Involvement of the Ryanodine Receptor in Morphologic Modification of *Hermisenda* Type B Photoreceptors After In Vitro Conditioning

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Kawai, Ryo, Tetsuro Horikoshi, and Manabu Sakakibara. Involvement of the ryanodine receptor in morphologic modification of *Hermisenda* type B photoreceptors after in vitro conditioning. J Neurophysiol 91: 728–735, 2004. First published October 15, 2003; 10.1152/jn.00757.2003. We examined whether Ca\(^{2+}\) induced Ca\(^{2+}\) release through ryanodine receptors is involved in the conditioning of specific morphologic changes at the axon terminals of type B photoreceptors in the isolated circumesophageal ganglion of *Hermisenda*. Calcium chelation by bis(2-aminophenoxy) ethane-N,N,N',N'-tetraacetic acid prevented the conformational change at the terminals after five paired presentations of light and vibration, which produce terminal branch contraction of B photoreceptors. Two ryanodine receptor blockers, dantrolene and micromolar concentrations of ryanodine, depressed the increase in excitability due to in vitro conditioning and the increase in intracellular Ca\(^{2+}\) in response to membrane depolarization. Although the ability to increase intracellular Ca\(^{2+}\) was depressed, synaptic transmission was preserved in the normal state from hair cells under dantrolene and ryanodine incubation. Ryanodine receptor blockers also prevented contraction at the B photoreceptor axon terminals. These results suggest that the ryanodine receptor has a crucial role in inducing the in vitro conditioning specific changes both physiologically and morphologically, including “focusing” at the B photoreceptor axon terminal.

INTRODUCTION

Morphologic changes at axon terminals or dendritic spines are part of the functional remodeling of neural circuits that occurs during recovery from injury and acquisition of learning. Although recent studies demonstrate many examples of functional remodeling, little is known about the mechanisms underlying the morphologic changes, especially those related to learning and memory. Motor learning increases dendritic arborization in rat motor cortex neurons (Adkins et al. 2002) and Purkinje cell spines (Kim et al. 2002). A large number of proteins are morphogenic for dendritic spines and regulate spine formation and morphology (Sala 2002). A recent study demonstrated that the dynamin gene type 3 is a postsynaptic dynamin that has a significant role in dendritic morphogenesis and remodeling (Gray et al. 2003). In dendritic spines of rat hippocampal neurons, Ca\(^{2+}\) increase via the ryanodine receptor (RyR) is thought to be involved in the morphologic changes (Korkotian and Segal 1999). In addition, glutamate-induced massive Ca\(^{2+}\) elevation contracts the spine (Segal et al. 2000). In *Aplysia* abdominal ganglia, neurotransmitter application induces elongation of the axonal branches of some neurons and an increase in the number of varicosities (Hatada et al. 2000; Sun and Schacher 1998).

In *Hermisenda*, classical conditioning with the pairing of light (conditional stimulus, CS) with rotation (unconditional stimulus, US) modifies the innate phototactic behavior to contract the foot in response to a flash of light (Alkon 1974; Crow 1983; Farley and Alkon 1982; Lederhendler et al. 1986; Matzel et al. 1990). After acquisition of learning, physiologic modifications are observed at type B photoreceptors, such as an increase in input resistance, prolongation of neuronal excitability, and inactivation of K\(^+\) currents (Alkon 1984, 1987; Alkon and Sakakibara 1985; Farley et al. 1983; West et al. 1982). The memory of the association is stored in the type B photoreceptors, which receive monosynaptic GABAergic input from statocyst hair cells. GABA released from hair cells onto photoreceptor terminals produces a Ca\(^{2+}\) increase that propagates from the terminal branch to the photoreceptor soma (Alkon et al. 1993; Blackwell 2002; Ito et al. 1994). Serotonin and GABA have important roles in the regulation of the two Ca\(^{2+}\) current types in the B photoreceptors (Yamoah and Crow 1996, 1994). In addition to these physiologic modifications, morphologic changes are observed as a contraction of the terminal branch of the type B photoreceptors (Alkon et al. 1990). The volume of the axon terminal in the type B photoreceptors decreases by 50% 3 days after Pavlovian conditioning. The interpretation of this contraction is that it eliminates redundant synaptic contacts during long-lasting memory formation; thus this modification was termed “focusing.” Furthermore, phosphorylation of G-proteins in the B cell membrane by protein kinase C has an important role in the structural changes of the soma in addition to terminal arborization (Lederhendler et al. 1990).

