Molecular, Biochemical, and Electrophysiological Characterization of Drosophila norpA Mutants*

(Received for publication, August 17, 1995, and in revised form, December 12, 1995)

Michael T. Peart$, Lydia L. Randall†, Randall D. Shortridge*, Martin G. Burg†, and William L. Pak‡

From the Department of Biological Science, Purdue University, West Lafayette, Indiana 47907 and the Department of Biological Science, State University of New York, Buffalo, New York 14214

Inositol phosphate signaling has been implicated in a wide variety of eukaryotic cellular processes. In Drosophila, the phototransduction cascade is mediated by a phosphoinositide-specific phospholipase C (PLC) encoded by the norpA gene. We have characterized eight norpA mutants by electrophoretinogram (ERG), Western, molecular, and in vitro PLC activity analyses.

ERG responses of the mutants show allele-dependent reductions in amplitudes and retardation in kinetics. The mutants also exhibit allele-dependent reductions in in vitro PLC activity levels and greatly reduced or undetectable NorpA protein levels. Three carry a missense mutation and five carry a nonsense mutation within the norpA coding sequence. In missense mutants, the amino acid substitution occurs at residues highly conserved among PLCs. These substitutions reduce the levels of both the NorpA protein and the PLC activity, with the reduction in PLC activity being greater than can be accounted for simply by the reduction in protein. The effects of the mutations on the amount and activity of the protein are much greater than their effects on the ERG, suggesting an amplification of the transduction signal at the effector (NorpA) protein level.

Transgenic flies were generated by germline transformation of a null norpA mutant using a P-element construct containing the wild-type norpA cDNA driven by the ninaE promoter. Transformed flies show rescue of the electrophysiological phenotype in R1-R6 photoreceptors, but not in R7 or R8. The degeneration phenotype of R1-R6 photoreceptors is also rescued.

Virtually all eukaryotic cells utilize the phosphoinositide (PI) signal transduction pathway to mediate such diverse cellular processes as metabolism, secretion, and cell growth and proliferation. Many types of more specialized functions, including muscle contraction, fertilization, sensory perception, and long term potentiation in neurons, are also subserved by PI signaling (reviewed by Berridge (1993)). The transduction cascade is initiated when phosphatidylinositol 4,5-bisphosphate (PIP2), a minor plasma membrane lipid, is hydrolyzed into two second messenger molecules, inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG). IP3 releases calcium from intracellular stores (Berridge, 1993) whereas DAG is an activator of protein kinase C (Nishizuka, 1988). Hydrolysis of PIP2 is catalyzed by the phospholipase C family of effector enzymes. The various transmembrane receptors known to activate these enzymes are responsive to hormones, neurotransmitters, growth factors, or external stimuli such as light and chemical ligands (Berridge, 1993).

PLCs have been isolated from a variety of mammalian tissues (reviewed by Rhee et al. (1989)), and complementary DNAs encoding some of these proteins, as well as complementary DNAs encoding two Drosophila PLCs and a yeast PLC (Takehiko et al., 1993), have been cloned and sequenced (see Meldrum et al. (1991) for references). Based on deduced amino acid sequences and immunological data, PLCs have been grouped into three classes: α, β, and γ (Rhee et al., 1989) that differ in primary structure, molecular weight, and mode of regulation. Activation of β class PLCs is mediated through a G-protein-dependent mechanism, whereas γ PLCs are activated by tyrosine residue phosphorylation (Morris et al., 1990). All known eukaryotic PLCs have two regions of extensive amino acid sequence homology called Box X and Box Y, which are approximately 120 and 150 amino acids in length, respectively (Rhee et al., 1989). This sequence conservation, along with in vitro biochemical data (Bristol et al., 1988), suggests that Boxes X and Y contain the catalytic site of these enzymes. Sequence homology and in vitro biochemical data have also been used to localize, tentatively and broadly, additional functional domains along the primary sequence of some β and γ PLCs (Bairach and Cox, 1990; Bristol et al., 1988; Cifuentes et al., 1993; Crooke and Bennett, 1989; Park et al., 1993; Schnabel et al., 1993; Wu et al., 1993a, 1993b). Much, however, remains to be determined about the structure-to-function relationship and regulation of this ubiquitous family of effector enzymes.

Since the mid-1980s, many studies have suggested that invertebrate phototransduction is mediated by a PI signaling cascade (reviewed by Bacigalupo et al. (1990) and Pak and Shortridge (1991)). Analysis of Drosophila norpA (no receptor potential) mutants has provided the most direct evidence for the role of this pathway in phototransduction (reviewed by Pak and Shortridge (1991)). The norpA gene encodes a β-class PLC (Bloomquist et al., 1988) that is predominantly expressed in the rhabdomeres of the compound eye (Schneuwly et al., 1991). It was one of the first PLC cDNA sequences to be elucidated. Photoreceptors in severely affected norpA mutants show essentially no electrophysiological response to light stimuli, and
therefore the flies are blind. Thus, the phototransduction cascade in Drosophila begins when light converts rhodopsin into its activated form, metarhodopsin. Metarhodopsin activates a G-protein which, in turn, activates the PLC effector enzyme to hydrolyze PIP$_2$. Specific roles for the PIP$_2$ hydrolysis, IP$_3$ and DAG, have not yet been clarified in the Drosophila phototransduction cascade. Nevertheless, calcium, presumably released by IP$_3$ binding to its receptor, is thought to be involved in photoexcitation and adaptation (Devery et al., 1987) (reviewed by Bacigalupo et al. (1990)), whereas an eye-specific protein kinase C, perhaps activated by DAG, appears to be required for adaptation through negative regulation (Hardie et al., 1993).

