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ORIGINAL PAPER

Cyclic nucleotide-gated channels are involved in phototransduction of dermal photoreceptors in *Lymnaea stagnalis*

Sabrina Pankey · Hiroshi Sunada · Tetsuro Horikoshi · Manabu Sakakibara

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Abstract Dermal photoreceptors in the pond snail *Lymnaea stagnalis* mediate the whole-body withdrawal response, including pneumostome closure, elicited by a shadow passing over the pneumostome area. The pneumostome closure response is part of the defense reaction in *Lymnaea*. The shadow or 'light-off' stimulus elicits activity in a higher order interneuron, RPeD11, which has a major role in mediating defensive withdrawal behavior elicited by noxious or threatening stimuli. Here, we tested our hypothesis that cyclic nucleotide-gated (CNG) channels are involved in the dermal photoreceptor-mediated transduction of the shadow stimulus. The response to the shadow stimulus recorded in RPeD11 was abolished by 500 μM *cis*-diltiazem, which blocks cGMP-activated conductance of CNG channels. On the other hand, the shadow response elicited in RPeD11 was not blocked by 2-amino ethyldiphenyl borate (2-APB), a transient receptor potential (TRP) channel blocker. Consistent with the electrophysiologic data, *cis*-diltiazem blocked the shadow-evoked withdrawal response, whereas 2-APB did not block the withdrawal response evoked by the shadow stimulus in intact freely behaving *Lymnaea*. Together, these findings support the hypothesis that the second messenger in dermal photoreceptors involves CNG and not TRP channels.

Keywords Dermal photoreceptor · Cyclic nucleotide-gated (CNG) channel · Transient receptor potential (TRP) channel · Withdrawal behavior · Right pedal dorsal 11 neuron

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S. Pankey and H. Sunada contributed equally to this work.

S. Pankey
Ecology, Evolution and Marine Biology,
University of California Santa Barbara,
Santa Barbara, CA 93106-6110, USA

H. Sunada · T. Horikoshi · M. Sakakibara
Graduate School of Bioscience, Tokai University,
Numazu, Shizuoka 410-0321, Japan

T. Horikoshi · M. Sakakibara (✉)
Laboratory of Neurobiological Engineering,
Department of Biological Science and Technology,
School of High-Technology for Human Welfare,
Tokai University, Numazu, Shizuoka 410-0321, Japan
e-mail: manabu@tokai.ac.jp; msakaki@chime.ocn.ne.jp

Introduction

The perception of light in many animals, even those lacking eyes, is mediated by a diffuse system of photoreceptive neurons, sometimes referred to as 'the dermal light sense' (Millot 1968; Steven 1963). Such nonvisual photoreception may represent an ancient mechanism predating the evolution of eyes (Fernald 2006). Indeed, nonvisual photoreceptors have been implicated in several biologic processes, ranging from predator avoidance to circadian rhythm and migration to bioluminescence regulation (Arikawa et al. 1991; Horne and Renninger 1988; Yamashita and Tateda 1986; Yoshida 1979; Young et al. 1979). The phototransduction mechanisms of ocular photoreceptors in vertebrate and invertebrates are well characterized. Ocular photoreceptor cells are subdivided into two types based on their electrophysiologic, morphologic, and molecular characteristics. One type is the vertebrate ciliary photoreceptor cell, characterized by a hyperpolarizing photoresponse involving cyclic nucleotide-gated (CNG) channels. The other type is the invertebrate rhabdomeric photoreceptor cell, characterized

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by a depolarizing photoresponse due to the generation of inositol 1,4,5-trisphosphate (IP_3), which results in the opening of a transient receptor potential (TRP) channel.