In molluscan preparations, cellular phenomena induced by in vitro conditioning are well correlated with in vivo conditioning, as in *Aplysia* (Nargeot et al. 1997), *Lymnaea* (Kemenes et al. 1997), and *Hermisenda* (Farley and Alkon 1987). Among them, *Hermisenda* has an advantage for investigation of short-term plastic changes, especially for morphologic study, because of the following: 1) the morphologic changes are specific to associative (in contrast to nonassociative) learning; and 2) the conditioning specific changes are localized to identified neurons, type B photoreceptors (Alkon 1980). Previous in vitro conditioning studies revealed that a few paired presentations induce a short-term increase in excitability that lasts less than an hour, while more than five paired...
presentations of light and vestibular stimulation produce a persistent increase in the B cell excitability and morphologic modifications. No changes in the morphology of the type B photoreceptors is observed after unpaired presentation of the CS and US or after CS or US presentation alone (Kawai et al. 2002; Matzel and Rogers 1993; Ramirez et al. 1998). Further, the persistent increase in excitability at the type B cells in the isolated nervous system involves calcium-dependent protein synthesis processes (Ramirez et al. 1998) that induce protein-synthesis morphologic changes (Kawai et al. 2003). One source of Ca\(^{2+}\) is from the endoplasmic reticulum through the RyR, which functions as intracellular channels for Ca\(^{2+}\)-induced Ca\(^{2+}\) release (CICR). Because the RyR in type B cells are necessary for the cellular changes underlying associative memory storage (Blackwell and Alkon 1999), the present study examines whether CICR through the RyR is involved in modifications of the morphology at the terminal branches in type B photoreceptors.

The purpose of this study was to investigate the importance of Ca\(^{2+}\) release from intracellular Ca\(^{2+}\) stores, particularly ryanodine (Ry)-sensitive stores, for contraction of the terminal arborization of type B cells after five presentations of in vitro conditioning. Here we report that specific RyR inhibitors, dantrolene (DANT) and Ry, blocked pairing specific morphologic modifications. This finding suggests that Ca\(^{2+}\) release, particularly CICR, is required for contraction of the terminal arborization in type B photoreceptors.

**Methods**

The methods were described in detail previously (Kawai et al. 2002) and are summarized below.

**Animals**

*Hermisenda crassicornis* were obtained from Sea Life Supply (Sand City, CA). Animals were maintained in artificial sea water, “Sea Life” (Marine-Tech, Tokyo) aquaria (Aqua, Tokyo), under subdued yellow light (20 \(\mu\)W/cm\(^2\) at 500 nm) at 13\(^\circ\)C on a 12-h light:dark cycle (on at 08:00) and fed dried clams.

**Preparation**

The circumesophageal ganglion was removed in artificial sea water (ASW) (430 mM NaCl, 10 mM KCl, 50 mM MgCl\(_2\), 10 mM CaCl\(_2\), and 10 mM Tris-HCl, pH 7.4). Preparations were immobilized on a glass slide by the weight of stainless steel pins whose ends were embedded in Vaseline. The isolated circumesophageal ganglion was incubated with protease solution (Sigma type VIII, 1 mg/ml in ASW) to digest the surrounding connective tissue for 7 min at room temperature.