In addition to the electrophysiological phenotype of norpA mutants, the photoreceptor rhabdomeres of these flies manifest light-dependent degeneration. It was first discovered in Drosophila that mutations in some genes encoding proteins involved in phototransduction can result in retinal degeneration (reviewed by Pak (1994)). Similarly, mutations in genes implicated in the mammalian phototransduction pathway can result in retinal degeneration. For example, some cases of retinitis pigmentosa, a heterogeneous class of human diseases involving retinal degeneration, are caused by mutations in the rod opsin gene (see, e.g., Dryja et al. (1990)).

Since Drosophila is readily amenable to molecular genetic analysis and its visual system can be examined by a wide variety of analytical techniques, the phototransduction pathway in Drosophila provides an excellent model system in which to study β-class PLCs and PI-mediated signal transduction. Furthermore, analysis of degeneration in norpA mutants may help elucidate some of the mechanisms of degeneration caused by defective signal transduction pathways. In this work we have characterized eight norpA mutants by molecular, electrophysiological, and biochemical analyses to gain better understanding of PLC function.

MATERIALS AND METHODS

Standard Molecular Techniques—Standard procedures were used for phage and plasmid DNA purification, subcloning, agarose gel electrophoresis, and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Sambrook et al., 1989).

Drosophila Stocks—Drosophila melanogaster Oregon-R wild-type strain and norpA mutants induced by ethyl methane sulfonate mutagenesis (Pak et al., 1989) on the Oregon-R background were used throughout these experiments. All wild-type flies and norpA mutants were placed in either a white (w) or brown; scarlet (bw;st) background to eliminate the screening pigments in the compound eyes. Flies were raised in a 12-h light/12-h dark cycle at 24 °C on a cornmeal-yeast-sucrose-agar medium. Seven-day-old adult flies were used throughout, except in electron microscopic examination of rhabdomeres where newly eclosed or 6-week-old adults were used. The eyes absent (eya) mutant (Sved, 1986), which completely lacks compound eyes, was used to compare NorpA protein expression and PLC activity in eyes versus heads.

Electrophysiology—Electroretinograms (ERGs) were recorded as described previously (Larrivee et al., 1981). A xenon arc lamp light source (Oriel) was used with infrared (Corning, CS1–75) and Wratten (Kodak) neutral density filtration to achieve the desired light intensity. Recordings were made over a 6 log unit range of stimulus intensity from six flies of each norpA mutant allele and six wild-type flies. At each stimulus intensity, the flies were first dark-adapted for 10 min and then given a set of three 4-s light pulses, one white light pulse followed by two blue light pulses, at 20-s intervals. All recordings were made at 25 °C.

R7 and R8 photoreceptor responses were tested by first saturating the R1–R6 photoreceptor response with a maximal prolonged depolarizing afterpotential (PDA) induced with a strong blue light stimulus and then applying a second blue light stimulus during the PDA. The response to the second blue stimulus, seen superimposed on the R1–R6 PDA, is due to the responses of R7 and R8 photoreceptors (Minke et al., 1975).

Tissue Homogenate Preparation—Compound eyes of wild-type or norpA mutant flies or heads of eye flies were isolated and immediately frozen on dry ice. Total eye or head protein was extracted by homogenization of tissue at 4 °C in a glass mortar and pestle in 50 mM Tris-HCl, pH 7.5, 0.05% sodium deoxycholate, 250 mM KCl, 0.1 mM dithiothreitol, 20% glycerol, 0.1 mM phenylmethylsulfon fluoride, 0.25 mM dicyclamine, 1.0 mM benzamidine, 2 μg/ml leupeptin, and 0.2 μg/ml pepstatin. Homogenates were briefly centrifuged to remove particulate material and immediately frozen on dry ice. Protein concentration of each homogenate was determined with the BCA protein assay method (Pierce) using bovine serum albumin as a standard.

Western Blotting—Total eye or head protein homogenates (prepared as described under “Tissue Homogenate Preparation”) were boiled for 5 min in sample buffer (62.5 mM Tris-Cl, pH 6.8, 2% SDS, 10% glycerol, 2% β-mercaptoethanol, 10 μg/ml bromphenol blue), fractionated by 7.5% SDS-PAGE, and electroblotted onto nitrocellulose membrane. Preincubation with 2% nonfat dry milk in phosphate-buffered saline buffer (150 mM NaCl, 10 mM sodium phosphate, pH 7.2) at room temperature served to block: unbound nitrocellulose. Blotted and blocked membrane was incubated with primary antiserum (1:3000 dilution) generated against a fusion protein expressed from a norpA cDNA restriction fragment (nucleotides 848–1340 in Bloomquist et al., 1988) doned in-frame with the glutathione S-transferase gene (Zhu et al., 1993). The final fusion protein consisted of the first 164 amino acid residues from the relatively poorly conserved amino-terminal region of the NorpA protein coupled to glutathione S-transferase. Immunoblotting was detected using horseradish peroxidase-conjugated protein A antibody (1:5000 dilution, Kirkegaard & Perry Laboratories, Inc.) and visualized by incubation with either tetramethylbenzidine or Lumiglo peroxidase substrate (Kirkegaard & Perry Laboratories, Inc.). Primary antiserum was preincubated with excess fusion protein prior to incubation with the nitrocellulose membrane as a control to test specificity (Zhu et al., 1993).

