Inhibitory Effect of Somatostatin-14 on L-Type Voltage-Gated Calcium Channels in Cultured Cone Photoreceptors Requires Intracellular Calcium

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Jian K, Barhoumi R, Ko ML, Ko GY. Inhibitory effect of somatostatin-14 on L-type voltage-gated calcium channels in cultured cone photoreceptors requires intracellular calcium. J Neurophysiol 102: 1801–1810, 2009. First published July 15, 2009; doi:10.1152/jn.00354.2009. The inhibitory effects of somatostatin have been well documented for many physiological processes. The action of somatostatin is through G-protein-coupled receptor-mediated second-messenger signaling, which in turn affects other downstream targets including ion channels. In the retina, somatostatin is released from a specific class of amacrine cells. Here we report that there was a circadian phase-dependent effect of somatostatin-14 (SS14) on the L-type voltage-gated calcium channels (L-VGCCs) in cultured chicken cone photoreceptors, and our study reveals that this process is dependent on intracellular calcium stores. Application of 500 nM SS14 for 2 h caused a decrease in L-VGCC currents only during the subjective night but not the subjective day. We then explored the cellular mechanisms underlying the circadian phase-dependent effect of SS14. The inhibitory effect of SS14 on L-VGCCs was mediated through the pertussis-toxin-sensitive G-protein-dependent somatostatin receptor 2 (sst2). Activation of sst2 by SS14 further activated downstream signaling involving phospholipase C and intracellular calcium stores. Mobilization of intracellular Ca2+ was required for somatostatin induced inhibition of photoreceptor L-VGCCs, suggesting that somatostatin plays an important role in the modulation of photoreceptor physiology.

INTRODUCTION

Somatostatin (SS), also known as somatotropin release-inhibiting factor, is a peptide hormone originally found in the hypothalamus that inhibits the release of growth hormone from the pituitary gland (Brazeau et al. 1973). SS is well known for its broad inhibitory effects in many physiological processes such as in secretion and cell proliferation and is present in the endocrine, gastrointestinal, immune, and nervous systems (Weckbecker et al. 2003). In the CNS, SS serves as an inhibitory neuromodulator, which has made it a potential therapeutic target to control hyperexcitability in epilepsy and other neuronal diseases (Weckbecker et al. 2003). In the retina, SS has neuroprotective properties against ischemia and diabetic retinopathy (Cervia et al. 2008a; Thermos 2003). Nevertheless, the function of SS in the retina largely remains unknown, and the molecular mechanisms underlying the effects of somatostatin in the retina are unidentified.

SS is synthesized and released from a subpopulation of amacrine cells known as enkephalin-, neurotensin-, and SS-like immunoreactive (ENSIL) cells in the chicken retina (Yang et al. 1997). There are two biologically active forms of SS, somatostatin-14 (SS14) and the N-terminal extended somatostatin-28 (SS28), and both forms are present in the retina (Ishimoto et al. 1986; Yang et al. 1997). While there are five classes of SS receptors (sst1-5; Hoyer et al. 1995), only four of these, sst2-5, are present in the chicken retina with sst2 as the most abundant (Chen et al. 2007). Both SS14 and SS28 bind to all SS receptor subtypes with varying affinities (Dryer et al. 1991; Rohrer et al. 1998). Even though SS has broad inhibitory effects in the nervous system, its actions in the retina appear to be more complex. SS enhances light-evoked activity of retinal ganglion cells (Adolph 1989; Zalutsky and Miller 1990), modulates ion channels in photoreceptors (Akopian et al. 2000; Chen et al. 2007), and affects neurotransmission in the retina that lead to changes in electroretinogram amplitudes (Cervia et al. 2008b; Dal Monte et al. 2003; Kouvidi et al. 2006; Petrucci et al. 2001; Zalutsky and Miller 1990).

