# The TRP $Ca^{2+}$ channel assembled in a signaling complex by the PDZ domain protein INAD is phosphorylated through the interaction with protein kinase C (ePKC)

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**Abstract Photoreceptors which use a phospholipase C-mediated signal transduction cascade harbor a signaling complex in which the phospholipase Cβ (PLCβ), the light-activated $Ca^{2+}$ channel TRP, and an eye-specific protein kinase C (ePKC) are clustered by the PDZ domain protein INAD. Here we investigated the function of ePKC by cloning the *Calliphora* homolog of *Drosophila* ePKC, by precipitating the TRP signaling complex with anti-ePKC antibodies, and by performing phosphorylation assays in isolated signaling complexes and in intact photoreceptor cells. The deduced amino acid sequence of *Calliphora* ePKC comprises 685 amino acids (MW = 78 036) and displays 80.4% sequence identity with *Drosophila* ePKC. Immunoprecipitations with anti-ePKC antibodies led to the co-precipitation of PLCβ, TRP, INAD and ePKC but not of rhodopsin. Phorbolester- and $Ca^{2+}$-dependent protein phosphorylation revealed that, apart from the PDZ domain protein INAD, the $Ca^{2+}$ channel TRP is a substrate of ePKC. TRP becomes phosphorylated in isolated signaling complexes. TRP phosphorylation in intact photoreceptor cells requires the presence of extracellular $Ca^{2+}$ in micromolar concentrations. It is proposed that ePKC-mediated phosphorylation of TRP is part of a negative feedback loop which regulates $Ca^{2+}$ influx through the TRP channel.**



*Key words:* $Ca^{2+}$ channel; Capacitative $Ca^{2+}$ entry; *Drosophila*; PDZ domain; Phototransduction; Phospholipase C; Phosphorylation; Protein kinase C; Rhodopsin; Vision

## 1. Introduction

The existence of diverse signal transduction cascades in each eukaryotic cell demands mechanisms that limit cross-talk between different signaling pathways. For example, in neurons and in sensory cells ion channels and the intervening signal transducing machinery must be organized in a way that guarantees the specificity of cellular responses to external and internal signals. This goal may be achieved by clustering of ion channels and proteins involved in the control of channel activity to defined sites of the plasma membrane. Recently, a new family of proteins has emerged which serve as a scaffold for multimeric protein complexes by providing sites for protein-protein interaction through PDZ domains (for review see [1]). Studies directed to unravel the molecular organization of the sensory transduction pathway in photoreceptors harboring a G-protein-coupled PLCβ signaling cascade provided evidence that the spatial localization of at least one light-activated ion channel, the transient receptor potential protein (TRP), and of other signal transducing proteins is maintained by a PDZ domain-mediated assemblage of proteins into a supramolecular complex [2–4]. TRP and its invertebrate and vertebrate homologs are proposed to constitute store-operated plasma membrane channels [5–8]. The *trp* genes which have been cloned from flies (*Drosophila*, *Calliphora*) encode integral membrane proteins located in the rhabdomeral photoreceptor membrane which constitutes the light-absorbing compartment of the photoreceptor cells in the compound eyes [2,9,10]. In addition, this membrane harbors a TRP-like protein (TRPL) which displays 39% sequence identity with TRP [11] and may form heteromultimeric channels with TRP [12]. Together, TRP and TRPL give rise to the light-dependent ion current across the photoreceptive membrane [13] which is activated upon light absorption by the G protein-coupled receptor rhodopsin [14]. The PDZ domain protein INAD anchors TRP in a complex with the transmitter-generating phospholipase C and an eye-specifically expressed protein kinase C (ePKC) [2]. The *norpA* (no receptor potential A)-encoded phospholipase C is a key enzyme of the signaling cascade operating in invertebrate photoreceptors because photoreceptors of *Drosophila norpA* null mutants lack any light-triggered electrophysiological response [15]. The eye-specific protein kinase C is encoded by the *inaC* (inactivation no afterpotential C) locus [16]. *Drosophila inaC* (ePKC) mutants display a similar phenotype as *inaD* mutants which is characterized by a slow deactivation of the light-induced current [17–19]. In addition, it has been suggested that calmodulin and rhodopsin are associated with this signaling complex [3].