**Drug application**

Bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid (BAPTA: A-9801, Sigma, 10 mM in 3 M KCl) was injected intracellularly into the type B photoreceptor soma with a glass microelectrode (inner resistance 100–150 M\(\Omega\)) by 10.220.32.246 on November 2, 2016 http://jn.physiology.org/ Downloaded from

Electrophysiology

Intracellular recordings were made from lateral type B cells. Type B cells were impaled with a 3 M KCl-filled glass microelectrode with an input resistance ranging from 25 to 35 M\(\Omega\). The glass microelectrode was connected by a silver chloride wire to a high-input impedance amplifier (7110A, Pelagic Electronics, Falmouth, MA). Voltage responses were monitored with a storage oscilloscope (DCL-7020, Kenwood, Tokyo) and recorded with a PC-AT compatible personal computer via an interface board (Digiteka 1200B, Axon Instruments, Foster City, CA) controlled by pCLAMP (Axon Instruments). The slope of the input resistance was assessed from the linear regression of the J-V curve made by measuring the steady-state voltage change due to constant current injection ranging from \(-0.2\) to \(+0.2\) nA in 0.1-nA steps before and after in vitro conditioning. To estimate synaptic strength, the inhibitory postsynaptic potential (IPSP) in type B photoreceptors was monitored in response to hair cell mechanical stimulation of 33 Hz for 2 s using a polished glass probe connected to a piezo-driver (DFS-255, Dia Medical, Tokyo).

**Ca\(^{2+}\) imaging**

To estimate the effect of DANT, we measured the intensity change in the fluorescence of the Ca\(^{2+}\) indicator dye, Oregon green 488 BAPTA-1 (O-6806, Molecular Probes, Eugene, OR; 10 mM in 0.1 M KCl) at the type B cell terminal in response to depolarization of the membrane potential to 0 mV by current injection to the soma. Oregon green 488 BAPTA-1 was injected intracellularly by iontophoresis with AC pulse of 3 nA for 15 min. After dye injection, the preparation was placed in the dark for 10 min to allow for dye diffusion. The fluorescence intensity of the Ca\(^{2+}\) indicator was recorded with a confocal microscope (TCS-NT, Leica, Heerbrugg, Switzerland) as follows: 1) the membrane was hyperpolarized to \(-60\) mV; 2) 40 s later the membrane potential was depolarized to 0 mV for 5 s; and 3) the fluorescence intensity was analyzed using the software package of the Leica TCS-NT and Origin 5.0 (Microcal, Northampton, MA). Fluorescence was expressed as the fluorescence increment ratio on the average intensity measured for 10 s at the resting membrane level before depolarization.

**Staining and morphologic observation of type B cell**

To evaluate morphology, type B cells were stained with Alexa 488 (A-10436, Molecular Probes, 1% in water solution). The tip of the glass microelectrode was filled with Alexa 488; the rest was filled with 3 M KCl. The resistance of the microelectrode was approximately 30 M\(\Omega\). After penetration into the cell, dye was injected iontophotically with AC pulses (\(-0.5\) nA, duty rate, 50%, 1 Hz) for 15 min. The morphology of type B photoreceptors was observed with a confocal microscope before and after the conditioning procedure.

**In vitro conditioning**

The timing of a light flash from a tungsten halogen lump (HL-100, Hoya Schott, Tokyo) was controlled by a solenoid mechanical shutter equipped in the lamp housing and the light was guided to the preparation by a fiber optic bundle. The unattenuated light intensity at the preparation was 5.8 mW/cm\(^2\) at 500 nm. The mechanical stimulus (US) was applied to the statocyst hair cell with a polished glass probe with a concave tip, approximately 50 \(\mu\)m in diameter. This probe was connected to a piezo-driver that gave a maximal vibration of 19 \(\mu\)m of travel/stroke operated at 33 Hz.

After microelectrode penetration into type B photoreceptors, only cells in which the resting membrane potential was less than \(-40\) mV after 10 min dark adaptation were used. Following another 10-min rest, five successive stimuli of 3-s light and 2-s vibration every 2 min were presented.
Because we previously reported that pairing specific contraction of the terminal arborization in type B photoreceptors occurs exclusively in the paired CS-US group (Kawai et al. 2002), we did not compare the morphology in the present study at the B cell terminals with unpaired CS-US or other control groups. In the paired presentation of the CS and US, the 2-s vibration was initiated 1 s after initiating the 3-s light; thus the presentations of light and vibration overlapped and coterminated.

In the present study, we assessed four measures to evaluate RyR involvement in the in vitro conditioning, one morphologic and three physiologic, as follows: 1) terminal length ratio at the axonal ending of type B photoreceptors; 2) IPSP amplitude at the synapse between hair cells and B photoreceptors; 3) depolarization (0 mV) induced intracellular Ca\(^{2+}\) elevation; and 4) input resistance of the B photoreceptor.