Relative quantities of the NorpA protein detected on Western blots were determined by laser densitometry. Biomax MR film (Eastman Kodak Co.) was exposed to Western blots stained with Lumiglo peroxidase substrate. Bands on the exposed film corresponding to the 130-kDa protein, the expected size of the norpA-encoded protein (Zhu et al., 1987) (recloned under the tight expression control and sequenced using, Watson-Cran XL 2222-020 laser densitometer with internal digital integrator (LKB). The area under the density curve (peak area) was then calculated for each 130-kDa band. A standard curve was constructed by plotting the peak area of the 130-kDa band against the corresponding quantity of protein homogenate loaded onto a SDS-polyacrylamide gel. Each Western gel was loaded with wild-type or mutant protein homogenate so that the relation of the area of the corresponding 130-kDa band fell in the linear region of the standard curve.

Reverse Transcription-Polymerase Chain Reaction (PCR)—Total RNA was recovered from isolated, frozen heads of wild-type or norpA mutant flies by phenol/chloroform extraction (Sambrook et al., 1989). Poly(A)$^+$ RNA was purified from total RNA by affinity chromatography (Pharmacia). Total RNA was reverse-transcribed using Moloney murine leukemia virus reverse transcriptase (Life Technologies, Inc.) and primer complementary to the 3'-untranslated region of the wild-type NorpA gene (nucleotides 4108–4127 in Bloomquist et al. (1988)). Using PCR and Taq polymerase (Perkin-Elmer), this cDNA was enzymatically amplified in five overlapping segments covering the entire NorpA open reading frame (nucleotides 653-3937 in Bloomquist et al. (1988)). Pairs of 19-mer oligonucleotide primers were designed from the published wild-type norpA cDNA sequence so that each amplified segment was approximately 800 base pairs in length.

Genomic DNA Polymerase Chain Reaction (PCR)—In some cases genomic DNA was used as template for the PCR. Genomic DNA was isolated from whole flies essentially as described by Bloomquist et al. (1988). Amplification primers were the same as those used for reverse transcription-PCR or DNA sequencing.

DNA Sequencing—PCR products to be sequenced were isolated by agarose gel electrophoresis (Sambrook et al., 1989) and purified by the Genomic Method (BIO 101). Double-stranded PCR products were either directly sequenced in both directions by the dideoxy chain termination method (Sanger et al., 1977) using T4 DNA polymerase (U. S. Biochemical Corp.) or first subcloned into the polylinker region of M13mp18 bacteriophage using Escherichia coli JM101 as the host strain. Subcloning was performed by using either standard methods (Sambrook et al., 1989) or the T-A PCR product subcloning method (Marchuk et al., 1990). At least three independent clones of each subcloned PCR product were sequenced in both strands.

In Vitro Phospholipase C Activity Assay—Total protein homogenate

4938 Characterization of norpA Mutants
Characterization of norpA Mutants

4939

RESULTS

Typical wild-type ERGs evoked by our standard stimulus protocol are shown in the fifth set of traces in Fig. 1 (only the responses to the white and first orange stimuli are shown for clarity; see "Materials and Methods"). Although several types of cells contribute to the ERG, the dominant component is generated primarily by photoreceptor cells. Two ERG parameters were examined in this work, the amplitude and initial slope. The amplitude (V) is defined as the peak response evoked by any given stimulus, and the initial slope is measured from the initiation of the response to half the amplitude. Both of these parameters are stimulus intensity-dependent and increase with increasing stimulus intensity until response saturation. Fig. 2A shows the amplitude (V) plotted against the log of the stimulus intensity (log I), and Fig. 2B shows the initial slope of the response plotted against log I (wild type is the uppermost curve in both figures). Wild-type flies also exhibit a PDA in response to strong white or blue light stimuli (e.g. log I = −1 in Fig. 1). This protracted response, which can persist well after the termination of the light pulse, is due to the incomplete inactivation of metarhodopsin by arrestin when a substantial amount of rhodopsin is photoconverted to metarhodopsin (Byk et al., 1993; Dolph et al., 1993). Reconversion of metarhodopsin to rhodopsin with an orange light stimulus will terminate the PDA (e.g. the wild-type response to log I = −1 in Fig. 1)

In comparison to wild-type flies, ERGs of norpA mutants display both reduced amplitude and much slower response kinetics (see examples in Fig. 1). Although the amplitude generally increases with the stimulus intensity, as in wild type, it is substantially reduced in comparison to wild type at each intensity (Figs. 1 and 2A). In addition, norpA mutants display slow developing responses, particularly at low stimulus intensities (log I = −6 to −5) (Figs. 1 and 2B). While the initial slope increases with increasing stimulus intensity, it never reaches the level attained by the wild-type ERG at any intensity (Figs. 1 and 2B). This general trend is seen even in the mild norpA mutant, P57, although the effect is not as marked (Figs. 1 and 2B). Two mutants, P16 and P79, differ from the others in that, at the highest stimulus intensities (log I = −2 to 0), both V and the initial slope actually begin to decrease (Fig. 2, A and B). The eight mutants may be ranked, in order of decreasing magnitude, with respect to both amplitude and initial slope, as follows: wild type > norpAP57 > norpAP16 > norpA79 > norpA12 > norpA42 > norpAP45 > norpAP70 > norpAP24. Thus, the mutants appear to fall into four groups. Group 1 consists of P57, which responds with a V and initial slope that are somewhat reduced compared to wild type. The amplitude of P57 also
Characterization of norpA Mutants