However, the transduction mechanisms of the non-ocular or dermal photoreceptors are not clear (Gomez and Nasi 2000; Hardie and Raghu 2001). In gastropod mollusks, dermal photoreceptors play a major role in escape behavior. For example, in the marine mollusk *Aplysia*, dermal photoreceptors mediate the siphon withdrawal response elicited by a 'light-on' stimulus (Lukowiak and Jacklet 1972). In another mollusk, the fresh water pond snail *Lymnaea stagnalis*, dermal photoreceptors mediate the shadow-induced withdrawal response, such as the pneumostome closure response to a shadow when animals perform aerial respiration at the water surface. It is thought that this behavior is a predator-avoidance response (Orr et al. 2007; Stoll 1972, 1973; Stoll and Bijlsma 1973). Chono et al. (2002) reported that photic stimulation of the foot (i.e., a 'light-on' response) significantly decreases extracellularly recorded afferent activity in pedal ganglion nerves innervating the foot. Based on those experiments, however, it is unclear whether locomotor activity is altered by the 'light-on' response.

CNG channels mediate the response of extraocular photoreceptors in the gastropod *Onchidium* (Gotow and Nishi 2007). Here, we attempted to characterize the phototransduction mechanisms of dermal photoreceptors in *Lymnaea*. We used specific channel blockers to determine the type of channel in the dermal photoreceptors that transduce this response and whether blockade of specific channels alters the monosynaptic electrophysiologic response in RPeD11 as well as the withdrawal response elicited by the shadow stimulus in intact freely moving *Lymnaea*. Both the electrophysiologic response in RPeD11 and the behavioral withdrawal response evoked by the shadow stimulus were abolished by selective blockade of CNG channels. Moreover, blocking the TRP channel mediated by IP_3 did not alter the behavior or the electrophysiologic response evoked by the shadow stimulus.

Materials and methods

Animals

Laboratory-reared fresh water adult pond snails, *L. stagnalis*, with shell lengths ranging from 21.0 to 26.0 mm were maintained at 20°C in well-aerated water, on a 12-h light:12-h dark cycle (lights on at 0800 hours), and fed with cabbage and goldfish/turtle pellets.

Semi-intact preparation

The circumesophageal ganglia attached to the pneumostome, mantle cavity, and internal organ without eyes or buccal ganglia (semi-intact preparation shown in Fig. 1) was dissected from *Lymnaea* with a microscissors and forceps in *Lymnaea* saline (51.3 mM NaCl, 1.7 mM KCl, 5.0 mM $MgCl_2$, 1.5 mM $CaCl_2$, and 5.0 mM HEPES, pH 7.9–8.1). Such semi-intact preparations show associative learning (McComb et al. 2005). All chemicals were purchased from Sigma-Aldrich (St Louis, MO) unless otherwise stated. The preparation was immobilized on a Silgard-coated culture plate using stainless steel pins.

Electrophysiology of RPeD11

Intracellular recordings were made from the RPeD11 with a 3 M KCl-filled glass microelectrode with input resistance ranging from 30 to 50 MΩ (measured with 3 M KCl-filled electrode). A silver chloride wire connected the glass microelectrode, fabricated with a Sutter Microelectrode Puller (P-2000, Sutter, Novato, CA) to a high-input impedance amplifier (Axoclamp 2B, Molecular Devices, Union City, CA). Voltage responses were recorded on a storage oscilloscope (511A, Tektronix, Beaverton, OR) and analyzed using a micro-computer with a Digidata 1322A board (Molecular Devices) with analysis software (p-clamp, Molecular Devices).

RPeD11 is identified by its characteristic inhibitory synaptic input to RPeD1, which is an easily identified neuron

