungsten-halogen lamp. The intensity of the light for these assays was measured using a power meter at the equivalent surface of the eye. In the standard ERG paradigm the light intensity was ~20 mW/cm^2.

The moderate light PDAs were performed by exposing flies (reared at 25°C and collected in the afternoons) to five light flashes of 2 s duration interrupted by dark adaptation periods of 4 s each. Blue and orange filters (Schott Optical Glass, Inc.; BG28 and OG590 respectively) were used in conjunction with the five light pulses according to the following sequence: orange, blue, blue, orange, orange. The intensity of the light for the moderate light PDA experiments was ~100 μW/cm^2. To achieve this light intensity with minimal variability between flies, the fiber optic light guide was positioned 18 mm from one of the eyes and not moved between experiments or when testing new flies. The flies were positioned in such a way that there was <1 mm variation in the distance of any eye to the light guide. As a result, the light intensity reaching the eye varied only ±5 μW/cm^2. No PDA or slow return after cessation of the light stimulus was detected in wild-type until the light intensity was increased to at least 150 μW/cm^2 (we typically use 2 mW/cm^2 of blue light to generate PDAs with wild-type). At 150 μW/cm^2 only some but not all wild-type flies showed a slow return or a PDA. PDAs were performed on a minimum of 12 flies from each stock.

M-potentials were performed as described previously (Minke and Selinger, 1992b). Briefly, white-eyed flies were immobilized as described above, adapted with intense blue light for 2 min and then exposed to a single bright orange flash from a strobe light. The extracellularly measured M-potentials were recorded on a Macintosh SE using a MacLab analog-to-digital convertor and the Chart/4 v3.1 program.

Co-immunoprecipitation of NinaC and calmodulin

Fly heads (100) were homogenized at 4°C in a microfuge mortar and pestle (Kontes) in buffer B (400 mls) which consisted of 10 mM imidazole pH 7.35, 350 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT, 1 mM ATP, 10 μg/ml PMSF and 1μg/ml leupeptin. In addition, different concentrations of EGTA and $CaCl_2$ were added to buffer B during the homogenizations to generate buffered free Ca^{2+} concentrations ranging from ~0 to 100 μM (see below). The homogenates were centrifuged at 100 000 *g* for 30 min. To reduce non-specific binding to the protein A–TrisAcryl beads (Pierce, Rockford, IL), the beads were first blocked by incubation with buffer B plus 1% BSA for 15 min at RT and then 50 μl of a 50% slurry of the blocked beads were combined with the supernatants in buffer B (0–100 μM free Ca^{2+}), gently mixed at 4°C for 15 min and centrifuged in a microfuge for 2 min at 14 000 r.p.m. The supernatant was then combined with 25 μl of undiluted p174-specific rabbit polyclonal antisera (raised to a β-galactosidase fusion protein that included p174 residues 1281–1501; Porter *et al.*, 1992) and incubated for 45 min at 4°C with gentle agitation. Then 50 μl of a 50% slurry of the blocked protein A–TrisAcryl beads in buffer B was added (0–100 μM free Ca^{2+}), the homogenate was gently mixed at 4°C for 15 min, the beads were allowed to settle, the majority of the supernatant removed and the bead supernatant slurry was gently overlayed onto a 1 ml 1 M sucrose/buffer B bed in a 1.5 ml Eppendorf tube. The microfuge tubes were centrifuged for 2 min at 14 000 r.p.m., the supernatants were gently removed, the beads were then resuspended in 50 μl of 2× SDS sample buffer containing 5 mM EGTA and the samples were boiled and fractionated by SDS–PAGE (12% gel). The top two-thirds of the gel was stained with Coomassie Blue while the lower one-third was transferred to a PVDF membrane and probed with calmodulin antibody as described (Hulen *et al.*, 1991) except that ^{125}I-labeled protein A was used instead of a secondary antibody. Briefly, the SDS–polyacrylamide gel was soaked for 15 min in KP buffer (25 mM KH_2PO_4/K_2HPO_4, pH 7.0) and transferred to a PVDF membrane (which was first pre-wet in methanol, immersed in water for 2–3 min and rinsed for 15 min in KP buffer) in KP buffer overnight at 4°C (~20 V). The PVDF membrane was then fixed in 0.2% (v/v) glutaraldehyde (EM Sciences) in KP buffer, rinsed in KP buffer, blocked in 5% (w/v) BSA in TBS (20 mM Tris, 500 mM NaCl, pH 7.5) for 1 h at 37°C (or overnight at 4°C), rinsed for 10 min in TBS at RT, probed with a rabbit anti-*Drosophila* calmodulin antibody (1:1000 dilution; gift of K.Beckingham) diluted in TBS with 1% BSA for 1 h at 37°C and rinsed three times for 10 min each at RT in washing buffer [1% BSA, 0.05% Tween-20 (w/v) in TBS]. The filter was then probed with ^{125}I-labeled Protein A (1:1000 dilution, 0.1 mCi/ml, 70–100 mCi/mg; New England Nuclear, Boston, MA) for 45 min at RT, diluted in TBS with 1% BSA, washed three times for 10 min each at RT in washing buffer, rinsed once in TBS and exposed to film.

To estimate the concentration of NinaC p174 in the immunoprecipitations, protein standards (BioRAD), which included myosin heavy chain (200 kDa) and β-galactasidase (116 kDa) were fractionated by SDS–PAGE alongside the immunoprecipitation samples at concentrations of 50–500 ng. The Coomassie Blue bands were scanned with a microcomputer Imaging device (Imaging Research Inc., St Catharine, Ontario, Canada) and the concentrations of p174 estimated using the standard curve generated from the molecular weight markers. The calmodulin concentration in the immunoprecipitates was estimated by loading different concentrations of retinal extracts alongside the immunoprecipitated proteins to generate a standard curve. The concentration of calmodulin in wild-type *Drosophila* retinas (~4 ng/retina) was based on the previous estimate that each rhabdomere contains 0.5 pg, that 80% of the calmodulin in the photoreceptor cells is in the rhabdomeres (Porter *et al.*, 1993), that ~90% of the retinal calmodulin is in the photoreceptor cells and that there are ~800 ommatidia per retina with seven photoreceptor cells (the R7 and R8 cells are counted as one cell since they each extend only half the depth of the retina). The samples were scanned by densitometry and compared with the standards to estimate calmodulin precipitated in nanograms.

To control the free Ca^{2+} concentrations in the immunoprecipitation experiments, we added ratios of EGTA (always 5 mM) and Ca^{2+} used previously (Conzelman and Mooseker, 1987) to generate conditions of 100, 10, 5, 1.0, 0.5 and <0 .001 μM free Ca^{2+}. These ratios were 1:1.1, 1:1, 1:0.95, 1:0.85, 1:0.65 and 1:0 EGTA to $CaCl_2$ respectively. To reduce the increase in free Ca^{2+} upon the addition of antisera (which contains ~2 mM free Ca^{2+}) 2 mM EGTA was added to the antisera prior to use. Ca^{2+} was also present in the tissue used to prepare the extracts. This source of Ca^{2+} introduced some error in estimating the Ca^{2+} concentration in the lower Ca^{2+} concentration buffers.

NinaC protein blots

Extracts from heads of wild-type and transgenic flies were prepared, fractionated by SDS–PAGE and probed with NinaC-specific antibodies as previously described (Porter *et al.*, 1992). The protein blots were performed in the linear range since we have previously shown, using identical methods, that NinaC heterozygotes show a 2-fold decrease in NinaC expression relative to wild-type (Porter and Montell, 1993).

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