Dependence of ERG parameters on stimulus intensity. A, ERG amplitude versus log stimulus intensity. The smooth curves were fit to the data points using the equation, \( \frac{V}{V_{\text{max}}} = 10^{a(n + \alpha)} \), where \( V \) = the ERG amplitude at any given intensity, \( V_{\text{max}} \) = the maximum obtainable amplitude at the highest intensities, \( I \) = stimulus intensity, \( \alpha \) = the stimulus intensity at half-maximal amplitude, i.e. \( \frac{1}{2} V_{\text{max}} \), and \( n \) = parameter adjusted to obtain a best fit (\( n = 0.45 \) and 0.56 for wild type and norpA\(^{57} \), respectively). B, stimulus intensity dependence of the initial slope of the ERG. The initial slope was determined by measuring the slope of the trace from the beginning of the response to \( \frac{1}{2} V_{\text{max}} \). This region of the ERG trace is essentially linear in all recordings. In both A and B, data were obtained from wild-type and eight norpA mutants each carrying a different allele. Each data point represents an average of measurements from six flies. The error bars represent S.E. values. P57, etc. = norpA\(^{57} \), etc. mutants.

saturates at a lower stimulus intensity (log1 = -3 as compared to -2 in wild type). Group 2 includes P16 and P79, which are similar to each other and have responses that are smaller and slower than either wild type or P57. Mutants P12, P42, P45, and P76 comprise group 3 and are comparable to each other, having very reduced amplitudes and initial slopes. The final group consists of P24, which shows virtually no response at any stimulus intensity.

All of these norpA mutants also exhibit a very prolonged response to white light stimuli of even low intensity (Fig. 1). This prolonged response can persist long after the termination of the stimulus but differs from the PDA of wild-type flies in that it can be generated by a low intensity stimulus and is not terminated by orange light.

Western analysis was used to determine the level of NorpA expression in total eye protein homogenates from the mutants. Polygonal antiserum generated against the amino-terminal region of the NorpA protein (see "Materials and Methods") detects a major protein of approximately 130 kDa on Western immunoblots of total head-protein homogenates from wild-type flies (Zhu et al., 1993) and eya mutants (Fig. 3) or of total eye-protein homogenates of wild-type flies and norpA mutants P16, P79, and P57 (Fig. 3). The size of the detected protein is consistent with the predicted size of NorpA from the work of Bloomquist et al. (1988), and the protein is not detected in strongly affected norpA mutants. However, more lightly stained, smaller proteins are also visible in some lanes of Fig. 3. Since the additional bands do not appear in any of the lanes where the 130-kDa protein is not detected, they are most likely related to the 130-kDa NorpA protein. For example, they could result from alternative splicing of norpA transcript (Zhu et al., 1993) or from degradation of the 130-kDa protein. An equal quantity of wild-type or mutant total eye protein homogenate was loaded into each lane of the Western blot shown in Fig. 3. Since the NorpA protein is much more highly expressed in wild type, a given quantity of total protein homogenate contains much more of this protein in wild type than in mutants. Consequently, the 130-kDa protein and the smaller proteins are especially prominent in this lane. NorpA expression is reduced to approximately 9, 9, 18, and 13% of wild type in P16, P79, P57, and eya, respectively (Fig. 4). Neither the 130-kDa band nor any smaller bands are detected in P12, P24, P42, P45, or P76 mutants (Fig. 3). Low expression of the 130-kDa protein in eya absence (13%) compared to wild-type eyes is consistent with the previous reports that NorpA expression is predominantly in the compound eye (see Yoshioka et al., 1993) and Zhu et al. (1993).

Next, we molecularly characterized the eight norpA alleles to determine if a mutation has been introduced into their coding sequences that might cause the reduced NorpA expression and observed ERG phenotype. DNA sequencing of the PCR products spanning the coding region (see "Materials and Methods") revealed 13 polymorphic differences between the published Canton-S wild-type cDNA sequence (Bloomquist et al., 1988) and the Oregon-R wild-type sequence, obtained in the present work. One of the polymorphisms results in an amino acid substitution (Arg-446 — His; numbering follows Bloomquist et al. (1988)). All of the polymorphisms were also found in the mutant sequences, which were induced in the Oregon-R background. Each norpA allele, however, also contains a nucleotide difference not found in the Oregon-R wild-type sequence. Each
of these mutations is expected to result in an alteration of the encoded amino acid sequence upon translation. Allele P57 contains a guanine (2955 in the numbering system of Bloomquist et al. (1988)) to adenine transition, which results in a missense mutation and the substitution of a glycine (768) with an aspartic acid. P16 and P79 are identical mutations containing a cytosine (1733) to thymine transition which creates a missense mutation resulting in the substitution of arginine 361 with thymine (nucleotides 1370, 2516, 2896, and 2468, respectively) that creates a premature termination codon, which is expected to result in early truncation of the protein product. Finally, allele P24 has a 28-base pair deletion (nucleotides 2710–2737), which causes a reading frameshift, resulting in the substitution of 24 amino acid residues, followed by a premature termination codon. Fig. 5 summarizes these data.

