Cellular/Molecular

Somatostatin Peptides Produce Multiple Effects on Gating Properties of Native Cone Photoreceptor cGMP-Gated Channels That Depend on Circadian Phase and Previous Illumination

Shih-Kuo Chen, Gladys Y.-P. Ko, and Stuart E. Dryer
Department of Biology and Biochemistry, University of Houston, Houston, Texas 77204-5001

A subpopulation of avian amacrine cells expresses somatostatin-14 (SS14) and somatostatin-28 (SS28), which provide a potential efferent limb for light-dependent regulation of photoreceptors. Here, we demonstrate that SS14 and SS28 modulate cone photoreceptor cGMP-gated channels (CNGCs) through multiple mechanisms. In chicken cones cultured in constant darkness for 2 d after previous entrainment to light– dark (LD) cycles or in cells maintained in LD, application of 100 nM SS14 or 100 nM SS28 for either 15 min or 2 h caused a decrease in the sensitivity of CNGCs to cGMP during the night, at circadian time 16 (CT16)–CT20 or zeitgeber time 16 (ZT16)–ZT20. SS14 had no effect during the day (CT4–CT8 or ZT4–ZT8). These effects persist in cells pretreated with pertussis toxin (PTX) and, like dopamine, may work to reinforce long-term circadian fluctuations in CNGCs driven by oscillators within the photoreceptors themselves. In contrast, a 15 min exposure to SS28 caused a seemingly paradoxical increase in the sensitivity of CNGCs to cGMP during the early day (ZT4–ZT6), but only in cones maintained in LD. This effect of SS28 desensitizes rapidly, is blocked by pretreatment with PTX, and is selectively mimicked by the cyclohexapeptide agonist MK-678. This transient response also requires activation of phospholipase C and protein kinase C. The transient response to SS28 may play a role in photoreceptor adaptation to rapid changes in ambient illumination. These data also show that photoreceptor responses to at least some peptide neurotransmitters depend on the previous history of light exposure.

Key words: **somatostatin; dark current; circadian; phospholipase C; protein kinase C; cone; photoreceptor**

Introduction

Cyclic GMP-gated cation channels (CNGCs) carry the photoreceptor "dark current" and are essential for visual phototransduction. Light induces degradation of intracellular cGMP as the endpoint of a G-protein mediated transduction cascade, resulting in deactivation of plasma membrane CNGCs, reduced cation influx, and membrane hyperpolarization.

Circadian oscillators control several aspects of photoreceptor physiology (Anderson and Green, 2000), including the activation of CNGCs by cGMP. Thus, we have previously shown that CNGCs in chicken cones are more sensitive to cGMP during the subjective night than during the subjective day, even in dissociated photoreceptors maintained in constant darkness (Ko et al., 2001a, 2003, 2004a,b; Chae et al., 2007). The output pathway leading from the intrinsic circadian oscillator to CNGCs in the plasma membrane is associated with increased activation of adenylate cyclase, Ras, and mitogen-activated protein kinase Erk during the early subjective night (Ko et al., 2004b). Moreover, blocking the oscillations in several of the components of this pathway suppresses the circadian rhythm in CNGC gating properties (Ko et al., 2004b).

These circadian output pathways persist in low-density cultures that are highly enriched in cone photoreceptors (Adler and Hatlee, 1989), and they appear to be driven by core circadian oscillators intrinsic to the photoreceptors themselves. However, photoreceptors in the intact retina occur in relatively close association with other cell types and are the targets of signals from the inner retina that could contribute to the robustness and persistence of circadian control (Manglapus et al., 1999). In this regard, we have observed that dopamine (DA), the secretion of which is under circadian control in the avian retina (Adachi et al., 1998, 1999), can produce complex circadian phase-dependent effects on the gating properties of cone CNGCs (Ko et al., 2003). The purpose of the present study is to examine a second potential efferent modulator of cone cell physiology, the neuropeptide somatostatin, which is synthesized and released from a subpopulation of chicken amacrine cells known as enkephalin-, neurotensin- and somatostatin-like (ENSLI) cells (Yang et al., 1997). In fact, two different somatostatin isoforms are released from ENSLI cells, somatostatin-14 (SS14) and the N-terminally extended somatostatin-28 (SS28), and their secretion is greater in the light than in the dark (Ishimoto et al., 1986; Dowton et al., 1994). Relatively little is known about somatostatin effects on photoreceptors. However, one report indicates that these pep-

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Correspondence should be addressed to Dr. Stuart E. Dryer, Department of Biology and Biochemistry, University of Houston, 4800 Calhoun Road, Houston, TX 77204-5001. E-mail: SDryer@uh.edu.
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tides can modulate voltage activated K^+ and Ca^{2+} currents in salamander photoreceptors (Akopian et al., 2000).