**Image processing**

The morphology of the terminal branch in type B cells was examined using a confocal microscope. The images obtained by confocal microscopy were processed by Scion-image beta 4 (Scion, Frederick, MD). Each image scanned 256 \( \times \) 256 pixels starting from the top of the branch to the bottom in 0.49-\( \mu \)m steps along the \( z \)-axis. Each pixel was represented as a brightness index value between 0 and 255. The maximum intensity projection (MIP) was defined by the maximum brightness index along the \( z \)-axis that yielded the largest cross-sectional image of the type B photoreceptor terminals. A binary image was obtained from the MIP image using a threshold operation; the threshold was adjusted visually for each preparation and maintained constant through the series of images. Although the binary image tended to produce a larger area than the MIP image, we analyzed binary images because it was difficult to distinguish the border of fine structures of the type B photoreceptor terminals from the background in the MIP image. Terminal length is one index of morphologic change that correlates with classical conditioning (Kawai et al. 2002). The terminal length ratio was calculated from images of post- to pre-conditioning.

**Statistical analyses**

All the statistical tests were performed with Origin 5.0 (Microcal). The difference between pre- and postconditioning was tested using a two-tailed paired \( t \)-test. A two-tailed independent \( t \)-test was performed for drug application group versus the control group. Multiple comparisons of the input resistance, the maximum fluorescence change ratio, and the terminal length ratio were analyzed using one-way ANOVA.

**RESULTS**

**Effect of BAPTA on terminal morphology**

According to previous studies, application of Ca\(^{2+}\) chelators such as ethylene glycol-bis(2-aminoethylether)-N\(_2\),N\(_3\),N\(_4\),N\(_5\)-tetraacetic acid (EGTA) and/or BAPTA on *Hermissenda* type B photoreceptors inactivate the light-induced and calcium-dependent potassium currents and decrease and eventually eliminate the light-induced delayed slow components of generator potentials of type B photoreceptors (Alkon and Sakakibara 1985; Sakakibara et al. 1998). For 20 min after injection of BAPTA into type B cells, the slow components of the light response to a 1-s flash of light gradually diminished while the fast sodium component remained intact (data not shown). After confirming the effect of BAPTA injection on the photoresponse, the fluorescent dye Alexa 488 was injected into the same photoreceptor through a different electrode before in vitro conditioning. Five presentations of paired in vitro conditioning trials did not induce pairing specific morphologic changes in the BAPTA-injected type B cells. Both the preconditioning and the postconditioning MIP image of a terminal branch are shown in Fig. 1. In five of five preparations, there was no change in the terminal branch morphology. The terminal length ratio, one index of morphologic change that correlates with classical conditioning, was 1.004 \( \pm \) 0.006 (mean \( \pm \) SD, \( n = 5 \)). This implied that there was no contraction between pre- and post-conditioning in BAPTA-injected type B cells (\( t = -1.518, \ P = 0.204 \)) and indicated that BAPTA inhibited the conditioning specific morphologic change. This finding suggests that focusing at the B cell terminal after in vitro conditioning is a Ca\(^{2+}\)-dependent process. A previous study demonstrated the importance of intracellular Ca\(^{2+}\) release through the RyR for associative memory storage (Blackwell and Alkon 1999). We examined the relevance of the RyR on the conditioning induced physiologic and/or morphologic modification at the terminals of type B photoreceptors.

**Effect of DANT and Ry on physiology**

A separate group of animals were used to assess the effects of DANT and Ry on physiology. DANT specifically blocks RyR and Ry inhibits RyR at micromolar concentrations in the cytoplasm (McPherson and Campbell 1993). We examined the physiologic effects of RyR blockers on type B photoreceptors by measuring IPSP size, intracellular Ca\(^{2+}\) concentration, and input resistance.

