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Effect of Rhodopsin C-terminal Peptide on Photoresponses in Functionally Intact Rod Outer Segments

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Summary

The protein-protein interactions that underlie shut-off of the light-activated rhodopsin were studied using synthetic peptides derived from C-terminal region of the rhodopsin. The photoresponses were recorded in whole-cell voltage clamp from rod outer segments (ROS) that were internally dialyzed with an intracellular solution containing the synthetic peptides. This was the first time that synthetic peptides have been used in functionally intact ROS. None of the tested peptides promoted the shut-off of the photolyzed rhodopsin (R*) by stimulating the binding of an activated arrestin to non-phosphorylated R*, contrary to what was expected from *in vitro* experiments (Puig *et al.* FEBS Lett. 362: 185–188, 1995).

Key words

Phototransduction – Photoresponse – Rhodopsin – Rod outer segment – Synthetic peptide

Abbreviations

ATP	adenosine 5'-triphosphate
cGMP	guanosine 3',5'-cyclic monophosphate
G protein	GTP binding protein
GTP	guanosine 5'-triphosphate
HEPES	N-(2-hydroxyethyl)piperazine-N'-(5-ethanesulfonic acid)
PDE	cGMP specific phosphodiesterase
R*	metarhodopsin II, an active photolyzed form of rhodopsin
ROS	rod outer segment

Introduction

Synthetic peptides have been used frequently in biochemical experiments to determine the structure of specific regions of the parent protein and to test protein-protein interactions. They have been shown to act as competitive inhibitors of protein-protein interactions by occupying a binding site on the target protein and by causing a conformational change that mimics the effect of the parent protein (Hamm and Rarick 1994). The possible use of peptides as therapeutic agents has been proposed (Martin *et al.* 1996) although nothing is known about how the peptides affect cell metabolism under physiological conditions. Some of these studies were conducted with proteins of phototransduction cascade.

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Yeagle *et al.* (1995, 1996) used synthetic peptides derived from rhodopsin cytoplasmic loops C-3 and C-4 for investigation of the high-resolution structure of these rhodopsin cytoplasmic domains. A study of Martin *et al.* (1996) provided detailed structural analysis of the requirements for Tα C-terminus - rhodopsin interaction by using Tα C-terminus derived peptides and their analogs. Konig *et al.* (1989) demonstrated by using rhodopsin loop derived peptides that transducin interacts with rhodopsin loop regions C-2, C-3 and C-4 *in vitro*. On the other hand, Osawa and Weiss (1994) using truncation and point mutations of rhodopsin demonstrated that the amino acid sequence of rhodopsin C-4 loop is not required for G protein activation or for the proper folding and expression of a functional rhodopsin molecule.

The lifetime of R* determines the gain of the first step of phototransduction cascade. The inactivation of R* is thought to be mediated by rhodopsin kinase catalyzed C-terminal phosphorylation and subsequent arrestin binding. The importance of the C-terminus for rhodopsin shut-off *in vivo* has been demonstrated by work of Chen *et al.* (1995) who studied transgenic mice expressing rhodopsin with C-terminus truncated by 15 amino acids before Ser^{334}. Rods containing truncated rhodopsin gave light responses that were prolonged and had increased sensitivity, consistent with disrupted R* shut-off by rhodopsin kinase phosphorylation.

Rhodopsin C-terminus has several potential serine/threonine phosphorylation sites. Growing evidence suggests, however, that only some of them play a major role in rhodopsin shut-off (Wilden 1995, Ohguro *et al.* 1995). Recently, Puig *et al.* (1995) showed that a synthetic fully phosphorylated heptaphosphopeptide (7P-peptide), corresponding to bovine rhodopsin C-terminal residues 330-348, triggered conformational changes in arrestin that caused arrestin binding to non-phosphorylated R* in a cell free system.

It is clear that the results from biochemical studies using synthetic peptides need to be verified by a reliable functional assay to test the physiological significance of the results. Therefore we decided to examine the protein-protein interactions necessary for R* shut-off by dialyzing functionally intact gecko ROS with synthetic peptides derived from rhodopsin C-terminus *via* whole-cell patch pipette. The peptides derived from gecko sequence were not available (McDowell, personal communication). Therefore synthetic peptides derived from bovine sequence, with the most important phosphorylation sites (Ohguro *et al.* 1995) conserved, were used.

