Phosphorylation of the InaD Gene Product, a Photoreceptor Membrane Protein Required for Recovery of Visual Excitation*

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Phototransduction by rhabdomeral photoreceptors, particularly of Drosophila compound eyes, has become an important model system for the ubiquitous phosphoinositide-mediated signal transduction. The progress achieved in this field is based on the powerful genetic and molecular biological techniques available for Drosophila, which have been successfully complemented by biochemical studies in other flies such as Calliphora and Musca. Despite the rapid progress that has been achieved in the understanding of sensory transduction mechanisms in recent years, the phototransduction cascade operating in this type of sensory cells has not yet been entirely resolved. In particular, the biochemical processes regulating the recovery and adaptation of the visual response in rhabdomeral photoreceptors are still obscure.

Extracellular Ca\(^{2+}\) enters the photoreceptors through ion channels and is required for rapid recovery of visual excitation (1–5). A major portion of the Ca\(^{2+}\) influx into the photoreceptor cell appears to be carried by a Ca\(^{2+}\)-selective class of channels that depend on, or may indeed be formed by, the transient receptor potential (trp)\(^1\) protein (6, 7). The primary structure of this trp protein was identified simultaneously by Wang et al. (8) and Montell and Rubin (9), and the trp gene product was shown to be localized to the rhabdomeral photoreceptor membranes. Direct measurements of the extracellular Ca\(^{2+}\) concentration revealed a decline of extracellular Ca\(^{2+}\) upon illumination in the eyes of wild type flies, which is significantly reduced in trp mutants (4). Signal transduction is also impaired in two other Drosophila mutants, inaC and InaD, which were originally classified as inactivation-no afterpotential mutants by Pak (10). While the InaD gene product is an 80-kDa protein of unknown function (11), the inaC gene was shown to encode an eye-specific protein kinase C (eye-PKC) (12, 13). Thus, it is tempting to assume that the Ca\(^{2+}\)-dependent deactivation of the visual response is controlled by phosphorylation of photoreceptor-specific proteins associated with the rhabdomeral photoreceptor membrane. Identified proteins that undergo light-dependent phosphorylation are rhodopsin and arrestin 2 (14–17). However, neither rhodopsin nor arrestin 2 were found to be phosphorylated by the inaC protein because phosphorylation of activated rhodopsin is not enhanced by Ca\(^{2+}\) (14, 15), and the Ca\(^{2+}\)-stimulated phosphorylation of arrestin 2 has been shown to result from the activation of a Ca\(^{2+}\)-calmodulin-dependent protein kinase (17). Accordingly, the target proteins of eye-specific protein kinase C have yet to be specified.

In an attempt to identify proteins that are part of the biochemical pathway in rhabdomeral photoreceptors, we have used an immunological approach to isolate Calliphora cDNA clones, which encode photoreceptor membrane proteins. Antibodies directed against purified rhabdomeral membranes (18)

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‡The abbreviations used are: trp, transient receptor potential; inaC, inactivation-no afterpotential C; InaD, inactivation-no afterpotential D; norpA, no receptor potential A; PAGE, polyacrylamide gel electrophoresis.
were generated and employed for the isolation of genes encoding rhodopsin-specific proteins. By this approach, we have cloned the Calliphora homologs of the Drosophila InaD, inaC, and trp genes. In the present paper we show that the InaD gene product is associated with the rhabdomeral photoreceptor membrane and that it is a putative substrate of eye-PKC. We also provide for the first time evidence for an interaction among eye-PKC, InaD protein, trp protein, and the norpA (non-receptor potential A) encoded phospholipase C.

**EXPERIMENTAL PROCEDURES**

Fly Stocks—Calliphora erythrocephala Meq, chalky mutant, was reared on bovine liver to maintain a high rhodopsin content in the eyes. Adult male flies were raised at 25 °C in a 12 h light/12 h dark cycle and were used for the experiments at an age of 8–10 days postecdysis.

Generation of Antibodies—Immunization of rabbits was performed according to standard protocols (19). Isolated rhodobes of 700 Calliphora eyes were used for each of the four injections. Final bleed sampling was 4 months after the first injection. The obtained antiserum was purified on protein A-agarose columns (Bio-Rad Life Technologies, Munich) as described (19).