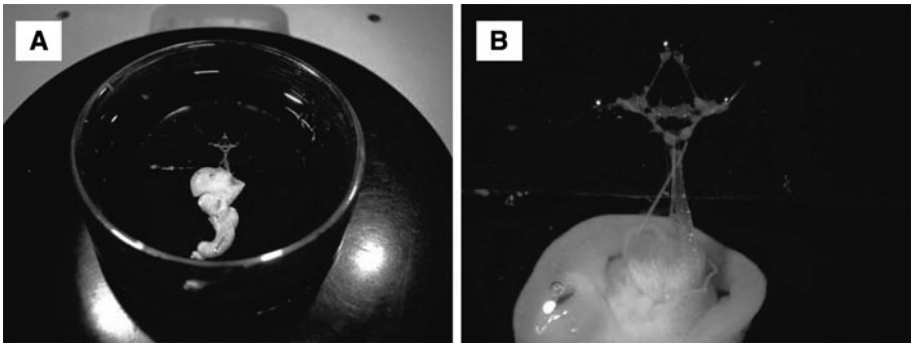

Fig. 1 The semi-intact preparation with the esophageal ganglia, mantle cavity, the skin around pneumostome, and the internal organ in the experimental dish (**a**). The esophageal ganglia and the other organs have intact neuronal connections and the border was isolated from the rest of the preparation using Vaseline gap (**b**)

involved in the mediation of aerial respiratory behaviors (Inoue et al. 2001; Sangha et al. 2003; Scheibenstock et al. 2002; Syed et al. 1990, 1992). Thus, we simultaneously recorded from RPeD1 and RPeD11. Activation of RPeD11 invariably led to an inhibitory postsynaptic potential in RPeD1.

To isolate the circumesophageal ganglia, including the right pedal ganglion where RPeD11 is located, from the pneumostome, mantle cavity, and internal organs, Vaseline was placed into the gap between the border of central ring ganglia and the other tissues, keeping neuronal connection intact. With this Vaseline gap, channel blocker agents did not make contact with the circumesophageal ganglia, including the RPeD11, where intracellular recordings were made.

Photic stimulation

The preparation was always under a light-adapted state because it was illuminated for at least 5 min before applying the shadow stimulus. The timing of a 500-ms light-off stimulus from a 50-W Halogen tungsten lamp (HL-10D, Hoya-Schott, Tokyo, Japan) was controlled using a solenoid-controlled mechanical shutter (EC-601, Copal, Tokyo, Japan) in the light path. The light stimulus illuminated the pneumostome area of the semi-intact preparation using a fiber-optic cable. The light intensity illuminating the preparation was ~700 $\mu W/cm^2$ at 510 nm, which was recorded using photopower meter (TQ8210, Advantest, Tokyo, Japan).

Behavioral observation

For the behavioral experiments in freely moving *Lymnaea*, we mainly used blinded animals to rule out the possibility that the shadow withdrawal behavior was mediated by ocular photoreceptors. In blinded animals, both eyes of the snails were enucleated with fine forceps 3 days before the behavioral experiments. Animal behavior was observed using a transparent container (10 × 10 × 10 cm, width × height × depth) made by Plexiglas filled with pond water to a depth of 8 cm. Animals were acclimatized in the container for 30 min under bright light conditions (see below) before the start of behavioral observation experiments. The output of the fiber-optic light was placed 10 cm above the surface of water, resulting in an intensity (measured using a photopower meter TQ8210) of 700 $\mu W/cm^2$ at 510 nm at the water surface (i.e., bright condition). To observe the shadow withdrawal response behavior, we presented a manually controlled shadow over an animal by moving a square black 2 × 2 cm plate just below the output of the fiber-optic light. The shadow response of an animal was evaluated as the percentage of withdrawal responses out of 20 shadow presentations (shadows were presented at 20-s intervals). We classified the shadow-evoked withdrawal behavior into three categories: local contraction-startle response (SR) with micro-motion, withdrawal till tentacle position (WTTP), and whole-body withdrawal response (WBWR). These behaviors are totally dependent on the stimulus intensity applied, or how noxious the stimulus was to the snails. In this study, the withdrawal response was defined as at least a micro-motion at presentation of the shadow. For example, if the snail withdrew in response to the shadow stimulus with at least a micro-motion 18 times out of 20 shadow presentations, it would receive a withdrawal score of 0.9.