We have recently shown that the PDZ linker protein INAD is phosphorylated in the membrane, at least in part due to the $Ca^{2+}$- and diacylglycerol-mediated activation of ePKC [20]. This finding raised the possibility that INAD participates in the control of TRP $Ca^{2+}$ channel activity through ePKC-catalyzed phosphorylation. An analysis of INAD function using *inaD* mutants of *Drosophila*, however, suggests that INAD serves primarily as a multivalent linker protein rather than as a regulator of TRP activity [4]. Accordingly, the main target protein of ePKC still awaits identification. Protein kinase C-mediated phosphorylation of bovine brain PLCβ and of PLCβ isoforms expressed in various vertebrate cell cultures has been suggested to alter the activation of PLCβ by the respective Gq protein [21] and phosphorylation of ion channels by protein kinases C is a well known mechanism for regulating channel activity. Ion channel phosphorylation has been reported for voltage-dependent sodium and calcium channels, for calcium-dependent potassium channels, and for ligand-gated channels, e.g. nicotinic acetylcholine receptors, $GABA_A$ channels, and glutamate receptors (for a review see

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[22]). Activation of protein kinase C was also shown to regulate the activity of store-operated channels which, among others, comprise the TRP protein family [23]. However, no biochemical evidence has been provided, to date, for a phosphorylation of store-operated channels. In the present study, we have specifically investigated whether or not members of the TRP signaling complex, i.e. phospholipase C and the TRP channel, are substrates of ePKC.

## 2. Materials and methods

Adult male files (*Calliphora vicina* Meig., chalky mutant) 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 post eclosion. The larvae were reared on bovine liver to achieve a high rhodopsin content in the eyes.

A partial cDNA clone (clone D38), encoding *Calliphora* ePKC, was previously isolated by immunoscreening an oligo(dT)-primed cDNA library with antibodies directed against rhabdomeral proteins [24]. Full-length clones were isolated from the same cDNA library by hybridization screening using clone D38 as a probe. The nucleotide sequence of the longest cDNA clone from this library screen was determined for both strands by the dideoxy chain termination method [25] using templates generated by nested deletions or appropriate sequence-specific primers.

Rhabdomeral photoreceptor membranes were isolated as described previously [26] and were then washed once in 25 mM Na-phosphate buffer, pH 6.2. For immunoprecipitation, membrane proteins were extracted for 20 min at 4°C with Triton X-100 buffer (1% Triton X-100, 150 mM NaCl, 50 mM Tris-HCl pH 8.0, 1 mM phenylmethylsulfonyl fluoride, 0.42 μg/ml leupeptin, 0.83 μg/ml pepstatin, 0.83 μg/ml aprotinin). Immunoprecipitation with anti-INAD(272–542) and anti-TRP(668–1183) was carried out using protein A-agarose beads (Bio-Rad) as described in [2]. Antibodies directed against *Calliphora* ePKC were generated by immunizing a rabbit with a recombinantly expressed ePKC fragment comprising amino acids 312–685 (anti-ePKC(312–685)). Polyclonal immunoglobulins were purified from the obtained antiserum using protein A-agarose. 1 ml of the purified antiserum was covalently coupled to 1 ml of NHS-activated Sepharose (Pharmacia). 30 μl of immobilized antiserum was used for the immunoprecipitation of ePKC which was extracted from isolated photoreceptor membranes of 40 *Calliphora* retinas. Immunoprecipitations were performed for 1 h at 4°C and were followed by four washes with 500 μl of washing buffer (0.1% Triton X-100, 150 mM NaCl, 50 mM Tris-HCl pH 8.0, 1 mM phenylmethylsulfonyl fluoride, 0.42 μg/ml leupeptin, 0.83 μg/ml pepstatin, 0.83 μg/ml aprotinin). Precipitated proteins were eluted with 15 μl 1×SDS-PAGE buffer (4% SDS, 2% 2-mercaptoethanol, 2 mM $CaCl_2$, 15% glycerol in 65 mM Tris-HCl, pH 6.8) for 10 min at 80°C and were subjected to SDS-PAGE on 8% polyacrylamide gels and Western blot analysis according to standard protocols [27,28]. Western blots were probed with anti-INAD(272–542), anti-TRP(668–1183), anti-ePKC(312–685), and anti-*Drosophila* NORPA [29].