Photoreceptors are nonspiking neurons, and the continuous release of glutamate in the dark is a result of depolarization-evoked activation of L-type voltage-gated calcium channels (L-VGCCs) (Barnes and Kelly 2002). There is a circadian regulation of L-VGCCs in bipolar cells (Hull et al. 2006) and cone photoreceptors (Ko et al. 2007, 2009), in which the L-VGCC current amplitudes and densities are greater at night. Somatostatin decreases L-VGCC currents in various neuronal tissues, including the pituitary gland (Chen et al. 1990), ciliary ganglion (Dryer et al. 1991), and cerebral cortex (Wang et al. 1990). In rod bipolar cell terminals, SS inhibits calcium influx through L-VGCCs (Johnson et al. 2001). Interestingly, the content of SS is under circadian control in the rodent retina (Peinado et al. 1990), and this rhythm correlates with the activities of ENSIL cells with a high sustained rate of activity in the dark and a low sustained rate of activity in the light (Morgan et al. 1994). Hence we postulated that there could be a circadian phase-dependent effect of SS in the modulation of L-VGCCs in retinal photoreceptors. In the present study, we examined the circadian phase-dependent effect of SS on L-VGCCs in cultured cone photoreceptors. Because SS14 and SS28 have different effects in the retina (Chen et al. 2007), we focused on the actions of SS14 in this study. We found that SS14 decreased the L-VGCC current density only during the subjective night, and the effect of SS14 on L-VGCCs was mediated through the pertussis toxin (PTX)-sensitive G-protein-coupled receptor sst2. Specifically, the action of SS on L-VGCCs required the activation of phospholipase C (PLC) and the mobilization of intracellular Ca2+. 
METHODS

Cell cultures and circadian entrainment

Fertilized eggs (Gallus gallus) were obtained from the Poultry Science Department, Texas A&M University (College Station, TX). Chicken retinas were dissociated at embryonic day 12 (E12) and cultured for 6–7 days as described previously (Ko et al. 2007). Cultures were prepared in the presence of 40 ng/ml cilostino- trophic factor (R&D Systems, Minneapolis, MN) and 10% heat-inactivated horse serum. Cell culture incubators (maintained at 39°C and 5% CO₂) were equipped with lights and timers, which allowed for the entrainment of retinal circadian oscillators to 12 h:12 h light-dark (LD) cycles in vitro. Zeitgeber time (ZT 0) zero was designated as the time when the lights turned on, and ZT 12 was the time when the lights went off. The following experiments were performed on the second day of constant darkness (DD), after 6 days of prior entrainment to LD cycles.

Electrophysiology

Electrophysiological recordings were performed at circadian time (CT) 4–7 or CT 16–19 on the second day of DD. Whole cell patch-clamp configuration of L-VGCC current recordings were carried out in an external solution containing the following (in mM): 110 NaCl, 10 BaCl₂, 0.4 MgCl₂, 5.3 KCl, 20 TEACl, 10 HEPES, and 5.6 glucose, pH 7.4 with NaOH. The pipette solution was (in mM) 135 Cs acetate, 10 CsCl, 2 MgCl₂, 0.1 CaCl₂, 1.1 EGTA, and 10 HEPES, pH 7.4 adjusted with CsOH. Both perforated and mechanically captured patches were used in this study. For perforated patches, beta-escin was prepared as a 25 mmol/l stock solution in water and added to the pipette solution to yield a final concentration of 25 µmol/l. Recordings were made only from cells with elongated cell bodies with one or more prominent oil droplets because these are the hallmarks of avian cone photoreceptors (Adler et al. 1984; Gleason et al. 1992; Goldsmith et al. 1984; Hart et al. 2006; Johnston and Hudson 1976; Lopez et al. 2005). Currents were recorded at room temperature (23°C) using an Axopatch 200B amplifier (Axon Instruments/Molecular Devices, Union City, CA). Signals were low-pass filtered at 2 kHz and digitized at 5 kHz with Digidata 1440A interface and pCLAMP 10.0 software (Axon Instruments). Currents were leak subtracted. After Gigaohm seals formed, the electrode capacitance was compensated. Current–voltage (I–V) relations were elicited from a holding potential of −65 mV in 200-ms steps (5 steps between steps) to test potentials over a range of −20 to +60 mV in 10 mV increments. The maximum currents were obtained when the steps depolarized to 0 −10 mV. The membrane capacitance, series resistance, and input resistance of the recorded photoreceptors were measured by applying a 5 mV (100 ms) depolarizing voltage step from a holding potential of −65 mV. Cells with an input resistance <1 GΩ were discarded. The membrane capacitance reading was used as the value for whole cell capacitance. The current densities (pA/pF) were obtained by dividing the current amplitudes by the membrane capacitances. To achieve better controls, we always compared groups by alternating recordings among the same set of cultures during the same period.