Pharmacology

We used two different specific channel blockers in this study: L-*cis*-diltiazem hydrochloride (*cis*-diltiazem: Sigma, D2521) and 2-amino ethyldiphenyl borate (2-APB: Sigma, D9754). *cis*-Diltiazem blocks the cGMP-activated conductance of CNG channels (Stern et al. 1986) and 2-APB is a remarkably effective probe for assessing the involvement of the IP_3 receptor in intact cells, as it functions by blocking the receptor-mediated release of Ca^{2+} from Ca^{2+} stores, thus acting as a TRP channel blocker (Chorna-Ornan et al. 2001; Maruyama et al. 1997). We assessed whether either or both of these blockers altered the electrophysiologic response in RPeD11 or the behavior of the snail to the shadow stimulus. Stock solutions of blockers were diluted with ethanol, and the agents were dissolved in snail saline for the electrophysiologic experiments involving RPeD11, and in the behavioral experiments the blockers were dissolved in pond water. As both agents are membrane permeable, they did not need to be injected into the snail. Though both of these agents rapidly penetrate the signaling region inside the cell, we incubated the snails with the channel blockers for at least 10 min before starting the experiment.

Statistical analysis

Results from the behavioral experiments were assessed using a two-factor repeated measures ANOVA (sighted/blinded; control/drug; withdrawal response). A sample size of six animals was used for each of four treatments (sighted/*cis*-diltiazem, blinded/*cis*-diltiazem, sighted/2-APB, blinded/2-APB).

Results

The light-off stimulus evoked a depolarizing response (shadow response) in RPeD11 (Fig. 2). The light-off-induced depolarizing response usually has a long latency

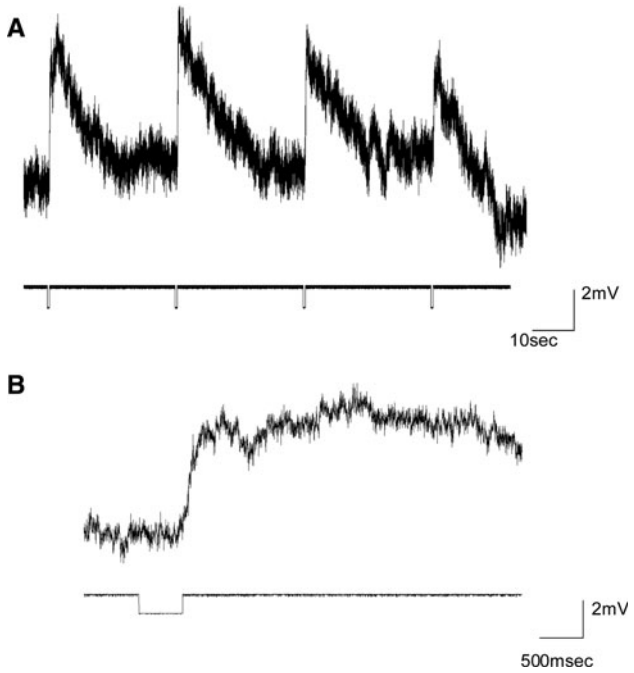

Fig. 2 Four typical successive light-off (*shadow*) responses recorded from RPeD11 (**a**). The resting membrane potential of this cell was −58 mV. The timing of the light-off signals is displayed in the *lower panel*. The first response is shown in a faster time scale in (**b**)