In the present study, we show that SS14 and SS28 cause a circadian phase-dependent decrease in the sensitivity of CNGC activation by cGMP that can be seen in either constant darkness or in light-dark cycles. However, SS28 produces an additional effect not seen with SS14. Specifically, SS28 also evokes a transient increase in the sensitivity of CNGCs to cGMP that is only seen in light-dark cycles, and only within the first 6 h after the lights come on. These two distinct effects appear to be mediated by different somatostatin receptor subtypes that act through different G-protein-coupled transduction cascades.

Materials and Methods

Cell isolation and culture. Chick retinas were dissociated at embryonic day 6 (E6) as described previously (Ko et al., 2001a,b). Retinal cells were grown for 5 d on poly-D-lysine-coated glass coverslips (molecular weight 276,000) in a medium consisting of Eagle's minimal essential medium supplemented with 10% heat-inactivated horse serum, 2 mM glutamine, 50 U/ml penicillin, 50 μg/ml streptomycin, and 40 ng/ml recombinant rat ciliary neurotrophic factor. Cell culture incubators (39°C and 5% CO_2) were equipped with lights and timers, which allowed for entrainment of retinal circadian oscillators *in vitro*. Sister cultures were maintained in two separate incubator chambers under 12 h light/dark (LD) cycles. The LD cycles in two separate chambers were maintained 12 h antiphase so that recordings from cells at different clock phases could be interleaved. Throughout, times of day are expressed as zeitgeber time (ZT; i.e., relative to the original entraining LD cycle), or as circadian time (CT) for cells free-running in constant darkness (DD). The time that lights come on is defined as ZT0, and the time that lights go off is ZT12. In most experiments, electrophysiology or biochemistry was performed on the fifth day of exposure to LD cycles or on the second day of DD, after 4 d of entrainment to the 12 h LD cycles. We refer to "subjective day" and "subjective night" in cells free-running in DD after previous entrainment, but simply refer to "day" and "night" when cells are kept on LD cycles.

Electrophysiology. Recordings were made from cells with elongated cell bodies, an outer segment, and one or more prominent oil droplets on the distal side of the soma, as described in detail previously (Ko et al., 2001, 2003, 2004b). Briefly, inside-out patches were excised into a saline free of divalent cations consisting of (in mM) 145 NaCl, 10 Na-HEPES, 1 EGTA, and 5 glucose, pH 7.4, and held at −60 mV. Pipette solution was the same as the bath saline. Recordings were performed in the light at room temperature (22–23°C). Channels were activated by gravity-fed bath application of varying concentrations of cGMP dissolved in bath saline. Cultures were typically pretreated with drugs 2 h or 15 min before recording, as indicated. Recording data were digitized, stored, and analyzed using pClamp v9.2 software (Molecular Devices, Foster City, CA) after low-pass digital filtering at 100 Hz. In initial experiments, concentration-response curves were fitted with the Hill equation $I_S = I_{Max}\,[S^n/(K_{(1/2)}{}^n + S^n)]$, where S is the concentration of cGMP, $K_{(1/2)}$ is the concentration at which half-maximal channel activation occurs, and n is the Hill coefficient. Fitting was done using the Levenberg–Marquardt routines implemented in Origin version 7.0 (Microcal, Northampton, MA). In most experiments, we also calculated the ratios of currents evoked by application of 20 and 200 μM cGMP to the cytoplasmic face of each patch. This ratio was used as an index of CNGC sensitivity to cGMP in the majority of these studies. Each group in every experiment contained data from at least eight patches obtained from at least three different preparations of retinal cells. Statistical analyses were performed using Origin version 7.0 (Microcal, Northampton, MA) and consisted of one-way ANOVA followed by an appropriate *post hoc* test, with $p < 0.05$ was regarded as significant. Error bars in the figures represent SEM.