Immunoblot analysis

Chicken embryos from E12 were entrained in LD cycles for 7 days in ovo. On the last day, retinas were dissected, dissociated, and cultured in DD for 2 day. On the second day of DD, cultured cells were treated with either vehicle (control) or SS14 (500 nM) for 2 h at CT 3 and 15. At CT 5 and CT 17, cells were harvested, washed in ice-cold PBS, and lysed in RIPA buffer, and samples were collected and prepared as described previously (Ko et al. 2007, 2009). Samples were separated on 10% SDS-PAGE gels and transferred to nitrocellulose membranes. The primary antibodies used in this study were anti-VGCCα1D (Alomone, Jerusalem, Israel) and a polyclonal antibody insensitive to the phosphorylation state of Erk (total Erk, used for internal control and loading control, Santa Cruz Biochemicals, Santa Cruz, CA). Blots were visualized using appropriate secondary antibodies conjugated to horseradish peroxidase (Cell Signaling Technology, Danvers, MA) and an ECL detection system (Pierce, Rock- ford, IL). All measurements were repeated five to six times.

Calcium imaging

Following culture of dissociated E12 retinal cells on coverslip chambered slides for 24 h under 12:12 h LD cycles, cells from the dark phase were loaded for 1 h with 3.0 µM Fluor-4 AM at 37°C in serum and phenol red-free medium and then washed with the same medium prior to measurement of cytosolic Ca²⁺. A Stallion Imaging workstation, equipped with a Zeiss Axiovert 200M microscope (Carl Zeiss Microimaging, Thornwood, NY) and slidebook software (Intelligent Imaging Innovation, Denver, CO), was used with a ×63 water objective 1.2 NA for acquiring images. Cellular Ca²⁺ levels were visualized with the cell-permanent probe Fluor-4 AM (Gee et al. 2000). Fluor-4 AM fluorescence was generated in the cells by argon laser excitation at 488 nm and fluorescence emission was monitored at 530 nm. Selected areas were scanned 5 times at 10 s intervals to establish basal calcium levels, and scanning continued for ~650 s. Thapsigargin was then added and scanning continued for another 600 s. At least two to five cells per field were analyzed. Two experiments were performed on different days.

Chemicals and statistical analysis

SS14 was obtained from American Peptide (Sunnyvale, CA) and Sigma-Aldrich (St. Louis, MO). MK-678 was from Tocris (Ellisville, MO). Cyanamid-154806 was from Sigma-Aldrich. PTX and BAPTA-AM were from Calbiochem (Gibbstown, NJ). ET-18-OCH₃ was from A.G. Scientific (San Diego, CA). Thapsigargin and Fluor-4 AM were from Invitrogen (Carlsbad, CA).

All of the data were presented as means ± SE. Student’s t-test or one-way ANOVA followed by Tukey’s post hoc test for unbalanced n was used for statistical analysis. Throughout, *P < 0.05 was regarded as significant.

RESULTS

SS14 inhibits L-type VGCCs during the subjective night through sst2 receptors

Chicken retinal cells were cultured from E12 and entrained to 12:12 h light-dark (LD) cycles for 5–6 days in vitro and then kept in constant darkness (DD) for 2 days. On the second day of DD, whole cell patch recordings were performed from cone photoreceptors during the subjective day at CT 4–7 or the subjective night (CT 16–19). Over 95% of cone photoreceptors express L-type VGCCs at this embryonic stage (equivalent of E18-E19) (Gleason et al. 1992). As described previously, there is a circadian regulation of L-VGCCs in chicken cone photoreceptors (Ko et al. 2007, 2009), and the maximal L-VGCC current density elicited at a voltage between 0 and 10 mV is significantly larger (*P < 0.05) when cells are recorded during the subjective night than during the subjective day (Fig. 1, A–F) (Ko et al. 2007, 2009). The circadian rhythm of L-VGCC current density is in part attributed to the circadian regulation of mRNA levels and protein expression of the L-VGCCα1D subunit, which peaks during the subjective night (at CT 16–17), as well as channel protein trafficking and membrane retention (Ko et al. 2007, 2009; Shi et al. 2009).
We observed that SS14 evoked a phase-dependent modulation of L-VGCCs in chicken cone photoreceptors. Application of SS14 (500 nM) for 2 h or 15 min prior to patch recordings caused a significant decrease in L-VGCC current density during the subjective night (CT 16–19; Fig. 1, C–F) but not during the subjective day (CT 4–7; Fig. 1, A and B). The 2-h treatment also decreased retinal L-VGCCα1D subunits during the subjective night (Fig. 1G). Even though our Western blot data showed a slight increase in L-VGCCα1D expression during the day after a 2 h treatment with SS14, we did not observe a significant increase in L-VGCC current density following this treatment during the day. Treatment with SS14 for 2 h did not shift the voltage-current relationship because the maximal currents were still elicited at 0–10 mV (Fig. 1, C and D). Hence our data suggested that there was a circadian phase-dependent effect of SS14 in which SS14 decreased L-VGCC activity only during the subjective night through a significant reduction of L-VGCCα1D subunits. Because chicken retinas express SS receptors 2–5 (sst 2–5) by embryonic day 11 with the sst2 receptor as the most abundant (Chen et al. 2007), we next examined whether the inhibitory effect of SS14 on L-VGCCs was mediated through sst2.