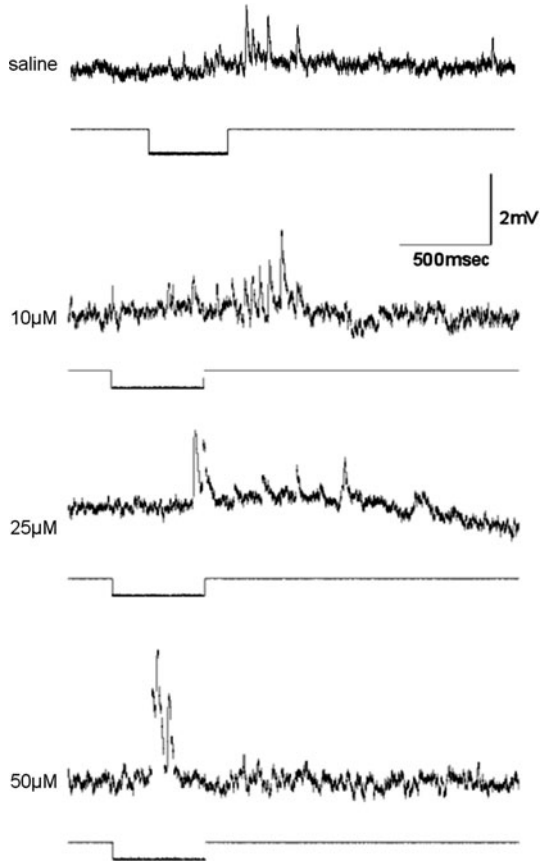

Fig. 3 The light-off response was preserved in saline containing 10, 25, or 50 μM 2-APB. Note that the membrane potential in 2-APB solution tended to become noisier compared with that in saline. The *top trace* demonstrated the control response obtained soon after 2-APB containing saline washing with normal *Lymnaea* saline

compared with the ocular photoreceptor response latency (see Table 1 in Sakakibara et al. 2005), i.e., sometimes it appeared after the cessation of the light-off stimulus and sometimes the depolarizing response was superimposed on the impulse generation.

The effect of the blockers on RPeD11 activity

We first evaluated whether 2-APB altered the shadow stimulus-evoked electrophysiologic response in the RPeD11 in the semi-intact preparation (Fig. 3). Three different concentrations of 2-APB were used: 10, 25, and 50 μM. The shadow-evoked response in the RPeD11 was not blocked by 2-APB at any of the concentrations tested; i.e., the shadow-induced responses were unaltered at all three concentrations across all six preparations (Fig. 3). Application of 2-APB, however, resulted in relatively unstable intracellular recordings, and the highest concentration used (50 μM) increased the excitability of the neuron, resulting in much noisier and sometimes spontaneous impulse generation (data not shown in the figure).

cis-Diltiazem at a concentration of 500 μM, but not 250 μM, abolished the electrophysiologic response in the RPeD11 induced by the shadow stimulus directed to the periphery (Fig. 4). The suppressive effect induced by 500 μM *cis*-diltiazem was reversible; the light-off response recovered after washing the drug out with saline. The blocking effect of *cis*-diltiazem on the shadow-induced response in the RPeD11 was observed in all six preparations tested.

Effect of the blockers on withdrawal in the behaving snail

We next tested whether either of the two compounds blocked the withdrawal response behavior of the snail to the shadow stimulus. Photographs of withdrawal behavior in 2-APB (5 μM; left panels) and *cis*-diltiazem (500 μM; right panels) are shown in Fig. 5. The upper photographs show the animal in 2-APB or *cis*-diltiazem 1 s prior to the 20th shadow presentation. Photographs in the middle panels were taken at the shadow presentation. The animal in 2-APB responded by withdrawing till tentacle, whereas the animal in *cis*-diltiazem did not show any obvious withdrawal response. The bottom photographs were taken 1 s after the 20th shadow presentation. The animal in 2-APB showed typical withdrawal behavior, but the animal in *cis*-diltiazem did not respond to the shadow stimulus. The behavior experiments are summarized in Fig. 6. Post-drug