First, we studied the effect on synaptic transmission at the inhibitory chemical synapse between the statocyst hair cell, which is one of the presynaptic elements to the B photoreceptor, and the type B photoreceptor. Periodic movement with a mechanical stimulus at 33 Hz for 2 s using a polished glass electrode applied to the statocyst induced an IPSP in ASW at...
the type B photoreceptor (Fig. 2A). There was no significant difference in the maximum amplitude of the IPSP at type B photoreceptors in DMSO containing ASW and/or DANT, Ry containing ASW (Fig. 2B and Table 1). This suggested that DANT and Ry in micromolar concentrations did not affect synaptic transmission between hair cells and type B photoreceptors. The light response from type B photoreceptors was also intact in DANT and Ry containing ASW (data not shown), as previously reported by Blackwell and Alkon (1999).

Second, we examined whether the increase in intracellular Ca\textsuperscript{2+} due to membrane depolarization to 0 mV was affected by the application of RyR antagonists. In the control group, bathing in ASW or 0.6% DMSO containing ASW, intracellular Ca\textsuperscript{2+} increased more than 50% in response to membrane depolarization, while that in DANT or Ry increased <20% (Fig. 3). This difference was statistically significant (Table 1). Incubation in DANT or Ry, which blocked the release of calcium through the RyR, significantly attenuated the increase in intracellular Ca\textsuperscript{2+} as compared with the controls (Fig. 4).

Third, we evaluated the DANT and Ry effects on the input resistance of type B photoreceptors. We previously demonstrated that an increase in the input resistance was one characteristic indicative of increased membrane excitability (Kawai et al. 2002). The input resistance was recorded before and after in vitro conditioning after incubating the preparation in either DMSO containing ASW, DANT containing ASW, or Ry containing ASW. The type B photoreceptors incubated in DMSO containing ASW had a marked increase in input resistance, as much as two times higher after five paired presentations of light and mechanical stimulation to statocyst hair cells as in normal ASW. This increase was similar to that observed previously in conditioned B photoreceptors (Blackwell and Alkon 1999; Kawai et al. 2002; Matzel and Rogers 1993). On the other hand, there was no significant change in input resistance in the DANT or Ry perfused preparations. These results clearly demonstrated that blocking calcium release through RyR prevented an increase in excitability due to conditioning of type B photoreceptors.

**Effect of DANT and Ry on morphology**

No morphologic changes were observed at the terminal branch of the type B photoreceptors in DANT or Ry containing ASW after five paired presentations of light and mechanical stimulation to statocyst hair cells (Fig. 5). The morphologic changes were observed as a contraction of the terminal branches along the centro-lateral direction of the B cell in DMSO containing ASW, as previously demonstrated (Kawai et al. 2002). This morphologic change in DMSO containing ASW was significant (Fig. 5B and Table 1). This finding indicates that RyR antagonists inhibited morphologic modification at the terminal branches of type B photoreceptors.

**DISCUSSION**

The main finding of the present study is that both calcium chelation by BAPTA and RyR blockers prevented specific conformational changes at type B photoreceptor terminals after in vitro conditioning together with physiologic correlations, even though the synaptic transmission from hair cells to type B cells was preserved.

Ry has dual effects on RyR, as follows: an agonistic action...
TABLE 1. Summary of statistical analyses of RyR blocking effects with DANT and Ry on physiology and morphology. The comparison between pre- and post-conditioning was tested by two-tailed paired t-tests. Two-tailed independent t-tests were performed to examine differences between the drug application group and control group. The ASW and DMSO containing ASW groups were combined to form the control group to compare the maximum fluorescence change ratio. Multiple comparisons of the input resistance, the maximum fluorescence change ratio, and the terminal length ratio were performed with one-way ANOVA. IPSP, inhibitory postsynaptic potential; DANT, dantrolene; Ry, ryanodine; SD, significantly different; NS, no significance.