The extent to which these mutations affect the enzymatic activity was investigated by in vitro PLC enzyme assay of total eye-protein homogenate. Eye homogenate from wild-type flies produces a β emission activity of approximately \(82 \times 10^6 \text{ dpm} / (\text{mg} \times \text{min})\) of PLC activity under our reaction conditions yielding specific activity of 4.6 nmol of \([^{3}H] \text{PIP}_2\) cleaved/(mg × min) (see "Materials and Methods"). Before any corrections are applied, P57 and P16/P79 eye protein homogenates have specific activities of approximately 4.7% (0.22 nmol \([^{3}H] \text{PIP}_2\) cleaved/(mg × min)) and 2% (0.091 nmol \([^{3}H] \text{PIP}_2\) cleaved/(mg × min)) of the wild-type activity, respectively (Fig. 6). The remaining five mutants have roughly similar activity levels of 0.9–1.5% (0.039–0.067 \([^{3}H] \text{PIP}_2\) cleaved/(mg × min)) that of wild type. Total head homogenate from the eye mutant has about 12% (0.56 nmol of \([^{3}H] \text{PIP}_2\) cleaved/(mg × min)) of the wild-type eye homogenate PLC activity.

To establish definitively that mutations in the norpA gene are entirely responsible for the norpA phenotype, we have rescued the norpA<sup>P24</sup> null mutant by P-element-mediated germline transformation (Rubin and Spradling, 1982; Spradling and Rubin, 1982) (see "Materials and Methods"). Similar experiments have also been reported by McKay et al. (1995). Western analysis showed that the expression of the NorpA protein in transgenic flies is at least as great as in wild-type flies (Fig. 7, A and B).

ERG responses from transformed flies are also similar to those of wild-type flies in both amplitude and time course (Fig. 7C). However, further examination reveals a difference between wild type and transformant in the responses of a minority subset of photoreceptor cells. Each ommatidium in the Drosophila compound eye contains a total of eight photoreceptors in three subtypes: R1–R6, the major subtype, and two minor subtypes, R7 and R8. The ninaE promoter used to drive
the expression of the NorpA protein encoded in the P-element construct (see "Materials and Methods") will express the protein in R1–R6 photoreceptor cells only (O'Tousa et al., 1985; Zuker et al., 1985). Therefore, it is expected that transformation will rescue photoreceptors R1–R6, but not R7 or R8. Although the ERG responses from R7 and R8 are usually masked by the much larger response from R1–R6 photoreceptors, their response can be isolated by inducing a maximal PDA in R1–R6 using strong blue light to saturate and inactivate these photoreceptors (Minke et al., 1975). Neither R7 nor R8 is inactivated with blue lights since they contain rhodopsins that absorb maximally at wavelengths distinct from that of the R1–R6 rhodopsin (Harris et al., 1976; Hardie and Kirschfeld, 1983; Montell et al., 1987; Zuker et al., 1987). Consequently, if a second blue stimulus is applied during the PDA, any response observed would be generated from R7 and R8 photoreceptors and would be seen superimposed on the R1–R6 PDA (Minke et al., 1975).

FIG. 7. Analysis of norpAP24 transformants. A, Western blot of an equal quantity of total eye protein homogenate from wild-type and transformant flies. Same procedure was used as in the Western blot shown in Fig. 3. B, peak area of the band corresponding to the 130-kDa protein in total eye homogenates of norpAP24 transformants compared to that in equal amounts of wild-type eye homogenates. C, ERG recording from a norpAP24 transformant compared to those of wild type and a norpAP24 mutant, using a white stimulus of log I = −2. D, ERG recordings showing the response to a blue light stimulus given during the blue light-induced PDA as a test for R7 and R8 function. WT, wild-type; P24 trans, w norpAP24 transformants.

In addition to the electrophysiological defect, norpA mutants undergo a slow, light-dependent degeneration of the photoreceptor that is particularly evident in rhabdomeres (Meyertoholien et al., 1987; Pak, 1994). Rhabdomere degeneration in transgenic flies was investigated by ultrastructural comparison of ommatidia from wild-type, norpAP24 and transformed animals. Transverse thin sections were cut through the compound eyes of both newly eclosed and 6-week-old flies and examined by electron microscopy. Ommatidia from newly eclosed P24 mutant and transformant flies show R1–R6 rhabdomeres that are similar in size and pattern to those of wild-type flies. The R7 rhabdomere is also present (Fig. 8, panels A, C, and E). At 6 weeks of age, however, P24 flies completely lack R1–R6 rhabdomeres but retain the R7 rhabdomeres (Fig. 8, panel D). The transformants, on the other hand, retain R1–R6 rhabdomeres in a size and pattern that appear similar to wild type. The R7 rhabdomere is also present; however, it was not possible to determine from these data whether R7 is showing any signs of degeneration or not (Fig. 8, panel F).