The novel approach of using synthetic peptides directly inside the functionally intact cell can be useful in three ways. 1. The results from the whole-cell experiments with intracellular dialysis of the synthetic peptides could be compared with the results of *in vitro* biochemical experiments. 2. The whole-cell experiments can determine if the peptides could become a new tool to manipulate cellular metabolism. 3. The activity of the peptides in physiologically intact ROS could also be considered in using synthetic peptides as therapeutic agents.

Materials and Methods

Animals and cell preparation

The animals used for the experiments were nocturnal lizards (*Gekko gekko*). The lizards were dark-adapted for at least 8 hours before an experiment and all subsequent procedures were performed in dark under infrared illumination using night-vision goggles and image converters.

Quarters of the dissected retina from the posterior eyecup were stored in oxygenated physiological solution in a light-tight container. ROS suspension was prepared from one retina quarter by mechanical dissociation in a drop of gecko saline solution immediately before an electrical recording.

Optical setup and light stimuli

The ROS were visually observed using an inverted microscope (Nikon Diaphot, 40x dry Neofluar Zeiss objective, NA 0.75) and an infrared-sensitive video camera (RCA, Lancaster, PA; Unicon model TC2055UC; infrared illumination >850 nm).

The ROS were uniformly illuminated using stimulus 20 ms flashes of 514 nm nonpolarized light. Light intensity was calibrated with a Picowatt digital power meter (Newport Corp., Irvine, CA; model 835-ST-01 with 818-ST detector) positioned in the normal location of the recording chamber. The unattenuated intensity of the stimulus light source was 7.57×10^5 photons/μm^2.s.

Stimulus intensities are reported as the number of R* (a number of photolyzed rhodopsin molecules). This was calculated from the measured intensity of light and the effective collecting area for gecko ROS of 22.8 μm^2 (Sather 1988). Effective collecting area takes into account the cell size and the concentration of rhodopsin and its quantum efficiency of light absorption and provides an estimate of the number of rhodopsin molecules that will be photoisomerized in a uniformly illuminated cell.

Electrical recording system and recording procedure

The ROS suspension was transferred in the recording chamber formed by two parallel coverslips that were attached to a plastic block with a reservoir of saline solution connected by a narrow channel to the solution containing cells. A ground electrode (Ag/AgCl) was placed in the reservoir.

Whole-cell pipettes were pulled from borosilicate glass (VWR Scientific, San Francisco, CA)

and were heat polished. When standard internal solution was used, the pipette resistance was 8–12 MΩ.

The recordings were made in voltage clamp mode using either a L/M-EPC 7 amplifier (Adams & List Associates, Westbury, NY) or an Axopatch 200A amplifier (Axon Instruments, Foster City, CA). The output signal was low-pass filtered at 30 Hz.

The pipette was brought into the chamber while applying slight positive pressure and 1 mV steps to test pipette resistance. The cell was quickly attached to the pipette tip by gentle suction and lifted the standard distance from the bottom coverslip. Typical seal resistances were 5–35 GΩ. A standard holding potential of −29 mV was applied with the correction for liquid junction potential (−9 mV; Rispoli *et al.* 1993).

The whole-cell recording was established using a brief pulse of suction to break the cell membrane at the pipette tip while applying −10 mV 50 ms voltage pulses. Breakthrough was characterized by large capacitive current transients. The access resistance between pipette and cell interior was evaluated from the peak of the uncompensated transient current induced by 50 ms 10 mV voltage step. Typical values of access resistances were 25–50 MΩ.

The dark current that developed after breakthrough stabilized after several minutes of recording. The testing flash protocols were applied after 10 minutes of whole-cell recording. All experiments were done at 16–18 °C. Indec Laboratory Data Acquisition System with Fastlab software (Indec Systems, Sunnyvale, CA) controlled the light stimulus application, the amplifier output and the data acquisition.

Chemicals and solutions.

Gecko saline solution was composed of 160 mM NaCl, 3.3 mM KCl, 1.7 mM $MgSO_4$, 1 mM $CaCl_2$, 2.8 mM HEPES, 10 mM glucose, pH adjusted to 7.4 with NaOH (Rispoli *et al.* 1993). The standard internal dialysis solution contained 120 mM KAsp, 7 mM KCl, 6.05 mM $MgCl_2$, 5 mM HEPES, 5 mM ATP, 1 mM GTP, pH adjusted to 7.4 by KOH (Rispoli *et al.* 1993) and for experiments 300 μM synthetic peptides or 100 μM calmodulin were added. The synthetic peptides were provided by the laboratory of Dr. Paul Hargrave. All chemicals were purchased from Sigma (St. Louis, MO).

Statistics and data expression.