<table>
<thead>
<tr>
<th>Measures</th>
<th>Statistics</th>
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<tbody>
<tr>
<td>1. Maximum amplitude of B cell IPSPs</td>
<td>$F_{(2,30)} = 0.299, P = 0.744, \text{NS}$</td>
</tr>
<tr>
<td>Control (16) vs. DANT (8) vs. Ry (6)</td>
<td>$t = -4.567, P = 1.509 \times 10^{-3}, \text{SD}$</td>
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<tr>
<td>Control (16) vs. DANT (8)</td>
<td>$t = -3.679, P = 0.001, \text{SD}$</td>
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<tr>
<td>Control (16) vs. Ry (6)</td>
<td>$t = 0.863, P = 0.405, \text{NS}$</td>
</tr>
<tr>
<td>2. Maximum fluorescence change ratio</td>
<td>$F_{(2,27)} = 15.219, P = 3.751 \times 10^{-3}, \text{SD}$</td>
</tr>
<tr>
<td>Control (8) vs. Ry (6)</td>
<td>$t = 0.0007, P = 0.999$</td>
</tr>
<tr>
<td>3. Input Resistance</td>
<td>$F_{(2,23)} = 0.234, P = 0.793, \text{NS}$</td>
</tr>
<tr>
<td>Control (8) vs. DANT (8) vs. Ry (10)</td>
<td>$F_{(2,23)} = 8.188, P = 0.002, \text{SD}$</td>
</tr>
<tr>
<td>Control (8) vs. DANT Post (8) vs. Ry Post (10)</td>
<td>$F_{(2,23)} = 2.016, P = 0.165, \text{NS}$</td>
</tr>
<tr>
<td>Control (8) vs. Ry Post (10)</td>
<td>$F_{(2,23)} = 2.514, P = 0.040, \text{SD}$</td>
</tr>
<tr>
<td>4. Terminal length ratio</td>
<td>$F_{(2,23)} = 5.309, P = 0.006, \text{SD}$</td>
</tr>
<tr>
<td>Control (8) vs. BAPTA (5) vs. DANT (8) vs. Ry (7)</td>
<td>$F_{(2,23)} = 3.141, P = 0.007, \text{SD}$</td>
</tr>
<tr>
<td>Control (8) vs. BAPTA (5)</td>
<td>$t = 2.267, P = 0.040, \text{SD}$</td>
</tr>
<tr>
<td>Control (8) vs. DANT (8)</td>
<td>$t = 2.487, P = 0.027, \text{SD}$</td>
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**FIG. 3.** Calcium increase in response to depolarization at the axon terminals of type B photoreceptors. Type B cells generate impulse activity and calcium increase due to membrane depolarization to 0 mV from the resting level by current injection to the soma (A). In controls, intracellular Ca$^{2+}$ increased more than 50% while that in DANT and Ry containing ASW increased <20%. Note that the Ca$^{2+}$ increase was significantly smaller in DANT and Ry compared with controls (B). **$P < 0.01$.**

**FIG. 4.** Five paired presentations of light and vibration resulted in significantly increased input resistance in type B photoreceptors, as much as 2 times higher than in the control conditions (in ASW or DMSO containing ASW). The input resistance of type B photoreceptors did not change significantly under DANT and/or Ry containing ASW.
evaluate the agonist effect on physiology and morphology because Ry is difficult to wash out completely; the effect of the compound in the cytoplasm is cumulative, and the elevated Ca\textsuperscript{2+} itself through RyR activation blocks RyR channels (Dettbarn et al. 1994). Therefore we did not examine the Ry agonistic effect.

Physiologic and morphologic plasticity has different dynamics with respect to associative memory formation; there was an increase in input resistance of the type B cells soon after the conditioning paradigm and it peaked at 10 min, while contraction of the terminal branch was initiated 5 min after conditioning and reached the final state 10 min after conditioning (Kawai et al. 2003). It is possible that BAPTA blocks the conditioning itself because it might block the light response of the calcium components instead of having direct effects on memory storage mechanisms. This is quite unlikely, however, because the effects of BAPTA on the response to light have a much slower time course (longer than 20 min) than the conditioning of specific conformational change.