DISCUSSION

In all eight norpA mutants characterized in this study, both the level of in vitro PLC activity and the amount of the NorpA protein expressed are found to be drastically reduced (Figs. 4 and 6). Moreover, the severity of the ERG phenotype (Figs. 1 and 2) is roughly correlated with the levels of NorpA expression and PLC activity. As noted under "Results," the eight norpA mutants examined fall into four groups on the basis of the ERG phenotype. In order of increasing severity, they are: Group 1, P57; Group 2, P16 and P79; Group 3, P12, P42, P45, and P76; and Group 4, P24. Each of these mutants falls into exactly the same group when examined on the basis of levels of NorpA protein expression or PLC activity, except that these measurements are not sensitive enough to distinguish Group 3 from Group 4. Thus, P57 (Group 1), with a NorpA level of ~18% (Fig. 4) and PLC activity level of ~5% that of wild type (Fig. 6), is the least affected. The next in order of severity are P16 and P79 (Group 2), with a protein level of ~9% of wild type and PLC activity level of ~2%. For the remaining five mutants (Groups 3 and 4), the NorpA expression cannot be detected by Western blot analysis (Fig. 3), and the PLC activity level is uniformly about 1.0–1.5% of the wild-type level (Fig. 6).
Generally, ERG responses observed in the mutants are much larger and more robust than might be expected from the levels of NorpA expression and PLC activity. For example, in P57 just 18% of the wild-type level of the NorpA protein (Fig. 4) and 5% of the wild-type level of PLC activity (Fig. 6) are enough to generate a response that, although reduced from that of wild type, is large and rapid (Figs. 1 and 2). Thus, at log I = 0, V obtained from P57 is approximately 72% of that of wild type (Fig. 2A), and the initial slope of the response is 69% that of wild type (Fig. 2B). Even in Group 3 mutants, in which the NorpA level is undetectable and the in vitro PLC activity is just 1.0–1.5% that of wild type, distinctly recognizable ERG responses are present, although much reduced in amplitude (–13% of wild type at I = 0) and very slow and prolonged in time course.

Since ERG responses are present in Group 3 mutants, some NorpA protein is likely to be present, even though it is not detected on Western blots. Calculations based on standard curves, plotting the peak area (see “Materials and Methods”) of the 130-kDa (NorpA protein) band versus the quantity of eye homogenate loaded onto a gel, show that as much as 2% of the wild-type NorpA protein could be present but not detected on Western blots (data not shown). However, even the maximum possible amount of the NorpA protein that could be present in these mutants (2%) is still much lower than what the ERG amplitude (–13%) generated in these mutants might suggest. As for the 1.0–1.5% wild-type level of PLC activity observed in these mutants, much of it is likely due to nonspecific background activity. At least one other PLC isozyme is known to be present in Drosophila heads in addition to the NorpA protein (Shortridge et al., 1991; Toyoshima et al., 1990). Since our PLC enzyme assay does not distinguish the activities of different isozymes, a small amount of non-NorpA PLC present in the eye homogenates could contribute to the observed PLC activity. Approximately 1.0% of the wild-type activity is detected in even norpA–/– mutants displaying virtually no ERG response, lending further support to the suggestion that this level of activity is largely or entirely due to non-NorpA PLC(s).

In light of the above discussion, a specific activity level of 0.053 nmol of [3H]PIP2 cleaved/(mg × min) (1.1% wild type activity) was considered a background PLC activity and subtracted from those of wild type, P57 and P16/P79 presented under “Results” and in Fig. 6. After this correction, the NorpA specific activities in total eye protein homogenates of wild type, P57, and P16/P79 are approximately 4.6, 0.16, and 0.04 nmol of [3H]PIP2 cleaved/(mg × min) total eye protein. However, the amount of the NorpA protein is reduced by factors of 0.18 and 0.09, respectively, in the total eye proteins of P57 and P16/P79 relative to that of wild type (Fig. 4). To assess reduction in specific activities of the mutant NorpA proteins independent of their reduction in the amount, the above specific activities were divided by the reduction factors, 0.18 and 0.09. With these corrections, the specific activities for P57 and P16/P79 are approximately 0.9 and 0.4 nmol of [3H]PIP2 cleaved/(mg × min) equivalent total eye protein, respectively, where equivalent total eye protein represents one having the same concentration of NorpA protein as in wild type (see “Materials and Methods”).

The above considerations, taken together, lead to the conclusion that the NorpA PLC need be activated by only a few percent of its normal capacity to yield a large and rapid light response from the photoreceptor. In the case of rhodopsin, activation of less than 1% of its normal amount is known to generate a maximal response of the photoreceptor (see, e.g., Johnson and Pak (1986) for Drosophila). In the case of vertebrates, this phenomenon has been attributed to the amplification of the response in the phototransduction cascade (see, e.g., Stryer (1983) and Lolley and Lee (1990)), i.e., each photoactivated rhodopsin molecule activates many G protein molecules, and each activated effector molecule (cGMP phosphodiesterase, in the case of vertebrates) hydrolyzes many cGMP. The present results suggest that similar amplification occurs at, or subsequent to, the effector molecule level in the PLC-based phototransduction cascade, i.e., each activated PLC molecule may generate many second messenger molecules to produce a sizable response even when the amount of PLC activated is very small.

Let us now consider the molecular defect identified in each of the norpA alleles and how it may relate to the ERG defect. In P16 and P79, the amino acid substitution occurs at an arginine residue (Arg-361), which is equivalent to Arg-358 in PLC-β1, a bovine brain PLC-β (Katan et al., 1988; Suh et al., 1988), and is conserved in Box X of all known eukaryotic PLCs (Fig. 5). Arg-361 is located within a cluster of several other highly conserved, contiguous residues that may be part of the catalytic site of the enzyme (Crooke and Bennett, 1989). Moreover, cysteine, which replaces arginine 361 in P16 and P79, is among the most reactive of amino acids. These data suggest that the mutation would have a significant effect on the function of the NorpA protein.