Transfection and live cell confocal imaging. Cultured cones were transfected with an expression vector encoding a PLCδ1-PH-GFP fusion protein, provided by Dr. Tobias Meyer of Stanford University. This construct is comprised of residues 1–170 of phospholipase C δ1 (PLCδ1), which contains a plextrin homology (PH) domain, which is fused to green fluorescent protein (GFP) to allow for fluorescent determination of its location in cells. The PH domain causes the fusion protein to bind to PtdIns[4,5]P_2, (Lemmon et al., 1995) and it consequently localizes to the plasma membrane in the resting state. Activation of PLC, which causes cleavage of membrane phosphoinositides in the plasma membrane, causes a portion of the fluorescent fusion protein to relocalize into the plasma membrane, and this can be monitored to infer activation of PLC. Transfection was performed using pn-Fect from Neuromics (Edina, MN). Briefly, retinal cells were grown for 4 d in LD cycles, until 24 h before analysis. At that time, coverslips with attached retinal cells were transferred to 400 μl of serum-reduced culture medium (Optimem; Invitrogen, Carlsbad, CA) in 24-well plates supplemented with 100 μl of solution containing pn-Fect with PLCδ-PH-GFP expression vector incorporated according to the manufacturer's instructions. In a series of pilot experiments, we determined that the maximum transfection efficiency occurred with a pn-Fect/DNA ratio of 1.4/1. After transfection, the cells were kept in LD for 24 h. Best results were obtained from cells expressing the construct at low levels as assessed by fluorescence intensity. To capture confocal images, coverslips were placed in a perifusion chamber containing 400 μl of Optimem medium, and observed using an Olympus (Tokyo, Japan) FV-1000 inverted stage confocal microscope using a Plan Apo N 60× 1.42 numerical aperture oil-immersion objective. Green fluorescence was evoked using an excitation wavelength of 488 nm while monitoring emission at 519 nm. Fluorescence images were captured from cells that have oil droplets visible under differential interference contrast optics, and which could therefore be identified as cones. The somatostatin receptor agonist MK-678 (2 μM) was added directly to the chamber. *Z*-stack images were captured from the same cells before and at various times after MK-678 treatment to ensure that the same focal plan was analyzed later on. All images were collected and analyzed by Olympus Fluoview software. To construct spatial intensity profiles, we drew an arbitrary line through the cell and measured fluorescence intensity along the line. Two peaks occurred at the periphery of the cell, where the line crossed the plasma membrane. We used the average of these two peaks to calculate the membrane intensity (I_{MB}). The cytosolic intensity (I_{CY}) in the same cell was obtained by averaging the fluorescence intensity along the length of the center plateau. Data were expressed as a function of these mean values $I_{CY}/(I_{MB} + I_{CY})$, before and after SS28 treatment. This ratio is expected to increase with activation of PLC (Stauffer et al., 1998; Varnai and Balla, 1998). Statistical analyses were performed using Origin 7.0 software (Northampton, MA) and consisted of Student's paired *t* test, with $p < 0.05$ was regarded as significant.

Reverse-transcriptase PCR. Retinal cells were cultured as described above, maintained in LD for 5 d, and total RNA was isolated from the cultures (RNeasy, Qiagen, Valencia, CA). Reverse transcription of 1.5 μg of DNase1-treated total RNA was performed using 10 U/μl Superscript 3 (Invitrogen) primed with 2.5 μM oligo-dT at 50° for 50 min. PCR was performed using the following primer pairs: 5′ACCATGGATCTG-GAATACGAGCT3′ and 5′TGCTCGTCTGCAG-GTCACCATT3′ (sst2); 5′ACCATGGACACTTCTGCTTTCA3′ and 5′GTAAATAGC-TGACATGCAACTTGC3′(sst3); 5′ACCATGAGCACCAAC-GCTGA′ and 5′GGAAGGTGGTGGTCTTTGCCA3′ (sst4); and 5′ACCATG-GACTCAAGTTCCAGTGAT 3′ and 5′CCTTGCTAGTCTGCATGT-GTCCATT3′ (sst5). PCR was performed using Platinum PCR SuperMix (Invitrogen) with the following cycling parameters: 95° for 2 min, followed by 35 cycles of 95° for 30 s; annealing at 58° (sst4 and sst5) or 55° (sst2 and sst3) for 30 s; and extension at 72° for 1 min. A final extension at 72° for 2 min occurred after the last cycle. PCR products were separated on agarose gels, cloned into a TOPO-TA vector (Invitrogen) and sequenced.

Results

Circadian modulation of cone CNGCs

In previous studies, we have shown that the $K_{(1/2)}$ of cone CNGCs for activation by cGMP is under circadian control (Ko et al., 2001a, 2003, 2004a,b; Chae et al., 2007). However, for most of the experiments in the present study we have adopted analytical procedures originally described by Gordon et al. (1992). As with our

previous studies, we observed that the $K_{(1/2)}$ for CNGC activation by cGMP is higher during the day than during the night in cells kept in LD or on the second day of DD (Fig. 1*A*). Because of this change in sensitivity, the ratio of current evoked by a given subsaturating concentration of cGMP to the maximum current observed at a saturating cGMP concentration should be higher during the subjective night than during the subjective day. In this study, we used the ratio of current evoked by 20 μM cGMP to that evoked by 200 μM cGMP to characterize the circadian behavior of CNGCs (as the later concentration produces maximal activation at all times of day). This ratio is proportional to the sensitivity of CNGCs to activation by cGMP. In pilot studies from 30 excised patches, we observed that this response ratio is highly inversely correlated with the $K_{(1/2)}$ measured from complete concentration response curves (Fig. 1*B*) ($r = -0.80274$; $p < 0.0001$). This ratio is significantly lower during the day and higher during the night (Fig. 1*C*), as is the sensitivity of CNGCs to cGMP.