Phosphorylation assays of immunoprecipitated proteins were carried out with immunoprecipitates obtained by precipitation with anti-INAD(272–542) [2]. The precipitated proteins coupled to protein A-agarose beads were washed in 500 μl of washing buffer containing 2 mM EGTA to remove residual amounts of $Ca^{2+}$ ions. Then the precipitates were suspended in 20 μl of HEPES-buffered saline (115 mM NaCl, 2 mM $MgCl_2$, 2 mM KCl, 1 mM DTT, 0.1 mM PMSF, 10 mM HEPES pH 6.8) containing 66 μM ATP (4 μCi [$\gamma$-$^{32}$P]ATP) and either 2 mM EGTA or 125 μM EGTA/250 μM $CaCl_2$ (60 μM free $Ca^{2+}$). When indicated, the phosphorylation buffer contained, in addition, 50 ng/μl phosphatidyl serine and 1 μM phorbol 12-myristoyl 13-acetate. The phosphorylation reaction was carried out for 10 min at 25°C. Subsequently, the precipitates were washed once with washing buffer and proteins were eluted with 1×SDS-PAGE buffer as described above. For measuring the time course of TRP and INAD phosphorylation the reactions were stopped by adding 5×SDS-PAGE buffer. Incorporated radioactivity was quantified by measuring the radioactivity of excised protein bands in a scintillation counter. For determining the stoichiometry of phosphorylation the amount of INAD and TRP present in the respective protein band was calculated by laser densitometry using bovine serum albumin as a standard.

Protein phosphorylation in intact photoreceptor cells was studied as described previously [30]. Forty isolated *Calliphora* retinas were incubated in 40 μl of HEPES-buffered saline supplemented with 10 mM glucose, 60 μCi carrier-free [$^{32}$P]orthophosphate (Amersham) and either 2 mM EGTA or 125 μM EGTA/250 μM $CaCl_2$ (60 μM free $Ca^{2+}$) for 20 min at 22°C. Then the retinas were washed two times with 1 ml HEPES-buffered saline and were homogenized in HEPES-buffered saline. After removing peripheral membrane proteins by extraction with 5 M urea in 50 mM Na-phosphate buffer, pH 6.2, integral membrane proteins were extracted with Triton X-100 buffer and were subjected to immunoprecipitation with anti-TRP antibodies. Proteins were separated by SDS-PAGE on 8% polyacrylamide gels and were stained with Coomassie blue or silver nitrate. Phosphate incorporation was detected by autoradiography.