In contrast, the glycine residue substituted in P57 (Gly-768 → Asp; Fig. 5) is located outside of both Box X and Box Y and is conserved in the β and δ subclasses of PLC only. It is, therefore, not likely to be directly involved in catalytic function. Instead, it could possibly be involved in β and δ class-specific regulation of the enzyme. The effect of this mutation on the ERG is relatively mild compared to P16/P79.
The remaining five alleles (P12, P24, P42, P45, and P76) contain nonsense mutations that result in premature termination codons. If translated at all, it is expected that the protein product from these alleles would be truncated and functionally defective (Fig. 5). None of the mutants carrying these alleles show detectable NorpA protein on Western blots (Fig. 3). Nevertheless, at least some ERG response does occur in four of these mutants (P12, P42, P45, and P76), and the responses are similar to each other in amplitude and time course (Fig. 2) irrespective of the expected extent of protein truncation (Fig. 5). The observation suggests that the abnormal ERG response is not due to the expression of mutant NorpA protein per se. It is possible that read-through of the termination codon in these four alleles allows expression of a very low level of protein. Since each contains a single point mutation, read-through would result in a wild-type protein or protein that differs from wild type by a single amino acid. The premature termination codon, UAG, found in P12, P42, and P45 and UGA in P76 are not the most commonly utilized termination codons in Drosophila (Brown et al., 1990) and may be more likely to allow read-through than the more common UAA codon. Because of the amplification of transduction signal at the PLC level, previously discussed, a very low level of PLC expression due to read-through may be enough to produce the observed ERG response.

In the case of P24, on the other hand, essentially no ERG response is observed (Fig. 2). P24 has a 28-base pair deletion that causes a reading frameshift, resulting in the substitution of 24 amino acids followed by a premature truncation of the protein. Read-through of the termination codon, therefore, would be in a wrong reading frame and would still result in a significantly altered, and presumably non-functional, protein product.

As discussed in the Introduction, the phototransduction cascade in Drosophila involves the activation of the NorpA protein (PLC) by photoactivated rhodopsin through a G-protein. Although details of the cascade have not yet been fully elucidated, calcium has been implicated in both photoexcitation and adaptation (Devary et al., 1987) (reviewed by Bacigalupo, 1990). Dark-adapted photoreceptors have a relatively low internal calcium concentration, which increases during the light response. This increase initially has a positive feedback effect on the transduction cascade facilitating the rapid onset of the light response. As the internal concentration continues to increase, however, this positive feedback transforms into negative feedback resulting in adaptation and rapid deactivation of the response (Hardie and Minke, 1993). The altered kinetics of the light response in norpA mutants may be due to the failure of this sequential positive then negative feedback effect of cytosolic calcium.

Since the amount of NorpA protein is reduced while the size of the rhodobemes, before their degeneration, is similar to wild type in the mutants (Fig. 8), whatever PLC molecules are present would be distributed at a lower density. This reduced PLC density, along with the decreased specific activity, would result in a diminished and, perhaps, slower activation of the effector enzyme at any given stimulus intensity, resulting in a diminished and slower rise in cytosolic calcium during the light response. Without the rapid calcium kinetics, the initial positive feedback mechanism would be attenuated, severely reducing the the rapid initial phase of the light response in mutants, as seen in the ERG responses of mutants (Fig. 1). With increasing stimulus intensities, however, the slope of the initial phase, although clearly not wild type, does increase in mutants (Figs. 1 and 2B). It may be that at higher stimulus intensities, the PLC is activated at a sufficiently high rate to activate the positive feedback mechanism even in the mutants.

In addition to the reduced amplitude and initial slope, the light response in norpA mutants is very prolonged relative to wild-type responses (Fig. 1). The response, in some cases, is maintained up to several minutes after the end of the light stimulus. This prolonged response differs from the PDA of wild-type flies in that it is not terminated by orange light and is generated by even low stimulus intensities. It is possible that normal adaptation or deactivation does not occur in the mutants because the concentration of cytosolic calcium does not reach the level required for activation of the negative feedback mechanism. An eye-specific protein kinase C has been implicated in adaptation (Hardie and Minke, 1993) and deactivation (Ranganathan et al., 1991; Smith et al., 1991). This protein kinase C may not be activated properly at the severely reduced calcium and/or DAG concentrations generated in norpA mutants.

The ERG recorded from transgenic norpAP24 flies carrying the cloned wild-type norpA coding sequence (Fig. 7C) shows that the PLC encoded by the cloned sequence is sufficient to restore the normal phototransduction process. Since the ninaE promoter (OTousa et al., 1985; Zuker et al., 1985) used in the transformation experiments drives the expression of the cloned PLC coding sequence in R1–R6 photoreceptors only, only R1–R6 photoreceptors respond to light normally in transgenic flies, while R7 and R8 photoreceptors do not respond (Fig. 7D). In addition to the ERG phenotype, strong norpA alleles cause light-dependent degeneration of photoreceptors (Meyertholen et al., 1987) (reviewed by Pak, 1994). As with the ERG phenotype, it might be expected that R1–R6 rhodobemes would be rescued from degeneration in the transfectants whereas the R7 rhodobemes would not. As shown in Fig. 8, R1–R6 rhodobemes, which degenerate completely in norpA by 6 weeks, appear normal in transgenic flies at the same age. On the other hand, because R7/R8 rhodobemes degenerate slowly even in strong norpA mutants, it was not possible to make a reliable assessment of their rescue, or the lack thereof, in the 6-week-old transgenic flies examined. Taken together, the above results establish definitively that mutations in the norpA gene are solely responsible for both the ERG and degeneration phenotypes of norpA mutants.

Acknowledgments—We thank Dr. Richard McKay for help with PLC enzyme assay. We also thank Ann Pellegrino for help in the preparation of the manuscript.