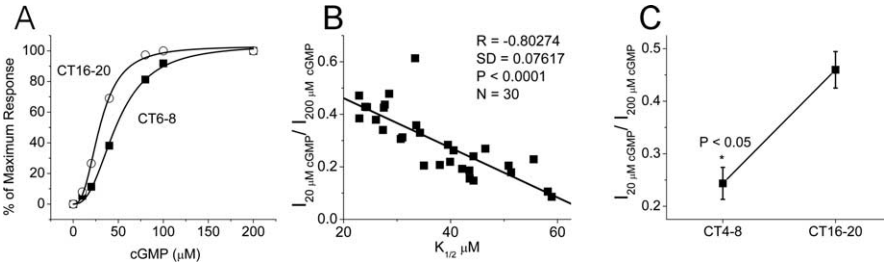

Figure 1. Circadian modulation of the sensitivity of CNGCs to cGMP in cultured cone photoreceptors. Data were obtained from inside-out patch recordings immediately after patch excision at various times of day on the second day of DD. ***A***, examples of concentration-response relationships in two patches excised during the subjective day (CT6–CT8) and during the subjective night (CT16–CT20), as indicated. Superimposed curves are nonlinear least squares fits to the Hill equation. ***B***, ratio of the currents evoked by 20 μM cGMP and 200 μM cGMP in individual patches is inversely correlated with the $K_{(1/2)}$ determined from Hill equation fits to complete concentration-response curves. Each data point on this graph was from a different patch excised at various times of day. ***C***, A statistically significant (*$p < 0.05$) circadian rhythm in cGMP sensitivity can be readily detected from the ratios of the currents evoked by 20 and 200 μM cGMP in excised inside-out patches.

Somatostatin-14 and somatostatin-28 produce different effects on the sensitivity of CNGCs to cGMP that depend on light and circadian phase

Somatostatin is expressed and secreted in a wide variety of tissues, including the avian retina. Because we found that antibodies prepared against mammalian somatostatin receptors are not useful in chicks, we used RT-PCR to determine which receptor subtypes are expressed in dissociated cultures enriched in cone photoreceptors. Using primers specific for all known avian subtypes, we amplified four types of somatostatin receptors, sst_2, sst_3, sst_4 and sst_5 (Fig. 2). The identity of these products was confirmed by sequencing. It bears noting that the gene encoding the vertebrate sst_1 receptor is not present in chicken genome databases.

We next determined whether somatostatin peptides cause modulation of cone CNGCs. To do this, we measured the effect of these peptides on the ratio of currents evoked by 20 and 200 μM cGMP in inside-out patches excised from cultured cones at various times of day in cells kept in LD or DD. We initially observed that treatment with 100 nM SS14 decreased channel sensitivity to cGMP during the night, but had no effect during the day. This was observed with either 15 min or 2 h of continuous exposure of intact cells to SS14 (Fig. 3), suggesting that this response does not desensitize rapidly. The same pattern was seen in cells maintained in LD (Fig. 3*A*) or on the second day of DD (Fig. 3*B*). It was also seen after 2 h treatment with 100 nM SS28 in cells kept in either LD or DD (Fig. 4). However, we obtained an unexpected result in cells maintained in LD. Under those conditions we observed that a 15 min treatment with 100 nM SS28 increased the sensitivity of CNGCs to cGMP, but only during the early part of the day (ZT4–ZT6) and only if the lights were on for 1–2 h before recording (Fig. 4*A*). It was not observed later in the day (after ZT8–ZT12). Notably, this increase in channel sensitivity was not detected in cells examined in DD at CT4–6 (Fig. 4*B*), suggesting that this effect of SS28 depends on both light and circadian phase (Fig. 4*A*). Finally, as already noted, this effect of SS28 was not seen if the peptide was present continuously for 2 h before electrophysiological analysis, even if lights were on during that period.