## 3. Results and discussion

An antibody generated on the basis of partial sequence information (anti-ePKC(677–685)) which was used previously to identify ePKC in the TRP signaling complex [2,20] did not precipitate the complex, contrary to antibodies directed against TRP and INAD. In order to be able to produce an ePKC antiserum suitable for immunoprecipitation we isolated cDNA clones encoding the complete ePKC. Sequencing of the gene was performed (i) in order to ensure the specificity of the transcript used for recombinant expression of ePKC and (ii) to be able to identify functionally important sites on ePKC which should be conserved between *Calliphora* and *Drosophila*. *Calliphora* cDNA clones encoding ePKC were isolated by using a partial cDNA clone obtained by immunoscreening a retinal library [24]. The longest clone obtained from that screen contained 86 bp of the 5′ untranslated region with in-frame stop codons, a 2055 bp open reading frame encoding a polypeptide of 685 amino acids (MW = 78 036), and a 214 bp 3′ untranslated region. Comparison of the amino acid sequence with *Drosophila* ePKC [16] revealed 80.4% sequence identity (Fig. 1). When compared to protein kinases C of vertebrates, both *Calliphora* ePKC and *Drosophila* ePKC displayed 48–53% amino acid sequence identity with the $Ca^{2+}$-dependent protein kinase C subtypes (PKC$\alpha$, PKC$\beta$I, PKC$\beta$II, PKC$\gamma$) but only about 20% sequence identity with $Ca^{2+}$-independent subtypes. In line with a classification among $Ca^{2+}$-dependent PKC subtypes, ePKC displays a putative $Ca^{2+}$ binding site (C2 signature) which is conserved between *Calliphora* and *Drosophila* ePKC. Other conserved features include a pseudosubstrate domain and two cysteine-rich, putative diacylglycerol binding domains in the regulatory region of ePKC as well as an ATP binding site and a catalytic core sequence in the catalytic region of this PKC (Fig. 1). Most proteins which are linked to PDZ domain proteins interact with the PDZ domain with their C-terminal region. The C-terminal tripeptide of ePKC, though not perfectly conserved between *Calliphora* (TMI) and *Drosophila* (TII), shows a sequence motif (S/TXI) which has been identified as a PDZ domain binding motif in the $K^+$ channels Kir 2.1 and Kir 2.3 [31]. Binding of the C-terminus of ePKC to INAD would also be in line with our finding showing that antibodies directed against the C-terminus of ePKC (anti-ePKC(677–685)) do not immunoprecipitate the complex. Alternatively, a conserved S/TXV motif (SQV at positions 299–301) located in the hinge region of ePKC might link ePKC to INAD.