REFERENCES
Bacigalupo, J., Johnson, E., Robinson, P., and Lismian, J. E. (1990) Transduction in Biological Systems (Hildago, C., Bacigalupo, J., Altmichow, E., and Vergara, J.-E. ed) pp. 27–45, Plenum Publishing Corp., New York.
Bairach, A., and Cox, J. A. (1980) FEBS Lett. 169, 454–456.
Berridge, M. J. (1993) Nature 361, 315–325.
Bloomquist, B. T., Shortridge, R. D., Schnewly, S., Perdue, M., Montell, C., Stafa, H., Rubin, G., and Pak, W. L. (1988) Cell 54, 723–733.
Bristol, A., Hall, M. L., Kriz, B. W., Stahl, M. L., Fan, Y. S., Byers, M. G., Eddy, R. L., Shows, T. B., and Knopf, J. L. (1988) Cold Spring Harbor Symp. Quant. Biol. 53, 915–920.
Brown, C. M., Stockwell, P. A., Tyszman, C. N. A., and Tate, W. P. (1990) Nucl. Acids Res. 18, 6339–6345.
Byk, T., Bar-Yaakov, M., Doza, Y. N., Minke, B., and Selinger, Z. (1993) Proc. Natl. Acad. Sci. U.S.A. 90, 1907–1911.
Cifuentes, M. E., Honkanen, L., and Rebecchi, M. J. (1993) J. Biol. Chem. 268, 11586–11593.
Crooke, S. T., and Bennett, C. F. (1989) Cell Calcium 10, 309–323.
Davies, O., Heschl, D., Bloor, M., Asscher, N., de Groot, R., Steiner, C. T., Minke, B., and Selinger, Z. (1987) Proc Natl. Acad. Sci. U.S.A. 84, 6939–6943.
Dokic, P. J., Ranganathan, R., Coyle, N. J., Hardy, R. W., Sosolich, M., and Zuker, C. S. (1993) Science 260, 1910–1916.
Dryja, T. P., McGhee, T. L., Reichel, E., Hahn, L. B., Cowley, G. S., Yandell, D. W., and Simon, M. A. (1990) Proc. Natl. Acad. Sci. U.S.A. 87, 6345–6349.
Dokic, P. J., Ranganathan, R., Coyle, N. J., Hardy, R. W., Sosolich, M., and Zuker, C. S. (1990) Nature 371, 364–366.
Hardie, R. C., and Kirschfeld, K. (1983) Biophys. Struct. Mech. 9, 171–180.
Hardie, R. C., and Minke, B. (1993) Trends Neurosci. 16, 371–376.
Hardie, R. C., Peretz, A., Suss-Toby, E., Ronn-Glias, A., Bishop, S. A., Selinger, Z., and Minke, B. (1993) Nature 363, 634–637.
Harris, W. A., Stark, W. S., and Walker, J. A. (1976) J. Physiol. 256, 415–439.

Downloads from <http://www.jbc.org/> by guest on April 27, 2019
Characterization of norpA Mutants

A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY

Sanger, F., Nicklen, S., and Coulson, A. R. (1977) Proc. Natl. Acad. Sci. U. S. A. 74, 5463-5467

Schnabel, E., Stein, D. M., and Parker, P. (1987) J. Comp. Physiol. A 170, 239-246

Spradling, A. C., and Rubin, G. M. (1982) Science 218, 341-353

Stryer, L. (1983) Cold Spring Harbor Symp. Quant. Biol. 48, 841-852

Suh, P. G., Ryu, K. H., Suh, H. W., and Rhee, S. G. (1988) J. Biol. Chem. 263, 161-169

Sved, J. (1986) Drosophila Information Service 63, 169

Takehiko, Y., Yasushi, M., and Hirotsu, Y. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 1804-1808

Thummel, C. S. and Pirotta, V. (1992) Drosophila Information Service 71, 150

Toyoshima, S., Matsumoto, N., Wang, P., Inoue, H., Yoshikawa, T., Hotta, Y., and Osawa, T. (1990) J. Biol. Chem. 265, 14842-14848

Wu, D., Jiang, H., Katz, A., and Simon, M. I. (1991) J. Biol. Chem. 266, 3704-3709

Wu, D., Katz, A., and Simon, M. I. (1993b) Proc. Natl. Acad. Sci. U. S. A. 90, 5297-5301

Yoshikawa, T., Inoue, H., Kasama, T., Seyama, Y., Nakashima, S., Nozawa, Y., and Hotta, Y. (1985) J. Biol. Chem. (Tokyo) 98, 557-562

Zhu, L., McKay, R. R., and Shortridge, R. D. (1993) J. Biol. Chem. 268, 15994-16001

Zuker, C. S., Cowman, A. F., and Rubin, G. M. (1985) Cell 40, 851-858

Zuker, C. S., Montell, C., Jones, K., Laverty, T., and Rubin, G. R. (1987) J. Neurosci. 7, 1550-1557
Molecular, Biochemical, and Electrophysiological Characterization of Drosophila norpA Mutants
Michael T. Pearn, Lydia L. Randall, Randall D. Shortridge, Martin G. Burg and William L. Pak

J. Biol. Chem. 1996, 271:4937-4945.
doi: 10.1074/jbc.271.9.4937

Access the most updated version of this article at http://www.jbc.org/content/271/9/4937

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 51 references, 24 of which can be accessed free at http://www.jbc.org/content/271/9/4937.full.html#ref-list-1