A 2-h treatment with MK-678 (100 nM), an sst2 preferred agonist, evoked a phase-dependent modulation of L-VGCCs (Fig. 2) and mimicked the effect of SS14 (SS14 for 2 h) groups show currents elicited from −65 to 0 mV. The maximum currents were obtained when the steps depolarized to 0–10 mV.

### Table 1

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Maximum Current (pA/pF)</th>
<th>n</th>
<th>P</th>
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<tr>
<td>Control CT 16–19</td>
<td>5.0 ± 0.2</td>
<td>24</td>
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<tr>
<td>SS14 for 15 min</td>
<td>2.6 ± 0.4</td>
<td>24</td>
<td></td>
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<tr>
<td>SS14 for 2 h</td>
<td>2.0 ± 0.3</td>
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* P < 0.05

**FIG. 1.** There is a circadian phase-dependent modulation of cone L-type voltage-gated calcium channels (L-VGCCs) by somatostatin-14 (SS14). The current density-voltage (I-V) relationship was obtained from whole cell recordings on the 2nd day of constant darkness (DD). A: the L-VGCC currents were larger during the subjective night [circadian time (CT) 16–19; solid square] than during the subjective day (CT 4–7; solid circle). B: treatment with SS14 (500 nM) for 2 h prior to recordings (○) did not have significant effects on the current density during the day. C: treatment with SS14 for 2 h (●) significantly decreased the L-VGCC current density during the night compared with the control (●). D: treatment with SS14 for 15 min prior to recordings (◇) significantly inhibited the L-VGCCs during the subjective night. E: 2 representative traces recorded during the subjective night from the control (con) and SS14-treated (SS14 2h) groups show currents elicited from −65 to 0 mV. The maximum currents were obtained when the steps depolarized to 0–10 mV. F: treatment with SS14 for 2 h or 15 min prior to recordings significantly decreased the maximum L-VGCC current density (Imax) elicited from −65 to 0 mV during the subjective night. The maximum current density of voltage step to 0 mV: control CT 4–7 (con CT 4–7): 5.0 ± 0.7 pA/pF, n = 24; control CT 16–19 (con CT 16–19): 7.4 ± 0.9 pA/pF, n = 21; treatment with SS14 for 2 h (SS14 for 2 h) CT 4–7: 5.5 ± 0.6 pA/pF, n = 26; SS14 for 2 h CT 16–19: 4.2 ± 0.8 pA/pF, n = 26; treatment for 15 min control (con) CT 16–19: 7.9 ± 0.8 pA/pF, n = 14; treatment with SS14 for 15 min (SS14 for 15 min) CT 16–19: 5.2 ± 0.7 pA/pF, n = 15, * P < 0.05. G: the protein levels of L-VGCCα1D in cultures harvested during the subjective night (CT 17) were significantly higher than during the subjective day (CT 5) in controls. Treatment with SS14 (500 nM) for 2 h significantly decreased the L-VGCCα1D during the subjective night, *, P < 0.05.
the inhibitory effect of SS14 on L-VGCCs during the subjective night was blocked (Fig. 3, B and C), while cyanamid-154806 itself did not affect L-VGCC current density (Fig. 3, A and C). Thus our data indicated that the inhibitory effect of SS14 on L-VGCCs during the subjective night was mediated through sst2 receptors. While somatostatin receptors can couple with both PTX-sensitive and –insensitive G proteins (Weckbecker et al. 2003), it was not clear whether the SS14 effect we observed was mediated through a PTX-sensitive G protein. We found that treatment with PTX (200 ng/ml) for 20–24 h prior to recordings blocked the inhibitory effect of SS14 on L-VGCCs (Fig. 4, B and C), but PTX did not interfere with calcium influx through the L-VGCCs (A and C). These data indicated that the effect of SS14 on cone L-VGCCs was through the sst2 receptor that coupled with a PTX-sensitive G protein. Because receptor-coupled G proteins activate multiple downstream intracellular signaling pathways, we subsequently investigated which of these pathways mediated the inhibitory effect of SS14 on L-VGCCs in cone photoreceptors.