Polyclonal anti-InaD antibodies were generated as follows: a DNA fragment encoding the C-terminal amino acids of the Calliphora InaD protein was amplified by polymerase chain reaction from cloned cDNA using sequence-specific primers. The polymerase chain reaction product was cloned into the expression vector pQE40 (Qiagen, Hilden/ Germany) in frame with six His codons and the dihydrofolate reductase gene. Fusion proteins were expressed in Escherichia coli M15 (pREP4), extracted with urea and purified on Ni²⁺-IDA-agarose columns (Bio-Rad Life Technologies, Munich). The purified antibodies were used for the experiments at an age of 8–10 days posteclosion.

The resulting antiserum was used to immunoscreen a Drosophila retinae cDNA library without poly(A) selection (Bio-Rad Life Technologies, Munich). The positive clones were rescreened, cloned as described, and sequenced—determined in the figure legends. If not indicated otherwise, the phosphorylation, analysis of the isolated protein was performed as described by Sambrook et al. (22). Expression of recombinant proteins was induced by applying n-isopropyl-β-D-thiogalactoside and was measured in a scintillation counter. The amount of InaD protein present in the InaD protein band was calculated by laser densitometry using bovine serum albumin as a standard.

**RESULTS**

Isolation and Characterization of Calliphora InaD cDNAs—Antibodies directed against proteins of the fly photoreceptor membrane had been generated by immunizing rabbits with rhodopsins (i.e. a subcellular fraction composed of the rhodobemal photoreceptor membranes and the intraommatidial matrix; see Ref. 18) isolated from 2800 Calliphora eyes. The resulting antiserum was used to screen a Calliphora retinae cDNA library. Out of 280,000 clones screened, 200 clones expressed polypeptides that reacted with the antiserum. Partial sequencing analysis revealed that the positive clones isolated so far encode at least six different proteins.

 Immunoprecipitation of the InaD Protein—Proteins were extracted from rhabdomeral membranes of 30 Calliphora eyes (10–20 μg protein each). The extract was added to 10 μl of protein A/G-agarose beads (Pierce), which had previously been incubated with anti-InaD-(272–542) for 1 h. Immunoprecipitation was performed for 2 h at 4°C and was followed by four washes with 500 μl of Triton X-100 buffer. Precipitated proteins were eluted from protein A/G-agarose beads with 15 μl of 1 × SDS-PAGE buffer for 10 min at 80 °C and were subjected to SDS-PAGE and Western blot analysis.

Protein Phosphorylation and Denvatartary—The standard assay for protein phosphorylation was carried out in a buffer containing Hepes-buffered saline (115 mM NaCl, 2 mM KCl, 15 mM Hepes, pH 7.8), 125 μM EGTA, 250 μM CaCl₂, and purified photoreceptor membranes from 10 fly retinae per sample. When indicated, phosphatase inhibitors were included: 1 mM sodium fluoride, 10 mM sodium pyrophosphate, 125 μM ethylenediaminetetraacetic acid, 250 μM phenylmethylsulfonyl fluoride, and 0.1 μg/ml chymostatin. For measuring phosphorylation reactions that contained nominally zero Ca²⁺, 10 mM EGTA and 0.1 μg/ml chymostatin were used. The soluble fraction of retinal proteins used in recombination experiments was obtained by homogenization in a small volume (about 0.5 μl retina) of 1 M phenylmethylsulfonyl fluoride in water and subsequently separating the soluble and particulate fraction by centrifugation at 50,000 × g for 10 min. Aliquots of 50 μl of soluble proteins of six retinae were added per sample in recombination experiments. Rhabdomeral photoreceptor membranes were prepared under dim red light. For activating light-dependent metarhodopsin phosphorylation, samples were illuminated with blue light for 2 min immediately before the reactions were started. In some cases blue light illumination was omitted as noted in the figure legends. If not indicated otherwise, the phosphorylation was carried out for 5 min at 20 °C in the dark. Thereafter, membranes were sedimented at 13,000 × g at 4 °C for 10 min, and proteins were extracted with high salt buffer, Triton X-100 buffer, or SDS-PAGE buffer and were subjected to SDS-PAGE as described above. For measuring phosphorylation time courses, reactions were terminated by adding 5 × SDS-PAGE buffer at the indicated times, and the whole sample was subjected to SDS-PAGE.