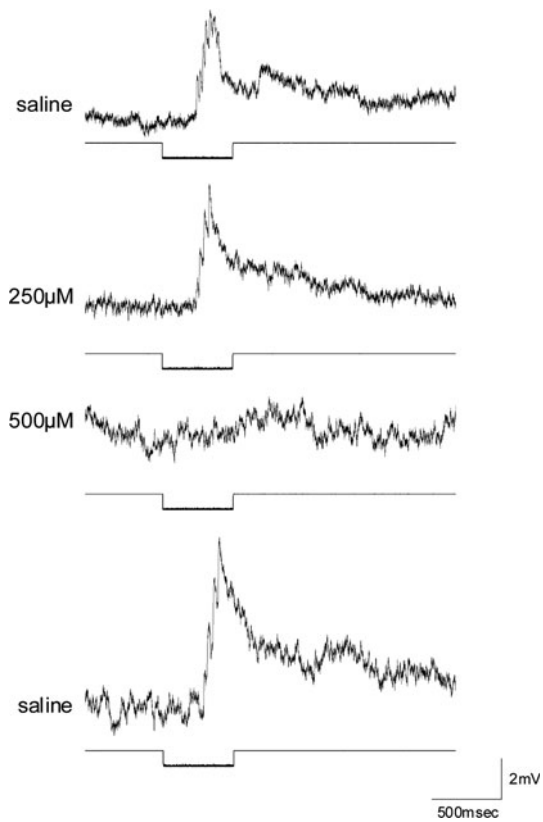

Fig. 4 The light-off responses in water containing either saline or *cis*-diltiazem at a concentration of 250 or 500 μM. The 500 μM concentration of *cis*-diltiazem abolished the light-off response and increased the noise recorded from the membrane; but the light-induced response recovered with the characteristic transient response after washing with saline. These responses are representative responses obtained from the same preparation and recorded successively

behavioral evaluations were also performed to verify animal recovery; a normal shadow-evoked response typically resumed within 2 h (data not shown).

Withdrawal behavior in response to the shadow stimulus in 24 *Lymnaea* was evaluated. The responsiveness of each of the snails to 20 shadow presentations was first evaluated prior to any pharmacologic treatment. Although mainly blinded snails were used, the responsiveness of blinded snails was compared to normal-sighted snails and the shadow-induced withdrawal behavior did not differ between blinded ($n = 6$) and sighted animals ($n = 6$) (Fig. 6). The specific drug to be tested was then dissolved in pond water and the snail was placed into it. Snails were evaluated for their responsiveness to 20 shadow presentations in water containing either 5 μM 2-APB or 500 μM *cis*-diltiazem. The shadow-induced withdrawal behavior in the presence of 2-APB did not differ significantly from that observed in normal pond water (Fig. 6). A 5 μM concentration of 2-APB had a weak effect on the shadow response, causing a 7.5% decrease in the response to the shadow (not significant). Higher concentrations of 2-APB, however, induced other behavioral abnormalities. For example, at concentrations of 10 μM 2-APB and higher in pond water, the snails showed twisting and writhing behavior. Despite this abnormal behavior, the animals continued to withdraw in response to the shadow stimulus.

The frequency of the withdrawal response was significantly lower in pond water containing *cis*-diltiazem compared to that in normal pond water (Fig. 6). The lowest concentration, 500 μM *cis*-diltiazem, had a very pronounced effect on shadow-evoked response behavior, causing at least a 60% reduction. These findings (i.e., a suppressed withdrawal response to the shadow stimulus in the presence of *cis*-diltiazem) indicate that the phototransduction mechanism in the dermal photoreceptors is disrupted by the cyclic nucleotide blocker, *cis*-diltiazem. Therefore, combined with the electrophysiologic data, these data suggest that CNG channels mediate the phototransduction process in *Lymnaea* dermal photoreceptors.