**FIG. 2.** The somatostatin receptor 2 (sst2) agonist, MK-678, mimics the effect of SS14. A: L-VGCC current densities were larger when photoreceptors were recorded during the subjective night (CT 16–19; ■) than during the subjective day (CT 4–7; □). B and C: application of MK-678 (100 nM) for 2 h prior to recordings had no effect during the subjective day (CT 4–7; □) but caused a significant decrease in L-VGCC current density during the subjective night (CT 16–19; H17040; C). D: MK-678 significantly inhibited the maximum L-VGCC current density (I_{max}) during the subjective night but not the subjective day. Control (Con) CT 4–7: 3.9 ± 0.7 pA/pF, n = 11; control CT 16–19: 6.4 ± 0.8 pA/pF, n = 13; MK-678 CT 4–7: 5.4 ± 1.4 pA/pF, n = 13; MK-678 CT 16–19: 2.5 ± 1.0 pA/pF, n = 8. *P < 0.05.

**FIG. 3.** The inhibitory effect of SS14 during the subjective night is mediated through somatostatin receptor 2 (sst2). A: application of the sst2 antagonist cyanamid-154806 (1 mM) for 2 h (○) had no effect on L-VGCCs compared with control (■). B: treatment with SS14 for 2 h (○) significantly decreased L-VGCC current density, while cyanamid-154806 reversed the inhibitory effect of SS14 on L-VGCC current density (■). C: SS14 significantly decreased the maximum current density of L-VGCCs. Treatment with cyanamid-154806 did not have any effect on the maximum L-VGCC current density (I_{max}), but it significantly reversed the inhibitory effect of SS14 on L-VGCC maximum current density. Control (Con): 6.2 ± 0.6 pA/pF, n = 15; cyanamid-154806: 5.6 ± 0.7 pA/pF, n = 15; SS14: 3.3 ± 1.0 pA/pF, n = 9; cyanamid-154806 + SS14: 6.5 ± 1.0 pA/pF, n = 13.
Intracellular calcium mobilization is required for somatostatin-14 inhibition of L-VGCCs

In chicken cone photoreceptors, somatostatin stimulates phosphatidylinositol-specific PLC (PLC) activation (Chen et al. 2007), so we examined whether the effect of SS14 on L-VGCCs was mediated through PLC. Treatment with 1-O-octadecyl-2-O-methyl-sn-glycerol-3-phosphorylcholine (ET-18-OCH₃), an inhibitor of phosphatidylinositol-specific PLCs, for 2 h increased the cone L-VGCC current density in a dose-dependent manner. At 50 μM, ET-18-OCH₃ significantly (*P < 0.05) increased the L-VGCC current density compared with the control during the subjective night but did not affect L-VGCC channel properties at a lower concentration (25 μM, Fig. 5A). Interestingly, 25 μM ET-18-OCH₃ was sufficient to reverse the inhibitory effect of SS14 on L-VGCC currents (Fig. 5B). We tested another PLC inhibitor, U73122, which showed similar results (data not shown). At 1 μM, U73122 did not affect L-VGCC channel properties but effectively reversed the inhibitory effect of SS14 on L-VGCC currents, whereas at 5 μM, U73122 alone significantly increased the L-VGCC current density compared with the control during the subjective night (data not shown). Therefore the inhibitory effect of SS14 on L-VGCCs in cone photoreceptors was mediated through the activation of PLC.