It has previously been shown that ePKC is co-precipitated

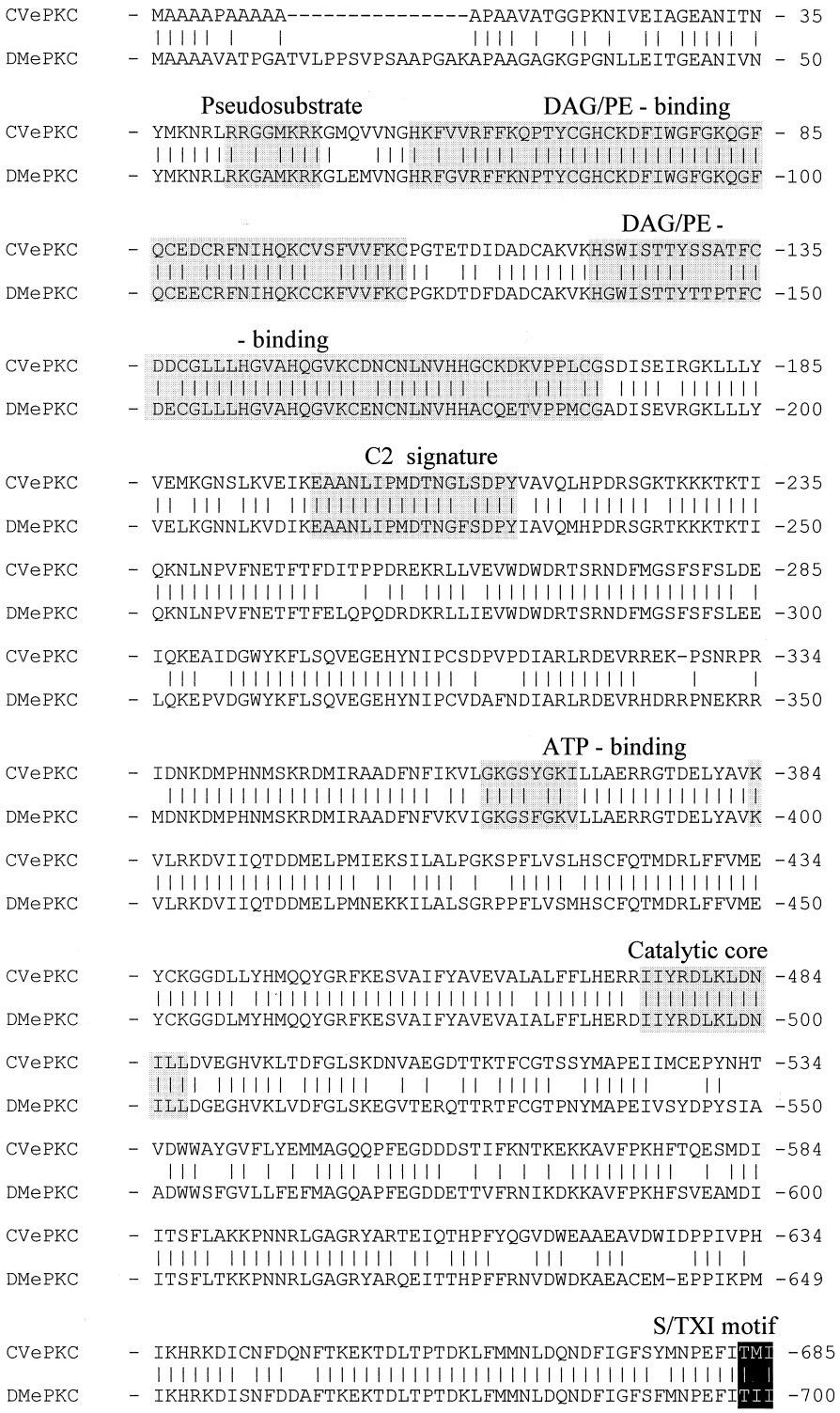


Fig. 1. Amino acid sequence alignment of eye-specific protein kinases C from *Calliphora* (CVePKC) and *Drosophila* (DMePKC). The amino acid sequences deduced from cDNA clones are shown in single letter code. Amino acid identities are indicated by vertical bars. Functional sites of ePKC are shaded in gray. Pseudosubstrate, pseudosubstrate domain; DAG/PE-binding, binding site for diacylglycerol and phorbol esters; ATP-binding, binding site for ATP; Catalytic core, catalytical core sequence. A S/TXI motif at the C-terminus is indicated by a black box.

with INAD, PLC and TRP from detergent extracts of isolated rhabdomeral photoreceptor membranes or from crude eye membranes when antibodies directed against INAD or TRP were used [2,4]. Given an interaction via a high affinity binding site such as a PDZ domain the identified members of the TRP signaling complex should also co-precipitate if the immunoprecipitation is performed with anti-ePKC. Fig. 2 shows that an antiserum generated against a recombinantly expressed fragment comprising amino acids 317–685 of ePKC co-immunoprecipitates INAD, PLC and TRP. Thus, the sig-

nal transduction complex of TRP, ePKC, and PLCβ, assembled by the PDZ linker protein INAD, is also retained if the immunoprecipitation is performed with anti-ePKC antibodies. Analysis of anti-ePKC immunoprecipitates did not reveal the presence of proteins other than the one we obtained previously under the same experimental conditions with anti-TRP and anti-INAD antibodies. Identical results were also reported with regard to the proteins co-precipitated by anti-INAD antibodies from *Drosophila* head extracts [4]. Within the complex, the linker protein INAD provides a total of five PDZ domains for interactions with other proteins. Domains 3, 4, and 5 have been identified as sites of interaction with TRP, ePKC, and PLCβ, respectively [4,32]. If additional proteins, e.g. rhodopsin or calmodulin [3], are bound to PDZ domains 1 and 2 of INAD, their binding affinity is probably too low to be unequivocally revealed by immunoprecipitation studies. In order to further elucidate the functional relevance of anchoring ePKC to the TRP signaling complex, we investigated protein phosphorylation in the isolated signaling complex. Immunoprecipitation was performed with anti-INAD antibodies and the precipitates were incubated with a phosphorylation buffer containing [γ-$^{32}$P]ATP and activators of PKC. Fig. 3 shows that INAD and, of primary significance here, also TRP become phosphorylated. The phosphorylation of INAD is in line with our previous results showing PKC-catalyzed INAD phosphorylation in rhabdomeral membranes [20]. The phosphorylation of TRP is a novel finding which suggests that the TRP family of $Ca^{2+}$ channels is affected by protein phosphorylation. In the isolated signaling complex the phosphorylation of TRP, and also of INAD, strictly depends on the presence of free $Ca^{2+}$ ions (compare Fig. 3A, lanes D′ and E′). In addition, phosphate incorporation into both proteins was enhanced in the presence of phosphatidylserine and was stimulated when the phorbolester phorbol 12-myristoyl 13-acetate was added to the reaction sample (Fig. 3A). These pharmacological characteristics are hallmarks of phosphorylation by $Ca^{2+}$-dependent subtypes of protein kinases C [33]. There is an obvious substrate specificity of ePKC towards TRP and INAD as neither PLC nor ePKC itself incorporates detectable amounts of phosphate. There is also no evidence for unspecific phosphorylation of antibodies which are present in the precipitates in significantly higher amounts than TRP. The time course of the in vitro phosphorylation of TRP and INAD (Fig. 3B) reveals that phosphate incorporation levels off after about 10 min. However, more than the half-maximal amount of phosphate is readily incorporated into TRP and INAD within 1 min. Light-dependent in vitro phosphorylation of Rh1 opsin and of arrestin 2 reaches half-maximal amounts after about 2 min and 0.5 min, respectively [20]. These findings suggest that the