To address the possibility that these two distinct effects of

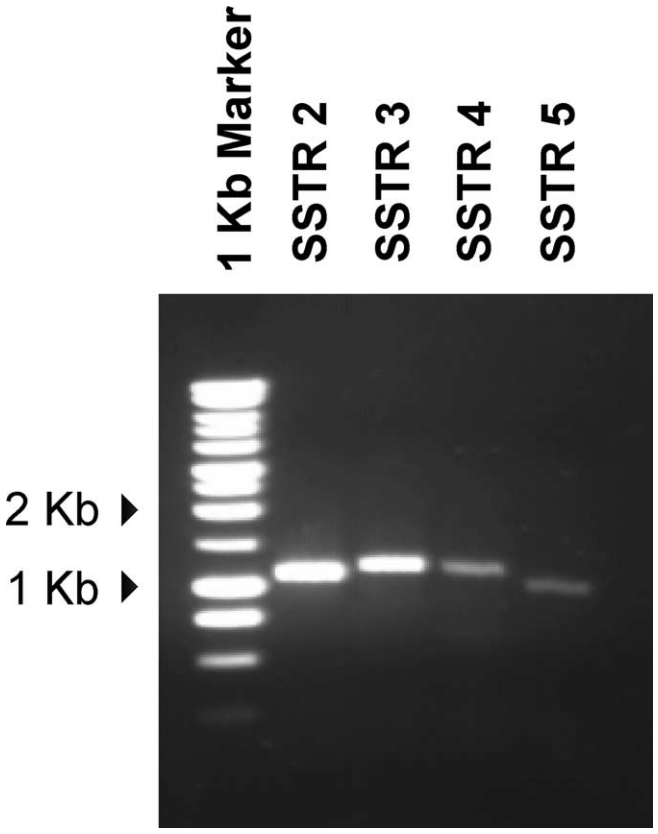

Figure 2. Expression of transcripts encoding somatostatin receptor subtypes as determined by RT-PCR in retinal preparations highly enriched in cultured cone photoreceptors. Primers were designed to amplify the complete coding sequences, and products of the expected size were identified by sequencing. We observed that transcripts encoding sst_2, sst_3, sst_4, and sst_5 are expressed in cone-enriched retinal cell cultures.

SS28 are mediated by different G-protein-mediated signaling cascades, we examined the effects of pretreatment with pertussis toxin (PTX). This toxin causes ADP ribosylation of $G_{i/o}$ α-subunits and thereby blocks their activation (Moss et al., 1983). As with our previous studies of dopamine D2 receptor agonists (Ko et al., 2003), we observed that the effects of SS14 on CNGCs persisted after 24 h pretreatment with PTX (Fig. 5*A*). The responses to a 2 h exposure SS28 were also unaffected by PTX

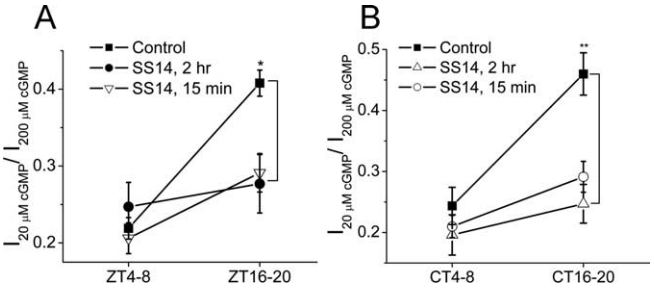

Figure 3. Modulation of native cone CNGCs by SS14. The peptide was applied to cultured retinal cells 15 min or 2 h before patches were excised from cones. Sensitivity to cGMP was measured from the ratios of the currents evoked by 20 and 200 μM cGMP. ***A***, ***B***, Treatment with 100 nM SS14 for 15 min or 2 h decreased the sensitivity of CNGCs to cGMP during the subjective night in cones maintained on LD cycles (***A***) or on the second day of DD (***B***). In all figures, asterisks indicate $*p < 0.05$ or $**p < 0.01$ compared to controls shown by brackets.

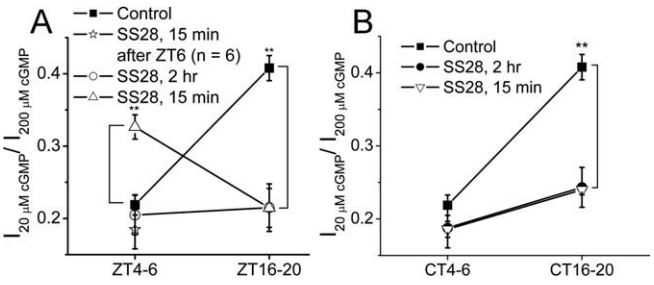

Figure 4. Modulation of native cone CNGCs by SS28. The peptide was applied to cultured retinal cells 15 min or 2 h before patches were excised from cones. ***A***, In retinal cells cultured under LD cycles, SS28 (100 nM) applied for 15 min or 2 h evoked a decrease in the sensitivity of CNGCs to cGMP during the subjective night. However, 15 min treatment with SS28 also caused an increase in channel sensitivity to cGMP early in the day (ZT4–ZT6), but not later in the day, after ZT6 (see data point with star). This increase in sensitivity effect was not seen after 2 h of SS28 treatment. ***B***, The effects of SS28 are identical to those of SS14 in cells maintained in DD. Note that 15 min exposure to SS28 does not produce an increase in channel sensitivity to cGMP early in the subjective day in cells free-running in DD.