Activation of PLC hydrolyzes membrane phospholipid phosphatidylinositol 4,5-biphosphate (PIP₂) into inositol 1,4,5-trisphosphate (IP₃) and diacylglycerol, and IP₃ further binds to its receptors that leads to Ca²⁺ release from IP₃-sensitive intracellular calcium stores (Berridge et al. 2000). Thus we further explored whether increased intracellular Ca²⁺ played a role in the effect of SS14 on L-VGCCs. We first verified the existence of intracellular calcium stores in chicken cone photoreceptors by calcium imaging. Dark adapted retinal cells were loaded for 1 h with 3.0 μM Fluo-4 AM at 37°C in serum and then washed with the same medium prior to measuring cytosolic Ca²⁺. Treatment with thapsigargin, an inhibitor of endoplasmic reticulum Ca²⁺ ATPase (Fischer et al. 1998), caused a sudden surge of intracellular Ca²⁺ followed by complete depletion of Ca²⁺ from intracellular stores (Fig. 6). The calcium-imaging result indicated the presence of intracellular calcium stores in chicken cone photoreceptors. We found that SS14 inhibition of L-VGCCs during the subjective night was completely blocked by thapsigargin (5 μM; Fig. 7, A and C), but thapsigargin alone did not affect the L-VGCCs (A and C). Furthermore, a 2-h incubation with BAPTA-AM, a membrane-permeable Ca²⁺ chelator, showed the same blockade of the SS14 effect on L-VGCCs as...
thapsigargin during the subjective night (Fig. 8, B and C), while BAPTA-AM alone only slightly decreased the L-VGCC current (A and C). These results indicated that Ca\(^{2+}\) release from intracellular stores was required for the inhibitory effect of SS14 on cone L-VGCCs during the subjective night.

**DISCUSSION**

In this study, we report that there was a circadian phase-dependent inhibitory effect of SS on L-VGCCs in cultured cone photoreceptors. Somatostatin caused a significant decrease in the L-VGCC current density as well as L-VGCC\(\alpha1D\)
significantly decreased L-VGCC current density. (Akopian et al. 2000). Our data showed that SS14 slightly cone photoreceptors but a decrease in rod photoreceptors retina, somatostatin elicits an increase of L-VGCC currents in receptors (Pavan et al. 2004). Interestingly, in the salamander only relies on different SS receptors coupling to different G proteins (Chen et al. 2007; Ho et al. 2001; Johansen et al. 2001; Siehler et al. 2001). In the retina, sst2 couples to both PTX-sensitive (Akopian et al. 2000; Pavan et al. 2004; Vasilaki et al. 2003) and -insensitive G proteins (Chen et al. 2007). The complex intracellular signaling elicited by SS not only relies on different SS receptors coupling to different G proteins, it also depends on the coupling between different SS receptors (Pavan et al. 2004). Interestingly, in the salamander retina, somatostatin elicits an increase of L-VGCC currents in cone photoreceptors but a decrease in rod photoreceptors (Akopian et al. 2000). Our data showed that SS14 slightly increased the protein expression of the L-VGCCα1D subunit at CT 5, and the sst2 agonist MK-678 also slightly increased the L-VGCC current density at CT 4–7 (Fig. 1 and 2). However, we did not observe an increase in L-VGCC current density by SS14 during the subjective day (CT 4–7). Because our recording data were the average across at least three circadian hours, it is possible that SS14 would have a more profound effect during the subjective day but at a different time period (i.e., CT 2–5 or CT 6–9). Currently, we are investigating the phase-dependent effect of SS14 during the subjective day at different time periods. In this report, we only focused on the effect of SS14 on L-VGCCs and the underlying signaling pathway in cone photoreceptors during the subjective night.

Activation of SS receptors further activates PLC (Akbar et al. 1994; Chen et al. 2007; Ho et al. 2001; Johansen et al. 2001; Murthy et al. 2004; Siffert et al. 1995), and all human SS receptor subtypes can stimulate different PLC isoforms (Akbar et al. 1994). We found that the inhibitory effect of SS on L-VGCCs was through activation of PLC. Interestingly, at a lower concentration, a PLC inhibitor was sufficient to reverse the effect of SS14 without affecting L-VGCC current density. However, at a higher concentration, the PLC inhibitor itself increased L-VGCC current density during the subjective night. There are two possible explanations. At higher concentrations, the PLC inhibitor might have some nonspecific effects that caused an increase in L-VGCC current density. However, we used two structurally different PLC inhibitors, ET-18-OCH3 and U73122, and both had similar effects on L-VGCCs. The higher concentrations used for both PLC inhibitors were within pharmacological ranges applied in other published studies on the inhibitory effects of PLC. Hence we exclude the possibility of nonspecific PLC inhibitor effects at higher concentrations on L-VGCCs. Alternatively, it is possible that there is a basal level of PLC activity in chicken cone photoreceptors at night, which might have slightly dampened the L-VGCC currents. The inhibitory effect of SS14 on L-VGCCs could be through a further activation of PLC.