The amount of protein loaded was visualized by staining the gels with Coomassie Blue, and protein phosphorylation was detected by autoradiography using Kodak Biomax MR films. Quantification of the relative amount of radioactivity present in a protein band was performed with a phosphor imager (FUJIX BAS 1000, Fuji). For determining the stoichiometry of phosphorylation the radioactivity of cut-out protein bands was measured in a scintillation counter. The amount of InaD protein present in the InaD protein band was calculated by laser densitometry using bovine serum albumin as a standard.
Work presented in this study focuses on clones that show homology to the recently published Drosophila InaD gene (11). We obtained 14 cDNA clones coding for the Calliphora homolog of InaD and determined the nucleotide sequence of the longest cDNA for both strands. This clone contained a 201-base pair 5'-untranslated region, a 195-base pair open reading frame encoding a polypeptide of 665 amino acids (M₀ = 73,349), and a 194-base pair 3'-untranslated region. The translation initiation site was assigned arbitrarily to the first AUG of the open reading frame at nucleotide 202, which is preceded by a stop codon at nucleotide 196, and fits well with the consensus sequence for translation initiation sites in Drosophila, (C/ A)AA(A/C)aug (26). Alignment of the deduced amino acid sequence of the Calliphora cDNA clone with the Drosophila InaD sequence (Fig. 1a) shows that the two proteins display 65.3% overall amino acid identity and 77.3% similarity if conservative substitutions are taken into account. Furthermore, both proteins share similar biophysical characteristics, i.e., the same predicted isoelectric point of 8.6, a high abundance of basic (Lys, Arg, His) and acidic (Asp, Glu) amino acids that together comprise more than 30% of the polypeptide, and similar hydrophilicity profiles (Fig. 1b) that reveal no stretches of hydrophobic sequences of 20 or more residues in length.

Two repeats of 40 amino acids (underlined in Fig. 1a) that were shown to share limited sequence homology with the Drosophila disc-large (Dl(g)), the rat post-synaptic density protein (PSD95), the vertebrate tight junction protein ZO-1, and the human ROS protein (see Ref. 11 and references therein), are highly conserved (90% similarity) between the Drosophila and Calliphora InaD proteins, implying a common functional role within the family of proteins that contain these repeats. On the other hand, repeats consisting of Gly-(Gln/Met), which are present in the Drosophila InaD sequence between amino acids 142 and 158, are not found in the Calliphora sequence. Indeed, the region between residues 106 and 183 is the least conserved part of the two proteins. The only common feature within this region is the relatively high abundance of glutamine residues. Another striking sequence motif, which is present in the Calliphora and the Drosophila InaD protein, is the highly hydrophilic cluster of lysine and glutamate residues between amino acids 454 and 473 (indicated by bars in Fig. 1b). While potential phosphorylation sites of cAMP- and cGMP-dependent protein kinase and tyrosine kinase present in the Drosophila sequence at Thr³⁶⁰ and Tyr²⁰³, respectively, are not found in the Calliphora sequence, eight potential phosphorylation sites of protein kinase C are conserved (Fig. 1a). Finally, two potential glycosylation sites at Asn₁⁶⁸ and Asn₃¹² (Drosophila) or Asn₁⁶² and Asn₃⁰³ (Calliphora) are found at similar positions in both sequences.