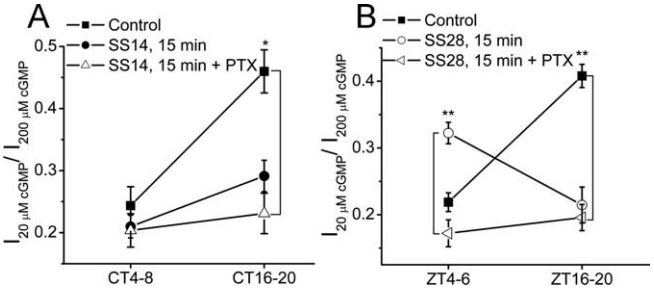

Figure 5. The effects of SS28 are mediated by multiple G-proteins. Cultured photoreceptors were treated with PTX (200 ng/ml) or with control medium for 16 h before application of 100 nM SS14 or SS28. Peptides were present for 15 min before patch excision. ***A***, The effects of SS14 persist in cells pretreated with PTX. These data are from cells analyzed on the second day of DD, but the same result was obtained for cells in maintained in LD (data not shown). ***B***, The nocturnal effect of SS28 also persists after PTX pretreatment. However, the increase of CNGC sensitivity to cGMP observed during the early day in cells maintained in LD cycles was completely blocked by PTX pretreatment.

(data not shown). In contrast, the transient light-dependent effect evoked by a 15 min treatment with SS28 was completely blocked by PTX pretreatment (Fig. 5*B*). Therefore, SS28 can modulate the sensitivity of cone CNGCs to cGMP by at least two different signaling pathways, including a light-sensitive pathway that is $G_{i/o}$-dependent, and a light-independent pathway through a different type of G-protein.

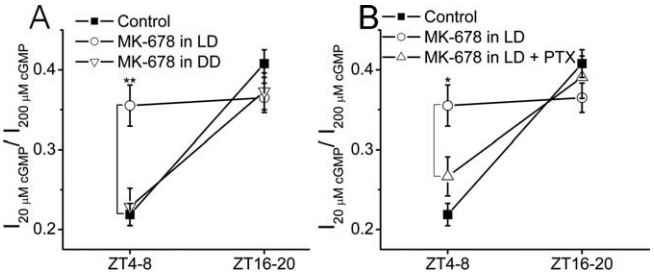

Figure 6. The selective SRIF1 agonist MK-678 evokes a light-dependent increase in CNGC sensitivity during the early part of the day. In these experiments, MK-678 (100 nM) or vehicle was added to cultured cells 15 min before patch excision. ***A***, Treatment with MK-678 evoked an increase in the sensitivity of CNGCs to cGMP during the early day (ZT4–ZT6) in cone photoreceptors cultured in LD cycles, but not in cells previously maintained in DD for 2 d before analysis. MK-678 differs from SS14 and SS28 in that it does not have any effect on the sensitivity of CNGCs to cGMP during the night. ***B***, The effect of MK678 is completely blocked by PTX pretreatment.

Somatostatin receptors can be divided into two families on the basis of their primary sequence and sensitivity to selective agonists. The SRIF1 family receptors (sst_2, sst_3, and sst_5) exhibit a high binding affinity for cyclohexapeptides such as MK-678. In contrast, the SRIF2 family (comprised of sst_1 and sst_4) does not respond to MK-678 (Raynor and Reisine, 1992). We observed that a 15 min exposure to MK-678 evoked an increase in CNGC sensitivity during the early part of the day in LD, but had no effect at all at other times of day in LD, or at any time of day in cells maintained in DD (Fig. 6*A*). Moreover, this effect is PTX sensitive (Fig. 6*B*). These data suggest that the transient light-dependent effect of SS28 is mediated by a receptor in the SRIF1 class that is coupled to $G_{i/o}$. Presumably, the other effects of SS14 and SS28 are mediated by a SRIF2 receptor acting through a different type of G-protein.

Light-dependent modulation of CNGCs by MK-678 entails activation of phospholipase C

Activation of PLC causes cleavage of plasma membrane PtdIns[4,5]P_2 and generation of Ins[1,4,5]P_3 and diacylglycerol (DAG), resulting in activation of multiple downstream effectors (Werry et al., 2003). Signaling by many PTX-sensitive $G_{i/o}$-coupled receptors, including sst_5, is known to occur via activation of PLC (Rebecchi and Pentyala, 2000). We therefore examined whether the effects of SS28 and MK-678 on cone photoreceptors are mediated by activation of PLC. In initial experiments, we examined the effects of 2,4,6-trimethyl-*N*-(*meta*-3-trifluoromethyl-phenyl)-benzenesulfonamide (*m*-3M3FBS), an agent that causes direct activation of all known PLC isoforms (Bae et al., 2003). We observed that 2 h treatment with 50 μM *m*-3M3FBS increased the sensitivity of CNGCs to cGMP throughout the subjective day, but not during the subjective night (Fig. 7*A*). However, unlike SS28 or MK-678, this effect occurred in either LD or DD, and the effect could be obtained over a greater portion of the day (i.e., both before and after ZT6). From this we conclude that PLC activation is sufficient to cause an increase in CNGC sensitivity during the subjective day. In a complementary set of experiments, we pretreated cones for15 min with 50 μM 1-*O*-octadecyl-2-*O*-methyl-*rac*-glycero-3-phosphorylcholine (ET-18-OCH_3), an inhibitor of phosphatidylinositol-specific PLCs (Seewald et al., 1990; Powis et al., 1992), and then examined the effects of MK-678 or *m*-3M3FBS between ZT4 and ZT6 in cells maintained in LD. We observed that ET-18-OCH_3 pretreatment caused a complete inhibition of the effects of MK-678 on CNGCs (Fig. 7*B*). Do the products of PLC modulate CNGCs directly, or