Activation of PLC elicits a production of inositol trisphosphate (IP3) and diacylglycerol, and IP3 then causes Ca2+ release from IP3-sensitive intracellular calcium stores (Berger et al. 2000; Hirata et al. 1985). There is a species- and spatial-dependent distribution of different intracellular calcium stores. Caffeine, a modulator of ryanodine receptor operant intracellular stores, readily evokes an intracellular Ca2+ rise in the inner segments of rods but not cones in tiger salamanders (Krizaj et al. 2003). While ryanodine receptor operant intracellular stores are more dominantly present in rod photoreceptors in rodents at both the outer and inner segments (Krizaj 2005; Shoshan-Barmatz et al. 2007), IP3 receptor operant intracellular stores are typically more prevalent in cone photoreceptors as well as at the synaptic terminals and inner segments of various vertebrate species (Peng et al. 1991; Wang et al. 1999). We found that thapsigargin (10 μM), an inhibitor of endoplasmic reticulum Ca2+ ATPase, evoked a robust surge of intracellular Ca2+ followed by its complete depletion, which provided evidence for the existence of intracellular calcium stores in chicken cone photoreceptors. Somatostatin has been shown to induce calcium release from intracellular calcium stores in different cell types such as pituitary cells and neuroblastoma × glioma NG108-15 cells (Akbar et al. 1994; Nunn et al. 2004; Rhie et al. 2003; Romoser et al. 2001; Siehler et al. 2005; Siffert et al. 1995). Here, we showed that the inhibitory effect of SS14 on L-VGCCs was blocked by both the Ca2+ chelator BAPTA-AM and thapsigargin. Hence, we demonstrated that through activation of PLC, SS triggered the release of calcium from intracellular stores that in turn decreased L-VGCC currents in cone photoreceptors. Calcium-dependent inhibition of L-VGCCs (by elevated intracellular Ca2+), also known as calcium-induced calcium
inactivation, has been demonstrated in various cells (Brehm and Eckert 1978; Peterson et al. 1999). There are two possible feedback regulatory mechanisms for calcium-dependent inhibition of L-VGCCs. When \( \text{Ca}^{2+} \) enters through L-VGCCs, it binds to calmodulin and calmodulin-like proteins (Lee et al. 2007; Peterson et al. 1999; Tippen and Lee 2007) and subsequently activates calcium-dependent kinases (Lee et al. 2007). Phosphorylation of L-VGCC subunits by calcium-dependent kinases can directly decrease L-VGCC currents and alter channel gating kinetics (Calin-Jageman and Lee 2008; Lee et al. 2007).

However, activation of calcium-dependent kinases can also further elicit \( \text{Ca}^{2+} \) release from intracellular calcium stores, and possibly through other mechanisms, that ultimately lead to the inhibition of VGCCs in the plasma membrane (Alvarez et al. 1991; Fischer et al. 1998; Mohr et al. 1995; Thayer et al. 1988). Our Western blot data indicated that the protein level of L-VGCCα1D was downregulated by treatment with SS14 for 2 h. We postulate that calcium released from the intracellular stores might trigger downstream signaling that ultimately affects the protein synthesis of L-VGCCα1D and causes a decrease in L-VGCCs. Such calcium-dependent inhibition of L-VGCCs in cone photoreceptors would have an impact on photoreceptor physiology that is waiting to be explored.

We showed that SS14 inhibited L-VGCCs during the subjective night but not during the subjective day in chicken cone photoreceptors. A similar circadian phase-dependent result was also observed in cGMP-gated channels (CNGCs) (Chen et al. 2007), in which SS14 decreased the sensitivity of CNGCs to cGMP during the subjective night but had no effect during the subjective day. Together, Chen et al. (2007) and our results demonstrate that SS14 inhibits \( \text{Ca}^{2+} \) influx through both CNGCs and L-VGCCs in a circadian-dependent manner. While CNGCs are essential for phototransduction, L-VGCCs are indispensable in other aspects of photoreceptor physiology including synaptic transmission and excitability, and therefore SS14 may have a tremendous impact on the modulation of visual processes.