Discussion

The aim of the present study was to elucidate the mechanisms by which the dermal photoreceptors in *Lymnaea* mediate the shadow-evoked withdrawal response. Aversive stimuli (e.g., a strong tactile stimulus) or stimuli that might signal the presence of a predator (e.g., the shadow stimulus) evoke a whole animal withdrawal behavior response (i.e., the 'escape response' of the snail) or, in the case of the shadow response, portions of that behavior. One of the *Lymnaea* predators is crayfish, and the scent of crayfish effluent consolidates memory in both adult and juvenile animals. It is important to note that the escape response of the snail is not an 'all or none' behavior. Rather it is graded; i.e., less aversive stimuli only elicit portions of the escape response. The escape-withdrawal response is mediated by a network of electrically coupled neurons, chief of which is RPeD11. We, therefore, evaluated whether specific channel blockers alter: (1) the electrophysiologic response evoked by the shadow stimulus in RPeD11; and (2) suppress the escape-withdrawal behavioral response evoked by the shadow.

The shadow stimulus evokes a depolarizing response in the RPeD11 (Fig. 2), resulting in excitation of the motoneuron to induce withdrawal (Inoue et al. 1996). This electrophysiologic response mediated by the dermal photoreceptors was unaltered by 2-APB, a TRP channel blocker (Chorna-Ornan et al. 2001; Maruyama et al. 1997). In contrast, the electrophysiologic response evoked by the shadow stimulus was abolished by 500 μM *cis*-diltiazem. Based on these findings, we suggest that the dermal photoreceptors rely on cyclic nucleotides as the second messenger, rather than a mechanism involving TRP channels as observed in *Lymnaea* ocular

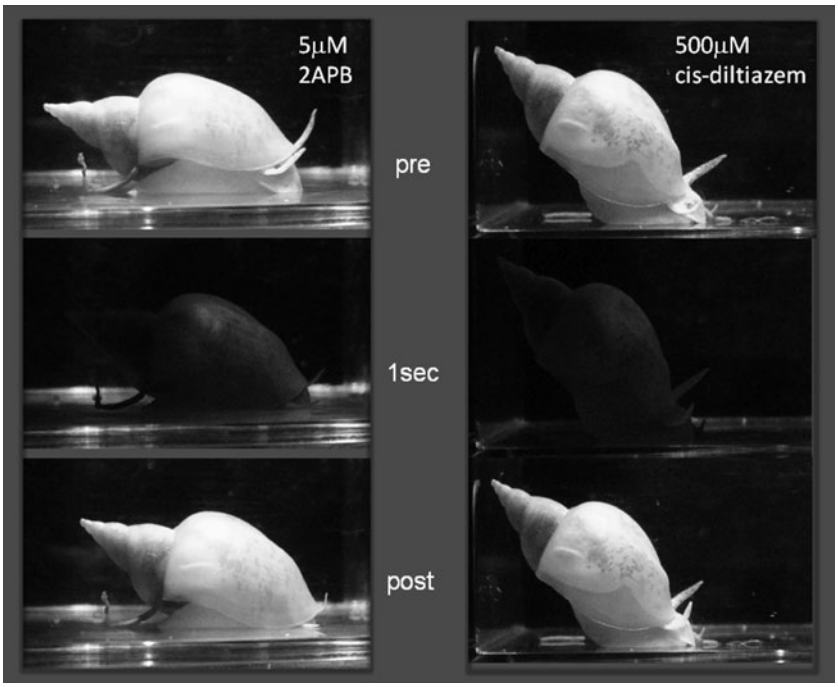

Fig. 5 Shadow-induced withdrawal behavior in water containing 5 μM 2-APB (*left*) or 500 μM *cis*-diltiazem (*right*). *Top panels* show animals moved with tentacles extended out of their shells during acclimatization (*pre*). The animal in 2-APB showed clear withdrawal behavior till tentacle position when a shadow stimulus was first presented (*middle-left panel*), whereas the animal in *cis*-diltiazem did not show the withdrawal response. Photographs were taken at 1 s after application of shadow. After the 20th presentation of the shadow stimulus, the animal in 2-APB continued to respond with withdrawal behavior, but the animal in *cis*-diltiazem did not respond to the shadow stimulus (*post*)