do they act through an intermediary enzyme such as protein kinase C (PKC)? To examine this, we added Ins[1,4,5]P_3 or the DAG analog DL-1,2-dioctanoyl-sn-glycerol (1,2-DiC8) to the bath solution after patch excision. Patch excision eliminates contact between CNGCs and most cytosolic enzymes, and an effect of 1,2-DiC8 under these conditions would reflect a direct effect on CNGCs or nearby proteins that remain associated with the plasma membrane. However, neither Ins[1,4,5]P_3 or 1,2-DiC8 changed the sensitivity of CNGCs to cGMP during the day (data not shown), suggesting that DAG does not act directly on CNGCs or adjacent membrane-associated proteins. In contrast, we observed an increase in the sensitivity of CNGCs to cGMP throughout the day if we pretreated intact cultured cells with 1,2-DiC8 for 30 min before patch excision. With that experimental design, cytosolic enzymes regulated by DAG, such as many isoforms of PKC, can remain in contact with CNGCs throughout the period of drug exposure. Moreover, the effect of 1,2-DiC8 on CNGCs was abolished when the PKC inhibitor Ro-31–8220 was simultaneously applied to intact cells. This pattern strongly suggests that modulation of CNGCs can occur through a PLC–PKC pathway, and argues against a direct effect of PLC products on CNGCs (Fig. 7*C*). These data also suggest that a PLC–PKC pathway is a necessary component of the signaling cascade activated by SS28 during the early day. As an additional test of this hypothesis, we compared the activity of PLC before and after MK-678 treatment in our culture system using an *in vivo* optical imaging procedure described by other workers (Stauffer et al., 1998; Varnai and Balla, 1998). Briefly, cone photoreceptors were transfected with an expression vector encoding a PLCδ1-PH domain/GFP fusion protein. The PLCδ1 PH domain binds to plasma membrane phosphoinositols, especially PtdIns[4,5]P_2, causing the fusion protein to associate primarily with the plasma membrane in resting cells. Activation of PLC causes a local decrease in membrane PtdIns[4,5]P_2 and an increase in cytosolic phosphoinositides, which together cause the fluorescent fusion protein to dissociate from the plasma membrane. The resulting redistribution of the fusion protein, which is an index of PLC activation, can be monitored in real time by confocal microscopy. As with other cell types (Stauffer et al., 1998; Varnai and Balla, 1998; Holz et al., 2000), we observed robust fluorescence of the fusion protein primarily in the plasma membrane, and low fluorescence in the cytosol in resting cones observed during the day time (Fig. 8*A*,*C*). However, after 15–20 min of MK-678 treatment, the intensity of cytosolic fluorescence in the same cells was increased when the measurements were made during the early day time (ZT4–ZT6) (Fig. 8*A*,*B*). In contrast, this change in the distribution of fluorescence did not occur if MK-678 responses were observed later in the day (ZT6–ZT8) (Fig. 8*C*,*D*). Data compiled from several cells during the day indicate that there is a significant difference between the effects of MK-678 on the distribution of the PLCδ1-PH domain-GFP fusion protein when measurements were made before and after ZT6 (Fig. 8*E*). Together, these data indicate that activation of PLC is both necessary and sufficient to induce an increase in the sensitivity of CNGCs for cGMP during the day time. These data also suggest that SS28 is only able to activate PLC during the early part of the day. As an aside, we noticed that SS28 treatment caused an elongation of most cone photoreceptors during the early day time that can be seen by comparing Figure 8, *A* and *B*. This is a consistent observation, and it is similar an effect of dopamine D_2