In the retina, the content of SS is affected by exposure to light. The content of SS is highest at night in the rat retina (Peinado et al. 1990), while a greater intracellular somatostatin-like immunoreactivity is observed in bipolar synaptic terminals during prolonged exposure to light in the chicken retina (Ishimoto et al. 1986). In the chicken retina, ENSLI amacrine cells that contain SS are more active in the dark phase (Morgan and Boelen 1996). Currently, it is not known whether the content of SS is under circadian control, and the physiological range of SS concentration is not clear in the chicken retina. It will be critical to re-evaluate the circadian regulation of SS content and release in the retina across different species, so the circadian phase-dependent effect of SS and its physiological significance and meaning can be properly interpreted. We observed inhibitory effects of SS14 on L-VGCCs only during the subjective night, but not during the day. This observation is interesting. Because there are four SS receptor subtypes expressed in the chicken retina, it is possible that some are regulated in a circadian manner while others are not. The effect of SS during the day might be mediated through a different SS receptor, coupled to a different G protein, leading to a different signaling pathway. We are currently investigating this hypothesis. Because visual systems have to anticipate daily changes in ambient illumination, the circadian oscillators in the retina provide a mechanism for visual systems to initiate more sustained adaptive changes throughout the course of a day (Cahill and Besharse 1993; Green and Besharse 2004). Somatostatin, along with dopamine and melatonin, has been proposed to serve as part of a “dark-light switch” within the retina neuronal circuitry in the diurnal changes of retinal physiology and function (Morgan and Boelen 1996). Therefore somatostatin plays an important role in fine-tuning light sensitivity of photoreceptors.

There are other factors that could contribute to the modulation of retina L-VGCC circadian rhythms in vivo. For example, dopamine levels in the retina are antiphase to those of melatonin (Adachi et al. 1998). Through D2 receptors, dopamine has differential effects on the L-VGCCs in rods and cones of salamander retinas (Stella and Thoreson 2000). Dopamine can entrain the photoreceptor circadian oscillators, induce light-like phase shifts, and contribute to circadian rhythms of rod-cone predominance (Hasegawa and Cahill 1999; Manglapus et al. 1998, 1999; Steenhard and Besharse 2000; Wang and Mangel 1996; Witkovsky et al. 1988). Dopamine is also known to modulate cGMP-gated cation channels of cone photoreceptors in a circadian phase-dependent manner (Ko et al. 2003). Therefore it is possible that dopamine may contribute to a circadian phase-dependent modulation of L-VGCC activities in cone photoreceptors. Another possible factor is the circadian regulation of retinal pH (Dmitriev and Mangel 2000, 2001, 2004; Harsanyi and Mangel 1993). In both fish and rabbit retinas, there is an increase in pH difference between the retina and superfusate at night in an in vitro whole retina preparation. The pH changes in the retina correlate to overall retinal energy metabolism and excitability measured by electroretinograms (ERGs) (Dmitriev and Mangel 2004). Because voltage-gated calcium channels, including L-types, are very sensitive to changes in extracellular pH (Iijima et al. 1986; Konnerth et al. 1987; Krafte and Kass 1988; Prod’hom et al. 1987), circadian rhythms in retinal pH may contribute to the modulation of L-VGCCs. Our in vitro dissociated retina culture system is a reduced system, as such the ability to simulate an in vivo environment is limited, and it is impossible to investigate all factors that potentially could contribute to the modulation of circadian oscillators in photoreceptors at once. Nonetheless, retinal photoreceptors possess self-sustained circadian oscillators that function independently in the absence of other retinal inputs (Cahill and Besharse 1993; Ko et al. 2001; Thomas et al. 1993). These photoreceptor oscillators lead to morphological, physiological, biochemical, and molecular changes that ultimately regulate photoreceptor function and physiology in a circadian fashion (Green and Besharse 2004), while other extracellular factors can further contribute to the modulation of photoreceptor oscillators, as well as stabilize the overall circadian rhythms of the retina.

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ROLE OF SOMATOSTATIN IN MODULATION OF PHOTORECEPTOR PHYSIOLOGY

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