The data statistics was performed using program SigmaPlot (Jandel Scientific). The data are presented as mean ± S.E.M., n equals to the number of samples. Student's t-Test was used to determine if there was a significant difference between the means of two samples.

Flash responses are expressed as fractional suppression of the maximum light sensitive current.

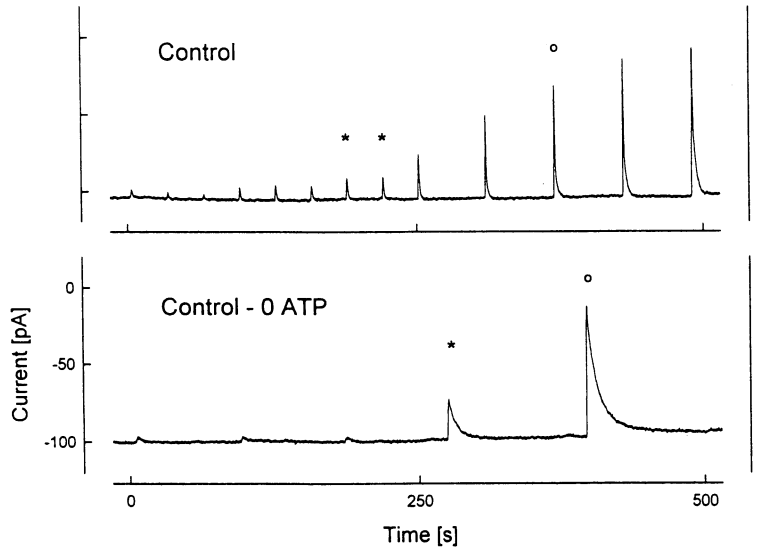

Fig. 1. *The effect of ATP removal on the flash responses. The traces compare flash response intensity series from two representative experiments in which ROS were dialyzed with standard internal solution containing 1 mM GTP and either 5 mM ATP (upper panel) or 0 ATP (lower panel). The * and o signs mark the flashes of corresponding intensities (* 40 R*/flash.ROS, o 471 R*/flash.ROS). Note the increased flash sensitivity and prolonged recovery of the responses in ROS lacking ATP.*

Results

Depleting ROS of endogenous ATP increases sensitivity and slows recovery kinetics, consistent with a reduction of R* phosphorylation (Fig. 1). Under these conditions one might expect that the 7P-peptide decreases sensitivity and accelerates response recovery by promoting arrestin binding to non-phosphorylated R*. The next step was the attempt to identify the crucial single phosphorylation sites for R* shut-off by using monophosphopeptides phosphorylated on different serine and threonine residues.

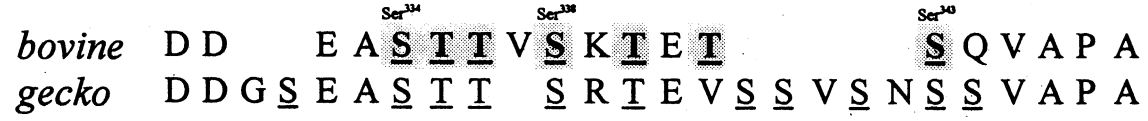

Fig. 2. *Comparison of gecko p521 and bovine opsin C-terminal regions. The comparison of the bovine and gecko sequences shows that there is 60 % homology. Note a different number of possible phosphorylation sites (7 in bovine, 11 in gecko, underlined). The bovine sequence is the 7P-peptide used for experiments. Shaded amino acids are the phosphorylation sites of 7P-peptide. The implied most important serines are pointed out. Amino acid abbreviations: A Ala, R Arg, N Asn, D Asp, C Cys, Q Gln, E Glu, G Gly, H His, I Ile, L Leu, K Lys, M Met, F Phe, P Pro, S Ser, T Thr, W Trp, Y Tyr, V Val.*

Cells were dialyzed with three peptides derived from bovine rhodopsin C-terminal residues 330–348 (Fig. 2). The first peptide was phosphorylated on all 7 serine and threonine residues (7P-peptide), the second peptide was phosphorylated on the residue Ser^{338} (MonoP-peptide) and the third peptide was not phosphorylated (NonP-peptide, control).