Identification and Localization of the InaD Protein by Monospecific Antibodies—In order to obtain more detailed information on the function of InaD in fly phototransduction, it is crucial to know whether the InaD gene product is a membrane protein, and if so whether it resides in the rhabdomeral membrane. Isolation of the InaD clone by means of an anti-rhabdom serum already suggests that the InaD protein is associated either with rhabdomeres or with the intracommatidal (extracellular) matrix of the photoreceptor cells. The InaD protein was identified on Western blots using monospecific anti-InaD (643–665) and anti-InaD (272–542) antibodies that were raised against peptides containing the 23 C-terminal amino acids and amino acids 274–542 of the Calliphora InaD protein, respectively. Both antibodies bind to a single protein with an apparent molecular mass of 75 kDa (Fig. 2). The apparent molecular mass of 75 kDa is in line with the molecular mass deduced from the cDNA sequence (73,349 Da). This demonstrates that the InaD protein is present in total eye membranes and in purified photoreceptor membranes (Fig. 2, lanes 1 and 3). It is detected neither in the fraction containing soluble proteins obtained from whole retinas after extraction with low salt buffer nor in extracts containing proteins of the intracom-
Maternal matrix (Fig. 2, lane 3) or transferred onto a polyvinylidene difluoride membrane for immunological detection with antibodies directed against the C-terminal region of the InaD protein (anti-InaD-(643–665)) or a peptide comprising amino acids 272–542 (anti-InaD-(272–542)). Membrane and soluble proteins from one Calliphora retina were analyzed in lanes 1 and 2, respectively. Extracted with 1 × SDS-PAGE buffer from purified photoreceptor membranes of 10 Calliphora eyes were loaded on lane 3. Lanes 4–6 show rhabdomeral proteins obtained after subsequently extracting purified photoreceptor membranes with a low salt buffer containing 3 mM EGTA (lane 4) with a high salt buffer containing 1.5 M NaCl (lane 5) and with 1 × SDS-PAGE buffer (lane 6).

Immunoprecipitation of the InaD Protein by Immunoprecipitation—In order to purify the InaD protein by immunoprecipitation rhabdomeral membranes were treated with a buffer containing 1% Triton X-100, which quantitatively extracted the InaD protein from nonsoluble material. Anti-InaD-(643–665) failed to immunoprecipitate the InaD protein. Thus, we generated an antisera that was directed against a different part of the InaD protein (anti-InaD-(272–542)) and could successfully be used for immunoprecipitation (Fig. 3). Resolving the immunoprecipitates obtained with anti-InaD-(272–542) by SDS-PAGE revealed that, in addition to the InaD protein band, two other protein bands with apparent molecular masses of 140 and 80 kDa were immunoprecipitated (Fig. 3a, lane 3). None of these proteins was precipitated in control experiments in which protein A/G beads alone were used (Fig. 3a, lane 4). The 140-kDa protein band turned out to represent a double band when resolved on 8% polyacrylamide gels (data not shown). Western blot analysis (Fig. 3b) showed that this protein band reacted with antibodies specific for the Calliphora trp protein and for the norpA-encoded phospholipase C. The 80-kDa protein represents the eye-specific protein kinase C (inaC protein). The immunoprecipitates were also probed with antibodies specific for the α- and β-subunit of an eye-specific G-protein (G\text{\textsubscript{a}} and G\text{\textsubscript{e}}, respectively). The immunoprecipitation of the InaD protein with an eye-specific protein kinase C prompted us to investigate whether or not the InaD gene product is a phosphoprotein. In order to test this hypothesis, we made use of the ability to enrich the protein by high salt extraction of purified photoreceptor membranes. In the experiment depicted in Fig. 4, the InaD protein was extracted with high salt buffer after performing phosphorylation of photoreceptor membrane proteins under the standard conditions described under “Experimental Procedures.” The extracted peripheral proteins, as well as integral membrane proteins, were subjected to SDS-PAGE and autoradiographed. Of the seven protein bands detected in the high salt extract after staining the gel with Coomassie Blue, four proteins are phosphorylated. The most prominent of these phosphoproteins shows an apparent molecular mass of about 75 kDa, a value corresponding to the apparent molecular mass of the InaD protein. Autoradiography of a duplicate blot and subsequent probing of the very same blot with anti-InaD-(643–665) demonstrated that the radioactively labeled protein band at 75 kDa represents the InaD protein band (Fig. 4b). In order to rule out that a phosphoprotein other than the InaD protein is present in the high salt extracts and has the same electrophoretic mobility as the InaD protein upon separation by SDS-PAGE, phosphorylated InaD protein was also purified by immunoprecipitation (Fig. 5). The presence of radioactive phosphate in the 75-kDa protein band, which was obtained by resolving the anti-InaD-(272–542) immunoprecipitates by SDS-PAGE and which was identified as the InaD protein with anti-InaD-(643–665), clearly demonstrated that the InaD protein is a phosphoprotein. The stoichiometry of InaD protein phosphorylation, determined for the InaD protein present in high salt extracts as described under “Experimental Procedures,” was 0.4–0.5 mol of phosphate/mol of InaD protein. Hence, a substantial fraction of the InaD molecules was not phosphorylated in the in vitro assays, which may indicate that a fraction of InaD molecules is isolated in a phosphorylated form or is compartmentalized in membrane vesicles to which externally added ATP or activators of the protein kinase have no access.