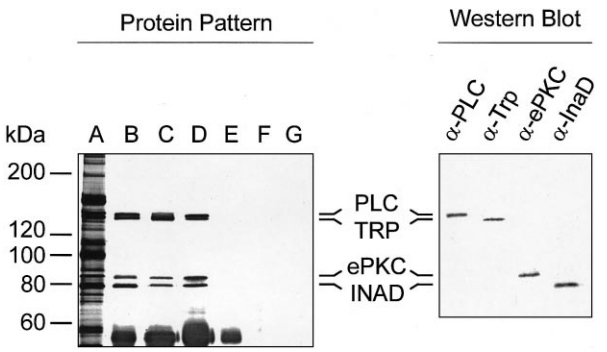


Fig. 2. Co-immunoprecipitation of PLCβ, TRP, INAD and ePKC with antibodies directed either against INAD, TRP or ePKC. Triton X-100-extracted photoreceptor membrane proteins were immunoprecipitated with antibodies directed against INAD, TRP or ePKC. Immunoprecipitations with anti-INAD and anti-TRP antibodies were carried out using protein A-agarose beads, ePKC antibodies were coupled to NHS-activated sepharose. Protein Pattern: Detergent-extracted membrane proteins (lane A), immunoprecipitates obtained with anti-INAD (lane B), anti-TRP (lane C), and anti-ePKC antibodies (lane D) were separated by SDS-PAGE and stained with silver nitrate. Lanes E, F, and G show controls: no probe was added to NHS sepharose-coupled ePKC antibodies in lane E. Sepharose (lane F) and protein A beads (lane G) without antibodies were used in order to rule out unspecific binding of proteins to the beads. Western Blot: Identification of the proteins precipitated with anti-ePKC antibodies was performed by subjecting ePKC immunoprecipitates to Western blot analysis using antibodies directed against PLCβ, TRP, ePKC, and INAD as indicated.