Flash responses were recorded in whole-cell voltage clamp from ROS internally dialyzed with standard solution containing no ATP and 300 μM of the tested peptide. The peptide concentration matched the estimated concentration of arrestin in ROS (Pfister *et al.* 1985). After 10 min of a whole-cell dialysis 20 ms test flashes of dim, moderate and bright intensity (2 R*/flash.ROS, 40 R*/ flash.ROS and 471 R*/ flash.ROS) were delivered. The traces in Figure 3 show a comparison of the normalized average responses evoked by dim, moderate and bright flashes that were recorded from ROS dialyzed with 0 ATP control solution (n=8) and of the average responses from ROS dialyzed with 0 ATP solution containing 300 μM of either 7P-peptide (n=6), MonoP-peptide (n=6) or NonP-peptide (n=6).

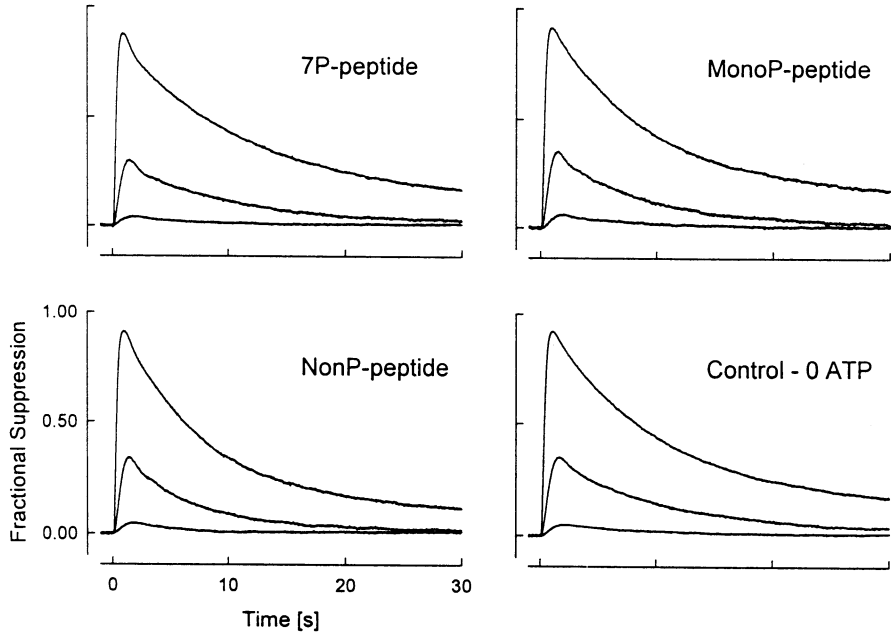

Fig. 3. *Effect of synthetic C-terminal peptides on the flash response recovery. The superimposed traces compare average responses to dim (2 R*/flash.ROS), moderate (40 R*/flash.ROS) and bright (471 R*/flash.ROS) 20 ms flashes of cells dialyzed with 0 ATP standard solution (control, n=8) and cells dialyzed with 0 ATP solution containing 300 μM of 7P-peptide (n=6), MonoP-peptide (n=6) or NonP-peptide (n=6). The responses were normalized as fractional suppression of the resting dark current. Two samples of the 7P-peptide dialyzing solution from three different experimental days were measured and the 7P-peptide content was found to be 349±7.5 μM.*