Previous studies suggest that various sources of Ca\textsuperscript{2+} are involved in conditioning: influx through voltage-dependent calcium channels (Alkon and Sakakibara 1985), IP\textsubscript{3}-mediated Ca\textsuperscript{2+} elevation (Sakakibara et al. 1986b; Talk and Matzel 1996), and calcium release from intracellular stores through RyR (Blackwell and Alkon 1999; Nelson et al. 1999). The calcium channels are distributed along the length of the axons of type B photoreceptors in a higher density compared with the soma and this high Ca\textsuperscript{2+} channel density might augment the conditioning signal (Tamse and Yamoah 2002). Furthermore, voltage-dependent facilitation of the P-type calcium current in the presynaptic hair cells produces a robust change in synaptic efficacy (Tamse et al. 2003). The role of IP\textsubscript{3} in B cells is to increase the release of Ca\textsuperscript{2+} from internal stores on a slower time scale. In addition, IP\textsubscript{3} might have a role in both light adaptation and conditioning specific changes at the type B photoreceptors (Sakakibara et al. 1986b). Thus the resultant increase in intracellular Ca\textsuperscript{2+} activates phosphorylation cascades involving activation of protein kinase C and/or CaM kinase II to inactivate K\textsuperscript{+} currents and increase in membrane resistance (Alkon et al. 1986; Sakakibara et al. 1986a). Similar to this effect of IP\textsubscript{3}, release of calcium through the RyR has a role in light adaptation and conditioning specific changes in type B photoreceptors. Furthermore, GABA-mediated calcium wave propagation requires the RyR (Blackwell 2002; Trafford et al. 1995; Williams et al. 1992). Thus the calcium wave that propagates from the terminal branches at the synapses of statocyst hair cells to the somata of the B cells (Ito et al. 1994) is probably prevented by DANT and Ry.

Prior work demonstrated the role of calcium in classical conditioning. The pairing specific increase in input resistance is blocked when type B photoreceptors are injected with EGTA prior to pairing, suggesting that this increase is Ca\textsuperscript{2+} depen-
dent. Moreover, because injection of BAPTA into B cells prior to conditioning blocks long-term enhancement induced by paired light and serotonin stimuli, a Ca$^{2+}$-dependent process for associative conditioning is clearly involved (Falk-Vairant and Crow 1992). Our experiments extend this finding and demonstrate that the morphologic changes also require calcium elevation through RyRs.

Recent studies demonstrated rapid long-term potentiation induced morphologic changes at the spines of rat hippocampal neurons (Engert and Bonhoeffer 1999). In other studies on rat hippocampal neurons, contrasting morphologic changes were reported: application of caffeine resulted in elongation (Korkotian and Segal 1999), while application of glutamate resulted in shrinkage (Segal et al. 2000). Further, changes in spine shape are a dynamic process that regulates synaptic function and changes in intracellular calcium are crucial for spine morphology (Segal 2001, 2002).

To change cellular morphology, the cytoskeleton would have to be affected by some intracellular molecular complex. Culture studies indicate that actin polymerization has a role in changing cellular shape (Hatada et al. 2000; Korkotian and Segal 1999). Fibroblast and neuronal culture studies suggest that one of the G-proteins (Rho-GTPases) contributes to reorganize cell shape, especially on cytoskeletal components (Hall 1998; Luo 2000; Maekawa et al. 1999). It was recently reported that actin-related protein is associated with intermediate memory in *Hermissenda* (Crow and Xue-Bian 2002). Calexcitin, extracted from the conditioned *Hermissenda* type B photoreceptors, is a potent signaling protein that binds calcium and GTP, inhibits potassium channels due to phosphorylation by PKC, and enhances membrane excitability (Nelson et al. 1996). Thus activation of the conditioning specific calcium sensor G-proteins, calexcitin, might contribute to contraction at the B cell terminal branches.

**Conclusion**

This is the first study to demonstrate that neuronal conformational changes induced by in vitro conditioning at an identified neuron, type B photoreceptors in *Hermissenda*, is mediated by an intracellular Ca$^{2+}$ rise via RyR. We demonstrate that neither conditioning specific morphologic changes at the terminals of the type B photoreceptors nor increases in neuronal excitability are observed in the presence of the Ca$^{2+}$ chelator, BAPTA, or the RyR antagonists, DANT, and micromolar concentration of Ry. On the other hand, when the nervous system is incubated in the control solution, either ASW or DMSO containing ASW, five paired presentations of light and mechanical stimulation to the statocyst hair cell result in both physiologic and morphologic modifications in type B photoreceptors of *Hermissenda*. These findings suggest that CICR through RyR has a crucial role in the formation of long-lasting modifications after associative learning in *Hermissenda*.

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