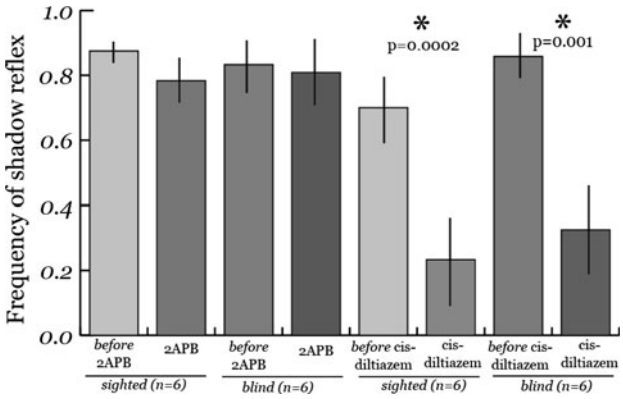

Fig. 6 Summary of responses to a shadow by blinded and sighted animals in the presence of channel blockers. Blinded and sighted animals were not significantly different from each other in their response to either drug. For both blinded and sighted animals, the presence of 2-APB did not change the frequency of shadow responses, whereas *cis*-diltiazem decreased the responsiveness to shadows by 67% in sighted and 63% in blinded animals (the difference between sighted and blinded animals was not significant). Data are presented as mean ± standard error

photoreceptors. The behavioral data obtained in freely moving *Lymnaea* support this conclusion. In both sighted and blinded *Lymnaea*, the shadow stimulus evoked the escape-withdrawal response. The escape-withdrawal response was blocked, however, by placing the snail in pond water containing *cis*-diltiazem, consistent with the electrophysiologic findings. Further, consistent with our electrophysiologic findings, 2-APB did not block the escape-withdrawal response evoked by the shadow stimulus. Thus, the electrophysiologic and behavioral data are in agreement.

We previously demonstrated that the phototransduction mechanism in the gastropod *Hermissenda*, which has rhabdomeric ocular photoreceptors, relies on an IP_3 pathway, synonymous with TRP channel involved phototransduction (Sakakibara et al. 1994, 1998). In rhabdomeric cells of ocular photoreceptors in *Lymnaea* (Sakakibara 2006), light-induced depolarization in two kinds of photoreceptors, T and A cells (Sakakibara et al. 2005), is also assumed to result from the generation of IP_3 and the opening of TRP channels. In support of this idea, intracellular recording of T photoreceptors showed that the response mediated by the ocular photoreceptors was abolished by perfusion of 2-APB (unpublished observation). The findings presented here are consistent with previous studies of extraocular photoreceptors in gastropods, such as *Onchidium* (Gotow and Nishi 2007) and some ocular photoreceptors in bivalves (Nasi et al. 2000) that have indicated the involvement of CNG channels. The findings of the present study also contribute to a growing body of evidence that CNG-type phototransducers exist outside of vertebrates (Arendt et al. 2004; Koyanagi et al. 2008; Velarde et al. 2005). Identification of opsin and G proteins outside of visual cells in invertebrates is also needed to understand if cyclic nucleotide-based phototransduction is ubiquitous and to assess the potential implications for the evolution of vision.

The withdrawal behavior in response to the shadow stimulus was only weakly affected in animals in water containing 5 μM 2-APB; however, abnormal behaviors such as twisting and writhing were observed at higher concentrations of 2-APB, but the animals still responded to the shadow stimulus with micro-motion. *C. elegans* OSM-9 is

a TRP-like channel and *Drosophila* has a TRPN channel (Lin and Corey 2005; Mutai and Heller 2003). Furthermore, recent findings indicate that even vertebrate preparations of *zebrafish* hair cells have TRPN channels that play a major role in hearing and body balance (Sidi et al. 2003). Though little is known about signal transduction in gastropod statocyst hair cells, the finding that *Lymnaea* showed abnormal writhing behavior in water containing 2-APB suggests that the TRP channels in hair cells are disrupted by this compound and further studies are required.

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