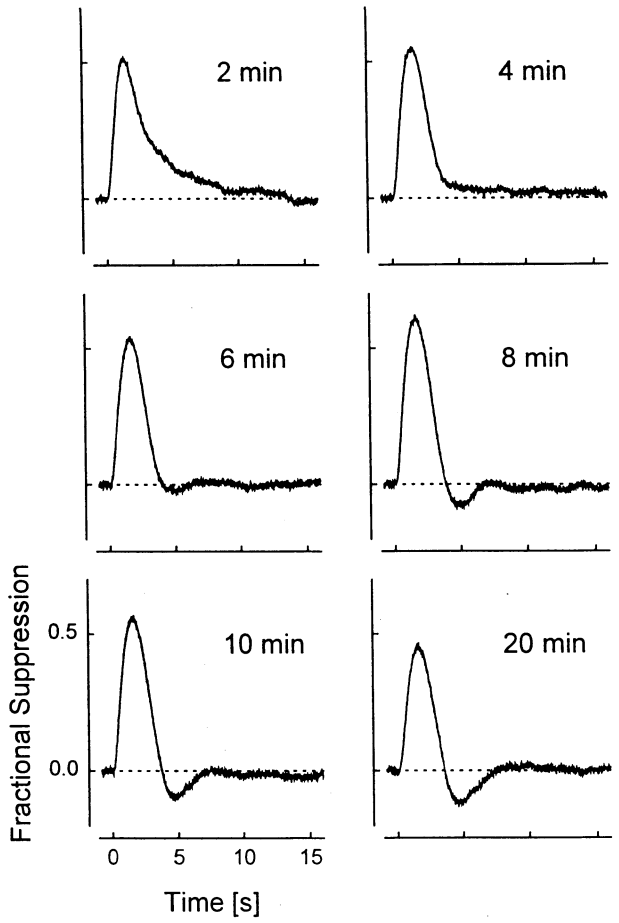

Fig. 4. *The effect of calmodulin on the photoresponse kinetics. ROS were dialyzed with standard control solution containing 100 μM calmodulin. Moderate intensity 20 ms flashes were given every 60 s. The traces show the progression of the effect of calmodulin on recovery kinetics with the characteristic undershoot appearing after 8–10 min of dialysis. The responses were normalized as fractional suppression of the resting dark current.*

The averaged resting dark current at 10 min of the dialysis was for the control 104.3±4.6 pA (n=8), for 7P-peptide 95.0±8.2 pA (n=6), for MonoP-peptide 81.0±6.0 pA (n=6) and for NonP-peptide 95.4±3.3 pA (n=6). The mean dark current values of 7P-peptide and NonP-peptide did not significantly differ from that of control at the 0.05 level as determined by independent t-Test. The MonoP-peptide and control dark currents are different at this level but the underlying mechanism for this effect is not known and it is unlikely to be connected with arrestin or rhodopsin kinase.

17 kDa protein calmodulin was dialyzed into the ROS as a control of the dialysis effectiveness (Fig. 4). Calmodulin is a calcium binding protein and causes the recovery phase of the photoresponse to undershoot (Gray-Keller *et al.* 1993). This is caused by an increased calcium buffering capacity (Torre *et al.* 1986) and is also observed in cells dialyzed with calcium chelators. The recording of a cell dialyzed with calmodulin (Fig. 4) shows that a prominent undershoot develops in the flash response after 10 min of dialysis. Similar results were observed in two other cells. The magnitude of the undershoot did not change over the next 10 min of dialysis which suggests that the calmodulin concentration inside the cell had equilibrated with its concentration in the pipette filling solution.

Discussion

The results in Figure 3 show that there is no pronounced effect of 7P-peptide, MonoP-peptide or NonP-peptide on the recovery phase of photoresponses evoked by dim, moderate or bright flashes. This suggests that, in the absence of ATP none of the peptides promoted R* shut-off by stimulating the binding of endogenous arrestin to nonphosphorylated R* as was expected on the basis of *in vitro* experiments by Puig *et al.* (1995).

It is clear that none of the three peptides had a substantial effect on photoresponse processes in ROS. An explanation for the lack of peptide effect could be that the peptides did not get inside the cell. This seems unlikely for the following reason. The experiments with exogenous calmodulin (Fig. 4) that were conducted under similar conditions showed calmodulin-dependent changes in light response. In addition, Gray-Keller *et al.* (1993) have demonstrated that under experimental conditions virtually identical with the conditions experiments 40 kDa fluorescein-labeled dextran enters ROS during whole-cell dialysis. These researchers also showed marked effects of dialyzed proteins including recoverin, visinin and gecko p26 on flash response kinetics.

An important factor to be considered is that the conformational state of the peptides under experimental conditions *in vitro* or in the dialyzing solution in our experiments is not known. The conformation of the rhodopsin loops is crucial *in vivo* since the only difference between activated and non-activated rhodopsin is the conformation of the molecule. The change of rhodopsin's cytoplasmic surface conformation upon photon absorption activates transducin.

The difference in the amino acid sequences of the C-termini of bovine and gecko opsin (Fig. 2) is another possible explanation for the lack of an effect. We have not done experiments with a phosphopeptide derived from the C-terminal region of gecko p521 (Kojima *et al.* 1992) because it was not available.

Our results with the synthetic peptides raise some questions about the validity of conclusions based

upon the use of synthetic peptides in biochemical experiments. This conclusion is supported by a report of Palczewski *et al.* (1988) who showed that synthetic peptides had nonspecific activity *in vitro*. The researchers found that 4 out of 11 tested synthetic peptides derived from rhodopsin sequence compete with rhodopsin for rhodopsin kinase, but two of these four peptide sequences corresponded to intramembrane regions of rhodopsin which do not interact with rhodopsin kinase *in vivo*.

For studying rhodopsin protein-protein interactions, other methods than the use of synthetic peptides appear to be more suitable, such as using genetically manipulated point and deletion mutant proteins for *in vitro* studies or using transgenic and knock-out mice for electrophysiological experiments.

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