Since eye-PKC co-immunoprecipitating with the InaD pro-
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Identification of phosphorylated InaD protein. In order to demonstrate phosphorylation of the InaD gene product, proteins of dark-adapted rhabdomeral membranes that remained in the membrane fraction after extraction with low salt buffer containing 3 mM EGTA were phosphorylated in the presence of 2 μCi of [γ-32P]ATP as described under "Experimental Procedures." After phosphorylation the InaD protein was extracted from the membrane with high salt buffer. Membrane proteins that were not extracted under these conditions were solubilized with SDS-PAGE buffer. The aliquots equivalent to purified photoreceptor membranes of 10 retinae for the SDS extract or 40 retinae for the high salt extract were subjected to SDS-PAGE. a, Coomassie-stained protein pattern and corresponding autoradiograph of the SDS-extracted proteins (lane 1) and the high salt-extracted proteins (lane 2), b, immunoblot of the same extracts as shown in panel a probed with anti-InaD-(643–665) and corresponding autoradiograph.

Coomassie-stained protein pattern and corresponding autoradiograph of the immunoprecipitates (lane 1) of the immunoprecipitates (lane 3) were resolved by SDS-PAGE and subjected to Western blot analysis and autoradiography. The Coomassie-stained protein gel, the immunoblot probed with anti-InaD-(643–665) and corresponding autoradiograph.

Phosphorylation of the InaD protein was extracted from the membrane with high salt buffer. The Coomassie-stained protein pattern and corresponding autoradiograph of the immunoprecipitates (lane 1) of the immunoprecipitates (lane 3) were resolved by SDS-PAGE and subjected to Western blot analysis and autoradiography. The Coomassie-stained protein gel, the immunoblot probed with anti-InaD-(643–665) and corresponding autoradiograph.

Identification of phosphorylated InaD protein. Protein of blue light-illuminated rhabdomeral membranes were phosphorylated in the presence of 2 mM EGTA were phosphorylated in the presence of 2 μCi of [γ-32P]ATP as described under "Experimental Procedures." After phosphorylation the InaD protein was extracted from the membrane with high salt buffer. Membrane proteins that were not extracted under these conditions were solubilized with SDS-PAGE buffer. The aliquots equivalent to purified photoreceptor membranes of 10 retinae for the SDS extract or 40 retinae for the high salt extract were subjected to SDS-PAGE. a, Coomassie-stained protein pattern and corresponding autoradiograph of the SDS-extracted proteins (lane 1) and the high salt-extracted proteins (lane 2), b, immunoblot of the same extracts as shown in panel a probed with anti-InaD-(643–665) and corresponding autoradiograph.

Ca2+-dependent phosphorylation of the InaD protein. a, purified photoreceptor membranes of 10 Calliphora retinae were phosphorylated in the presence of 2 mM EGTA (lanes 1, 3, 5, and 7) or 60 μM calculated free Ca2+ (lanes 2, 4, 6, and 8). b, immunoblot of the same extracts as shown in panel a probed with anti-InaD-(643–665) and corresponding autoradiograph. Under the conditions described under "Experimental Procedures," and protein phosphorylation was detected by autoradiography or quantified by using a phosphor imager.