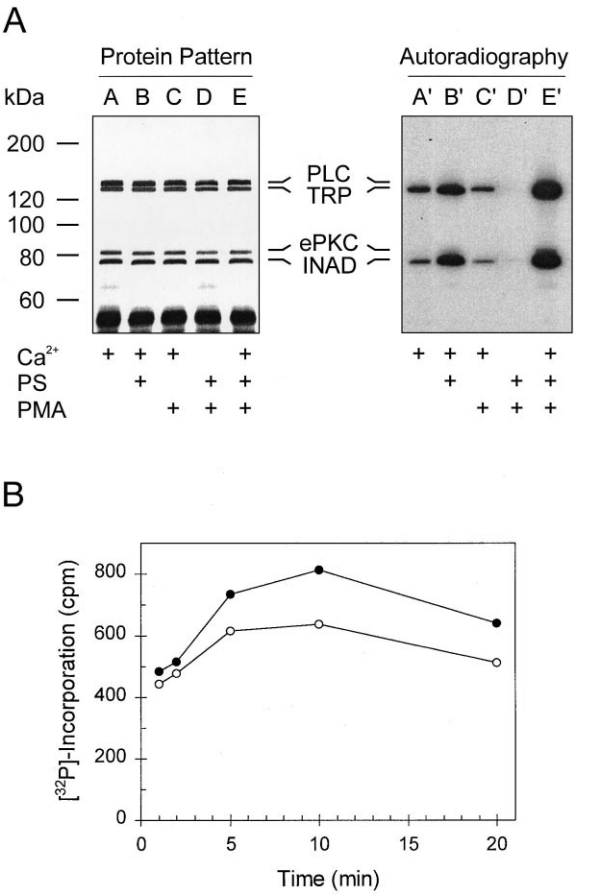


Fig. 3. Phosphorylation of TRP and INAD in precipitated TRP signaling complexes. A: TRP signaling complexes were immunoprecipitated with anti-INAD antibodies. Phosphorylation reactions were carried out in the presence or absence of $Ca^{2+}$, phosphatidylserine (PS), and phorbol 12-myristoyl 13-acetate (PMA) as indicated. Following phosphorylation the proteins were eluted from protein A beads with SDS-PAGE sample buffer and were separated by SDS-PAGE. After electrophoresis the proteins were stained with silver nitrate (Protein Pattern). Incorporation of radioactive phosphate into proteins was visualized by autoradiography. B: Time courses of TRP (●) and INAD (○) phosphorylation in immunoprecipitated TRP signaling complexes. Phosphorylation reactions containing $Ca^{2+}$, phosphatidylserine, and phorbol 12-myristoyl 13-acetate were terminated at the indicated time points by adding 5×SDS-PAGE buffer. Phosphorylated TRP and INAD were separated by SDS-PAGE and the radioactivity present in excised TRP and INAD protein bands was counted in a scintillation counter.

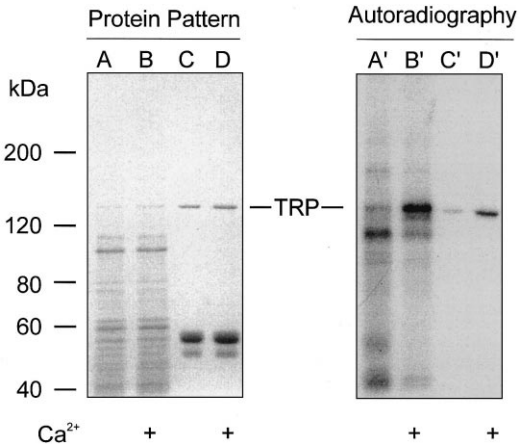


Fig. 4. Phosphorylation of TRP in intact photoreceptor cells. The phosphorylation assays were carried out in the presence or absence of $Ca^{2+}$ as described in Section 2. After removing peripheral membrane proteins including PLCβ, ePKC, and INAD with 5 M urea, integral photoreceptor membrane proteins including TRP were extracted with Triton X-100 and subjected to immunoprecipitation with anti-TRP antibodies. Detergent-extracted integral membrane proteins (lanes, A, B, A′, B′) and immunoprecipitates (lanes C, D, C′, D′) were separated by SDS-PAGE, stained with Coomassie blue (Protein Pattern) and subjected to autoradiography for detecting incorporation of radioactive phosphate.

phosphorylation of TRP and of INAD is almost as fast as the phosphorylation of arrestin 2 which has been reported to undergo the earliest light-induced phosphorylation in *Drosophila* photoreceptors in vivo [34]. The stoichiometry of phosphorylation, determined for phosphorylation in the presence of all PKC activators and $Ca^{2+}$ (Fig. 3A, lane E′) was 0.1–0.2 mol phosphate per mol of TRP. The substoichiometric phosphorylation may result from a decreased activity of ePKC in detergent micelles and absence of ePKC molecules in individual signaling complexes obtained by immunoprecipitation. The generally high abundance of ePKC in this precipitation and the $Ca^{2+}$ and phorbolester dependence of INAD and of TRP phosphorylation strongly suggest that TRP phosphorylation is catalyzed by ePKC.

