Available online at www.sciencedirect.com
ScienceDirect
Neuroscience Letters 406 (2006) 6–10
Neuroscience Letters
www.elsevier.com/locate/neulet

Testing the role of calmodulin in the excitation of *Limulus* photoreceptors

Alexander V. Garger [1], Edwin A. Richard *, John E. Lisman

Department of Biology, Brandeis University, Waltham, MA 02454-9110, USA

Received 5 April 2006; received in revised form 2 June 2006; accepted 27 June 2006

Abstract

The phototransduction cascade in *Limulus* ventral photoreceptors involves multiple second messengers, including Ca^{2+} and cGMP. Light-induced Ca^{2+} release from intracellular stores is an intermediate step, but the subsequent Ca^{2+}-activated reaction remains to be determined. The possibility that Ca^{2+}/calmodulin (Ca^{2+}/CaM) might be involved is suggested by the high calmodulin content of the transducing lobe. To test whether CaM can excite the transduction cascade we injected a 25 μM Ca^{2+}/CaM solution. This produced a rapid, brief depolarization similar to that produced by light, suggesting a role for CaM in the cascade. However, an important caveat is that Ca^{2+} dissociating from the Ca^{2+}/CaM complex might excite this process. Several control experiments argue against, but do not entirely eliminate this possibility. To test whether endogenous CaM has a function in excitation, trifluoperazine was pressure injected into the rhabdomeric region. The response to brief flashes was not affected, but the response to steady illumination was transiently attenuated by each injection. We conclude that calmodulin should be considered a candidate to couple intermediate and late stages of the transduction cascade.

Keywords: *Limulus polyphemus*; Invertebrates; Phototransduction; Calmodulin

Rhodopsin molecules are coupled to cellular signaling pathways by diverse G proteins. In arthropods and cephalopods, light leads to activation of G_q-alpha and subsequent activation of phospholipase C [6,8,14,19,37]. In *Drosophila* the phospholipase C product diacylglycerol and its metabolites appear to trigger downstream transduction processes [9,12,18]. In *Limulus*, a second phospholipase C product, inositol 1,4,5-trisphosphate, triggers the release of Ca^{2+} (>150 μM) from intracellular stores [7,11,29]. Buffering the resulting rise in free Ca^{2+} blocks the light response [16,22,34], whereas injecting Ca^{2+} can mimic the light response [27,32,34]. These results indicate that elevation of cytosolic Ca^{2+} is a critical intermediate stage in the transduction cascade (but see [28]). One line of evidence supports a model in which a late stage of the transduction cascade involves guanylate cyclase [17] and the activation of the light-dependent channels by cGMP [2,15,20]. A second line of experiments suggests that diacylglycerol has a role during excitation in *Limulus* similar to that in *Drosophila*, but the analogy so far is limited [3,13].

A critical unresolved question is how light-induced Ca^{2+} elevation is coupled to activation of late stage processes. An attractive candidate, calmodulin, is enriched to high levels, estimated to be as much as 0.5 mM, in the transducing lobes of Drosophila photoreceptors [31]. The rhabdomeric lobes (R-lobe) of *Limulus* are large enough to allow a clear demonstration that this enrichment is associated with the light-transducing machinery [5]. Substantial evidence supports the conclusion that calmodulin modulates light adaptation in both organisms [1,30,32], however there have been no reports on the role of calmodulin in the excitation of *Limulus* photoreceptors.

Limulus polyphemus were obtained from the Marine Biological Laboratory (Woods Hole, MA) and maintained in aquaria at 16 °C with a 12 h light/12 h dark cycle. Ventral nerves were dissected, desheathed, and treated with a 20 mg/ml solution of Pronase dissolved in artificial sea water (ASW) for 45 s. Nerves were then left in ASW in the dark at room temperature for at least 4 h before use. Artificial seawater contains (in mM): 423 NaCl, 10 KCl, 22 $MgCl_2$, 26.2 $MgSO_4$, 10 $CaCl_2$, and 20 Tris–HCl, pH 7.8. The arrangements for observing cells with infrared illumination and stimulating with light have been described previously [17]. The intensity of the white light source was approximately 1 mW/cm^2 and was modulated by neutral density filters rated by log(10) attenuation (ND). Intensity of a 20 ms test flash was adjusted to produce a 10–20 mV depolarization (typically 5–6 ND). Intracellular recording with double-barreled theta and standard electrodes has been described previously [20]. Standard

* Corresponding author. Tel.: +1 781 736 3146; fax: +1 781 736 2398.
E-mail address: richard@brandeis.edu (E.A. Richard).
[1] Present address: New York Medical College, Valhalla, NY 10595, USA.

0304-3940/$ – see front matter © 2006 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.neulet.2006.06.056

recording electrodes contained 3 M KCl and had 10–16 MΩ resistance. Electrodes used for intracellular pressure injection and current injection, 10–20 MΩ resistance, were filled with intracellular solution (170 mM KCl, 0.001% TX-100, 10 mM HEPES, pH 7.2) with the addition of chemicals or calmodulin as indicated in the text. A pressure pulse was previously estimated to eject a volume less than 0.1% of the cell volume [34]. Calmodulin was dissolved in intracellular solution at 0.5 mM and frozen at −80 °C until use and not re-frozen. EDTA and intracellular solution was added and the final diluted solution filtered before use. Trifluoperazine (TFP) and calmodulin were obtained from Calbiochem. Di-bromo-BAPTA was obtained from Molecular Probes. Theta glass was obtained from Warner Instrument Co.