Protein is assumed to be a Ca2+-dependent protein kinase, we tested the effect of Ca2+ on the phosphorylation of the InaD protein and compared its phosphorylation with phosphorylation of rhodopsin and arrestin, which has been studied previously (14–17) (Fig. 6). Lowering of internal Ca2+ by the addition of 2 mM EGTA to the phosphorylation assay significantly reduced the incorporation of radioactive phosphate into the InaD protein as compared with standard phosphorylation assays performed at a calculated free Ca2+ concentration of 60 μM. Under these conditions, the phosphorylation of two other proteins involved in signal transduction is affected by lowering the free Ca2+ concentration. First, the amount of phosphorylated arrestin 2 associated with the rhabdomeral photoreceptor membrane is decreased. This finding is consistent with earlier reports indicating that arrestin 2 is phosphorylated by a Ca2+-calmodulin-dependent protein kinase (17). Secondly, the phosphorylation of rhodopsin is affected by calcium concentration. There the amount of radioactive phosphate attached to metarhodopsin is reduced in the presence of Ca2+, presumably due to dephosphorylation by a Ca2+-dependent rhodopsin phosphatase (27, 28). We also investigated whether the light conditions to which the photoreceptor membranes were subjected before the reaction was started, might affect the incorporation of phosphate into the InaD protein. Under the in vitro conditions used here, the already intensively studied light activation of
rhodopsin phosphorylation (14, 15) was reproduced, but light
dependence of InaD protein phosphorylation was not revealed
(Fig. 6a, lanes 1 and 2 and lanes 3 and 4). Thus, we can exclude
the possibility that activated rhodopsin (metarhodopsin), pre-
sent in the membranes, directly promotes (or suppresses) the
phosphorylation of the InaD protein. Furthermore, the addi-
tion of soluble proteins did not enhance the incorporation of
phosphate into the InaD protein, but rather suppressed its
phosphorylation (Fig. 6a, lanes 5–8). This indicates (i) that the
protein kinase that catalyzes InaD phosphorylation resides in
the photoreceptive membrane, and (ii) that soluble cofactors
are not required for InaD protein phosphorylation.

Time courses of the protein phosphorylation revealed similar
phosphorylation kinetics for the InaD protein and for opsin
with no further increase in net phosphate incorporation 10 min
after the reactions were started (Fig. 6b). Arrestin 2 phos-
phorylation, described as the most rapid protein phosphorylation
observed in Drosophila eyes (29), saturated 2 min after starting
the reactions. The Ca\textsuperscript{2+}-dependence of the phosphate incorpo-
ration into the InaD protein, arrestin 2, and metarhodopsin is
evident throughout the entire phosphorylation time course,
except in the initial phase of metarhodopsin phosphorylation
(Fig. 6b). The Ca\textsuperscript{2+}-enhanced phosphorylation of the InaD gene
product suggested that this reaction is catalyzed either by a
protein kinase C or by a Ca\textsuperscript{2+}-calmodulin-dependent protein kinase.
In order to discriminate whether the InaD protein is
phosphorylated by a protein kinase C or a Ca\textsuperscript{2+}-calmodulin-de-
pendent protein kinase, protein kinase C was hyperactivated
using a phorbol ester or specifically inhibited with bisindolyl-
maleimide I. As shown in Fig. 7, the addition of phorbol
12-myristate 13-acetate to the phosphorylation reaction enhances
the phosphate incorporation into the InaD protein by 25%. In
the presence of bisindolylmaleimide I InaD protein phos-
phorylation is reduced by 25%. These effects are statistically sig-
nificant (see legend of Fig. 7), and they are comparable with
those observed in studies with other photoreceptor membrane
proteins, for example the protein kinase C-dependent phos-
phorylation of bovine rhodopsin (30). The protein kinase C activa-
tor and inhibitor used here had no significant effect on the
phosphorylation of arrestin 2 and opsin (Fig. 7), indicating that
the addition of the phorbol ester or of bisindolylmaleimide I
modulated specifically the phosphorylation of the InaD protein
but did not generally enhance or quench the phosphorylation of
rhabdomal proteins.

**DISCUSSION**

This study describes the molecular and biochemical charac-
terization of the Calliphora InaD protein. The experiments
have been performed to understand the function of this protein
in the deactivation of light-triggered responses of photoreceptor
cells. Cumulative evidence suggests that the biochemical reac-
tions involved in phototransduction are identical in Drosophila
and Calliphora. The eyes of both species have the same mor-
phological architecture, they contain photoreceptors with iden-
tical absorbance characteristics, and the photoreceptor cells
respond to light stimuli in the same way. Biochemical studies
show that identified proteins of the phototransduction path-
way, including Rh1 opsin (31–33), arrestin 2 (17, 27, 34), and
phospholipase C (35, 36), perform identical functions in both
species. However, these functionally homologous proteins are
less conserved than they are between photoreceptors currently
used as model systems of vertebrate phototransduction, for
instance bovine, rat, and mouse. These differences in the over-
all homology allow us to identify conserved regions as probable
sites of functional importance within the protein.