Finally, we have investigated whether or not TRP is also phosphorylated in intact photoreceptor cells, i.e. in a preparation unaffected by extraction procedures which may lead to the loss of proteins interacting with PDZ domains 1 and 2 of INAD and of possible soluble regulator proteins lost during the isolation of photoreceptor membranes. Isolated *Calliphora* retinas were incubated with [$^{32}$P]orthophosphate in the presence or absence of external $Ca^{2+}$. After the phosphorylation reaction, TRP was extracted from retinal membranes and immunoprecipitated with anti-TRP antibodies. Prior to the extraction of TRP with detergent, peripheral membrane proteins, including ePKC, INAD, and PLCβ [2] were extracted from the membranes with 5 M urea such that the anti-TRP immunoprecipitate contained only TRP. Separation of integral retinal membrane proteins and immunoprecipitates by SDS-PAGE and subsequent autoradiography revealed that TRP is phosphorylated not only in the isolated signaling complex but also in intact photoreceptor cells (Fig. 4). As is the case in phosphorylation assays with isolated signaling complexes, TRP phosphorylation is enhanced in the presence of external $Ca^{2+}$, suggesting a calcium dependence of the protein kinase which phosphorylates TRP. Notably, among the integral membrane proteins with molecular masses between 40 and 200 kDa TRP is the most prominent phosphoprotein (Fig. 4, lanes A′, B′).

The existence of a PKC isoenzyme (ePKC) which is specifically expressed only in photoreceptor cells [16,17] is an indication that this enzyme serves a specific function in the visual cascade. A role in phosphorylation of the light-activated ion channel TRP clearly is in line with a function of ePKC in the phototransduction pathway. Indeed, *Drosophila* ePKC mutants show a defect in the deactivation of the light response, i.e. the receptor potential evoked by a short light pulse is prolonged as compared to wild-type flies [17]. Unitary depolarization events, known as quantum bumps which are activated by single photon absorptions and are estimated to correspond to the opening of a few hundred ion channels, display a delayed repolarization in ePKC mutants [18]. In view of this we propose that ePKC-catalyzed phosphorylation of TRP is a mechanism for TRP channel inactivation. Mutations in ePKC would then result in a prolonged opening of TRP. Such a function of ePKC would constitute a logical negative feedback loop: opening of TRP channels results in $Ca^{2+}$ influx and hence in a local elevation of the intracellular $Ca^{2+}$ concentration. The rise in intracellular $Ca^{2+}$ activates ePKC which in turn results in TRP phosphorylation and closure of the ion channel. Since electrophysiological measurements of capacitative $Ca^{2+}$ entry channels of *Xenopus* oocytes showed a biphasic effect after pharmacological activation of protein kinases C [23], it is important to note that the control mechanisms of ePKC activation also have input from the light-dependent, PLCβ-catalyzed generation of diacylglycerol. Apart from a function in modulating channel activity, TRP phosphorylation might regulate TRP binding to PDZ domain 3 of INAD as has been demonstrated for the $K^+$ channel Kir 2.3, which dissociates from a PDZ domain of the post-synaptic density protein PSD-95 upon stimulation of protein kinase A [31]. Uncoupling of TRP from INAD could in principle result in the macroscopic phenotype observed in ePKC mutant, i.e. in a seemingly delayed deactivation of the visual response. But so far, immunoprecipitation studies have not revealed any evidence for an effect of TRP phosphorylation on binding to INAD. Moreover, recording of quantum bumps reveals a difference between *inaD* and ePKC mutants. In *inaD* mutants these events are indistinguishable from quantum bumps of wild-type flies with respect to termination kinetics, but the latency of bump generation is increased [4]. In ePKC mutants the termination of quantum bumps is markedly delayed [18]. These differences suggest that the delayed repolarization observed in macroscopic recordings of both *inaD* and ePKC mutants results from separate defects at the molecular level, e.g. from a mislocalization of TRP in *inaD* mutants [4] as opposed to a defect in TRP inactivation in ePKC mutants.

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