In preliminary experiments, calmodulin antagonists were tested for their effects on a test flash response in dark-adapted photoreceptors. Ca^{2+}/CaM-binding peptides were found not to be suitable for these photoreceptors as a combination of physiological and biochemical tests indicate their primary mode of action is by direct inhibition of phospholipase C activity, rather than CaM [32]. Several calmodulin antagonists were not used either because their properties as dyes would complicate use in photoreceptors or they have been suggested to be cyclic nucleotide-gated channel antagonists. Calmidazolium (1 μM), *N*-fluphenazine mustard (30 μM), phenoxybenzamine (25 μM), and TFP (60 μM) were bath applied in ASW in the dark for more than an hour without effect. In each case the solubility limit in ASW was less than that typically used to ensure complete inhibition of calmodulin. As irreversible antagonists *N*-fluphenazine mustard and phenoxybenzamine might be expected to slowly deplete calmodulin. However, useable concentrations were approximately half those used *in vivo* and the cells had to be maintained at 20 °C rather than 37 °C. These drugs have been reported to either require or be much more effective against Ca^{2+}/CaM compared to the apo-CaM. Extensive illumination was used to raise intracellular Ca^{2+} levels in the presence of TFP, *N*-fluphenazine mustard, and phenoxybenzamine. All three showed some desensitization of the test flash response after treatment (not shown); TFP showed the strongest and longest lasting effects. As our goal was to study the effects of calmodulin antagonists on excitation rather than adaptation, we concentrated on trifluoperazine and developed the tests reported here.

The light-sensitive rhabdomeric lobe of *Limulus* ventral photoreceptors can be identified in living cells and directly impaled with an electrode. Previous work has shown that the injection of $InsP_3$, Ca^{2+}, and cGMP into the R-lobe can excite the transduction process [14,20,27]. If CaM has a role in excitation, intracellular pressure injection of Ca^{2+}/CaM into the R-lobe should mimic the depolarizing effect of light. The calmodulin used was lyophilized with excess Ca^{2+} by the manufacturer. This Ca^{2+} might act to directly stimulate the cell [34] and promote clogging in electrode tips [27]. Therefore, EDTA was added to lower free Ca^{2+} in the injection solution (25 μM calmodulin, 60 μM EDTA, 170 mM KCl, 0.001% TX-100, and 10 mM HEPES, pH 7.2). Using a standard reaction scheme and binding equilibria ([21] and the product literature), the concentration of saturated calmodulin (four Ca^{2+} bound) in this solution can be estimated at 1–2 μM. The bulk of the remaining calmodulin is predicted to have two Ca^{2+} bound to the carboxy-terminal sites. The effects of 30 ms pressure pulses used to inject this solution are illustrated Fig. 1A. The injections caused a brief depolarization that resembled the response to a dim flash of light. Typically, responses were 2–5 mV in amplitude, but could be as large as 15 mV. Responses lasted 100–300 ms. Responses were reproducible from trial to trial ($N \geq 4$ in each cell). Increasing pressure pulse duration was tested in three cells from two animals using a solution of 50 μM CaM/75 μM EDTA (about 15 μM saturated calmodulin). Increasing pulse duration initially increased the response amplitude and then prolonged the response (Fig. 1B).

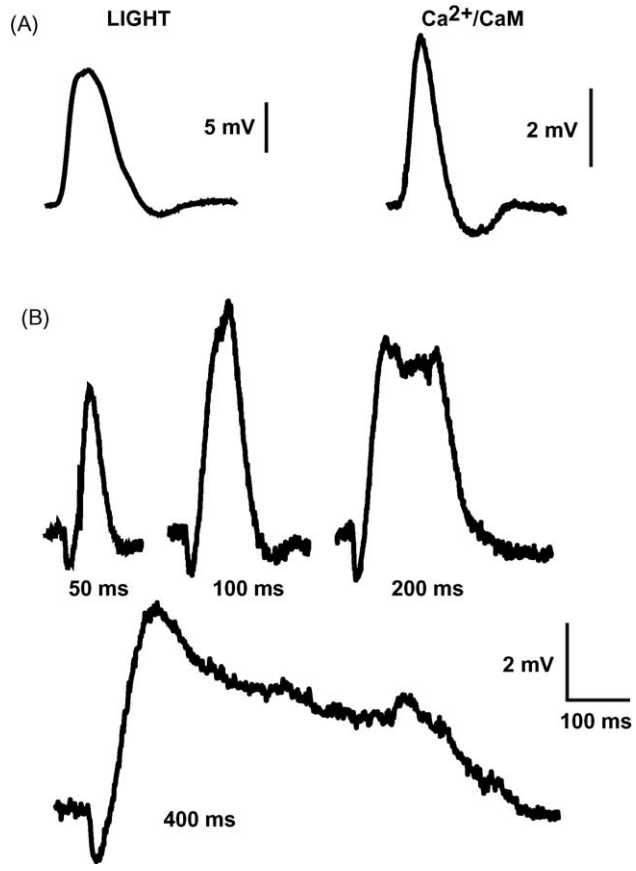

Fig. 1. CaM directly excites *Limulus* photoreceptors. (A) Averaged response to Ca^{2+}/CaM injections (right) are compared to the averaged light response (left). (B) Responses to Ca^{2+}/CaM injections became larger as the duration of injections was increased. In this cell a series of four injection pulses was used: 50, 100, 200 and 400 ms in duration. A tip potential-dependent hyperpolarization marks the onset of the pressure pulse.

We next tested whether the responsiveness to the 25 μM Ca^{2+}/calmodulin solution was specific to the R-lobe. Cells were penetrated adjacent to the R-lobe in the light-insensitive remainder of the cell, the arhabdomeric lobe (A-lobe). While the microelectrode tip remained in the A-lobe, the cell did not respond to injection. As the tip was advanced into the R-lobe, a response developed over a few microns advance. This positional dependence for excitation was documented in four cells, and is consistent with experience in positioning the microelectrode tip for other agents that excite the transduction cascade