The sequence alignment of the Drosophila and Calliphora
InaD proteins (see Fig. 1) highlights the weakly, as well as the
highly, conserved regions of the protein sequences. The N-
terminal region (amino acids 1–14 of the Drosophila sequence)
and the stretch between amino acids 106 and 183 show little if
any sequence homology, suggesting that these regions are func-
tionally less important and were, therefore, subject to extensive
mutation during the evolution of both fly species.

Other sites of the InaD protein are well conserved. With
respect to the phosphorylation of the InaD protein investigated
in the present study it is particularly striking that five out of
eight conserved potential protein kinase C phosphorylation
sites (at positions 19, 194, 329, 330, and 553) reside within
stretches of 10–16 amino acids that are identical in the Dros-
ophila and Calliphora InaD protein.

Despite the fact that there are some poorly conserved regions
in the Drosophila and Calliphora InaD protein, the overall
biophysical characteristics (for example the isoelectric point at
8.6, the high abundance of acidic and basic amino acids, and
the hydrophilicity profile of both proteins) are nearly identical.
The apparent molecular mass of about 75 kDa of the Calliphora
InaD protein, as estimated by SDS-PAGE, fits the molecular
mass calculated from the sequence data (73.4 kDa). The dis-
crepancy between the calculated and apparent molecular mass
of the Drosophila InaD protein (80 and 90 kDa, respectively)
reported by Shieh and Niemeyer (11) is not evident in Cali-
phora. Due to the hydrophobic nature of the InaD protein, it has
been proposed that the Drosophila InaD gene product is not an
integral membrane protein (11). Our results obtained with the
Calliphora homolog of InaD are in agreement with this pre-
novation.

In the present study we show that the InaD protein is asso-
ciated with the rhabdomal photoreceptor membrane, from
which it is extracted by buffers of high ionic strength. The
attachment to the photoreceptive membrane may be crucial for
InaD function, because functional impairment of the Drosoph-
illa InaD mutant (InaD\textsuperscript{D215}); Ref. 10) results from a single point
mutation in which a methionine (Met\textsuperscript{442}), located within a
small stretch of hydrophobic amino acids, is replaced by lysine
(11). In the Calliphora InaD protein leucine is present at the
corresponding position, indicating that Met\textsuperscript{442} is not necessar-
ily required for normal InaD function, and may be exchanged
with another hydrophobic amino acid. Distortion of the hydro-
phobic character of the region by a highly polar amino acid, for
example lysine of InaD \(^{225}\), however, might lead to the mutant phenotype, because the Met\(^{442}\) to Lys mutation may render a soluble InaD protein that is nonfunctional. Alternatively, the nonpolar character of this region may be crucial for hydrophobic protein-protein interactions. A significant contribution to the hydrophilic character of the InaD protein results from a conserved stretch of lysine and glutamate residues (see bars in Fig. 1b). Interestingly, similar lysine/glutamate-rich clusters are found in the bovine and mouse rod photoreceptor cGMP-gated channels (37, 38). Analysis of the structure-function relationship of cGMP-gated channels has not yet established the function of this hydrophilic cluster.

The biochemical experiments of the present study were designed to investigate whether or not the function of the InaD gene product might be controlled by phosphorylation. The striking conservation of several putative protein kinase \(C\) phosphorylation sites between the Drosophila and Calliphora InaD sequence (Fig. 1), the localization of the InaD protein and the InaC encoded eye-PKC in the rhabdomeral photoreceptor membranes (Fig. 2 and Ref. 13), and, most importantly, the co-immunoprecipitation of the InaD protein and eye-PKC (Fig. 3) suggest that the InaD protein is a likely candidate for phosphorylation by eye-PKC. Moreover, Drosophila InaD and InaC mutants show a similar phenotype, which is characterized by a defect in photoreceptor deactivation and by abnormal light adaptation (1, 2, 11, 13, 39), indicating that the respective gene products are acting, or even interacting, in closely related steps of the transduction cascade. The phosphorylation studies presented here reveal that the InaD protein is a phosphoprotein (Figs. 4 and 5). The \(Ca^{2+}\)-dependence of the InaD protein phosphorylation (Fig. 6) and the findings that the incorporation of phosphate into the InaD protein is moderately enhanced in the presence of a phorbol ester and quenched by the protein kinase C inhibitor bisindolylmaleimide (Fig. 7) are in line with the assumption that this phosphorylation is catalyzed by eye-PKC.

Despite this evidence, the data do not yet allow us to unequivocally rule out the possibility of phosphorylation of the InaD protein by other protein kinases. \(Ca^{2+}\)-dependent phosphorylation of arrestin 2, reported to result from a \(Ca^{2+}\)-calmodulin-dependent protein kinase (17), is observed in parallel to the \(Ca^{2+}\)-dependent phosphorylation of the InaD protein, indicating that the corresponding protein kinase is present in the same membrane preparation used in the assays. Also, Matsumoto and colleagues (17, 40, 41) reported on the phosphorylation of an 80-kDa protein present in the photoreceptor cell layer of Drosophila eyes. The molecular mass of this phosphoprotein suggests that it might represent the Drosophila InaD protein. However, the phosphorylation of this Drosophila 80-kDa protein was shown to be activated by cAMP but not by calcium (17). At least the Calliphora InaD protein lacks consensus sites for phosphorylation by a cAMP-dependent protein kinase. In dark-adapted Drosophila eyes, this 80-kDa protein is in the nonphosphorylated state, but it rapidly (within 3 s) becomes phosphorylated when the flies are exposed to a 1-ms light flash (41). Furthermore, the light-dependent phosphorylation of this protein is not observed in Drosophila norpA mutants (40), indicating that it depends on the activation of the phototransduction cascade and occurs downstream of the norpA-encoded phospholipase C.

Phosphorylation by protein kinase \(C\) is shown to be involved in the desensitization of a number of vertebrate G-protein-mediated transduction cascades, e.g. vertebrate phototransduction (30, 42) and \(\beta\)-adrenergic receptor signaling (43). These desensitization is achieved by a protein kinase \(C\)-dependent phosphorylation of the respective receptor (rhodopsin or \(\beta\)-adrenergic receptor), which in contrast to the phosphorylation by rhodopsin kinase or \(\beta\)-adrenergic receptor kinase occurs in the activated and the nonactivated state of the receptor. Protein kinase \(C\)-mediated phosphorylation was shown to uncouple the receptor from its G-protein (44), thereby terminating the signal response. The proposed deactivation of the visual response via phosphorylation of the InaD protein by eye-PKC would act at a different site of the transduction cascade.

Towards a model for the \(Ca^{2+}\)-dependent response inactivation in fly photoreceptor cells, we propose that the InaD protein is modulated via phosphorylation by eye-PKC, which itself should be activated by the transient rise of the intracellular \(Ca^{2+}\) concentration upon visual excitation. Phosphorylated InaD protein in turn may be a subunit of, or act on, a third protein, e.g. an ion channel, in order to regulate \(Ca^{2+}\) influx into the cytosol. In this respect it is important to note that the trp protein, which is proposed to represent a novel \(Ca^{2+}\)-channel responsible for light-dependent inositol triphosphate-mediated \(Ca^{2+}\) entry (6, 7), co-immunoprecipitates with the InaD protein. Alternatively, the activated InaD protein could be part of a feedback control mechanism that acts on upstream members of the transduction cascade. One of these may be the norpA-encoded phospholipase C (11). Our finding that key proteins of the phototransduction cascade investigated here co-immunoprecipitate with the InaD protein may indicate that proteins that provide a control mechanism of visual excitation are associated into a functional protein complex.

In conclusion, we have for the first time provided evidence that the Calliphora homolog of the InaD protein is phosphorylated by the InaC-encoded eye-PKC. InaD protein phosphorylation may be part of the mechanism that regulates the deactivation of the light response in invertebrate photoreceptors, in a way that is distinct from the protein kinase \(C\)-mediated desensitization of vertebrate phototransduction or \(\beta\)-adrenergic receptor signaling. However, a similar mechanism may operate in other vertebrate and invertebrate signaling pathways in which trp homologs are used as part of a store-operated \(Ca^{2+}\) entry (45).

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