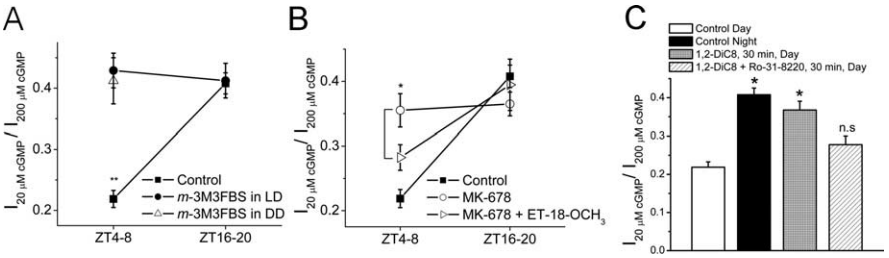

Figure 7. Activation of PLC is sufficient and necessary to cause an increase in CNGC sensitivity to cGMP during the early day time. ***A***, Application of the PLC activator *m*-3M3FBS (50 μM) causes an increase in the sensitivity of cone CNGCs to cGMP throughout the day and this effect can be observed in cells maintained in either LD and DD. ***B***, Pretreatment with the PLC inhibitor ET-18-OCH_3 (50 μM) can block the effects of MK-678 or *m*-3M3FBS on CNGCs observed during the day. ***C***, Treating intact cells with the short-chain DAG analog 1,2-DiC8 (25 μM) during the day mimics the effect of *m*-3M3FBS, as it causes an increase in the sensitivity of CNGCs to cGMP. However, this effect is antagonized by simultaneous administration of the PKC inhibitor Ro-31–8220 (500 nM).

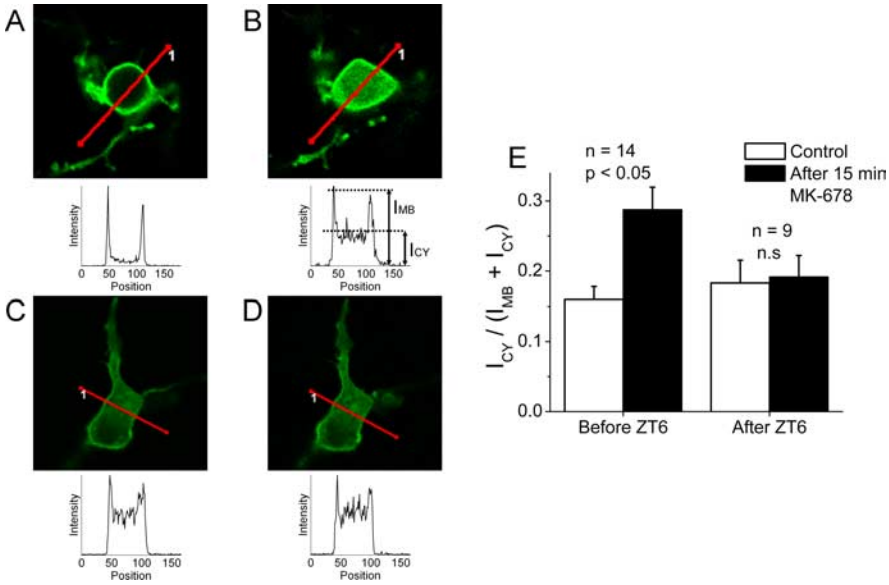

Figure 8. A live-cell imaging assay indicates that MK-678 causes activation of PLC during the early part of the day (ZT4–ZT6). Cultured cone photoreceptors were transfected with an expression vector encoding a PLCδ1-PH domain/GFP fusion protein, which dissociates from the plasma membrane with activation of PLC and cleavage of PtdIns[4,5]P_2. Confocal micrographs of GFP fluorescence were taken from the same cells before and after application of MK-678. ***A***, fluorescence observed in a photoreceptor during the early day (ZT4) in a cell maintained previously in LD. The graph below the micrograph shows fluorescence intensity as a function of distance along the arbitrarily drawn red line, indicating that the most intense fluorescence is in the periphery of the cell. ***B***, Micrograph of the same cell 15 min after application of MK-678. The fluorescence intensity in the interior of the cell is markedly increased, as indicated by the higher plateau between the two peaks in the fluorescence intensity plot. ***C***, ***D***, A similar experiment in a cell observed later in the day, at ZT8 is shown before (***C***) and after (***D***) application of MK-678. Note that there is no rearrangement of fluorescence at that time of day. ***E***, Quantitative results from several cells shows that MK-678 causes a statistically significant rearrangement of fluorescence, measured as a change in the fraction of signal in cell periphery, during the early part of the day (ZT4–ZT6), but not later in the day (after ZT6).

agonists reported previously in the same cells (Stenkamp et al., 1994).

Discussion

Paracrine signals play a role in preparing the vertebrate retina for long-term changes needed to accommodate the changes in ambient illumination that occur over the course of a day. For example, melatonin secreted from photoreceptors produces several effects that appear to be adaptive for dim illumination (Adachi et al., 1998; Anderson and Green, 2000), whereas dopamine secreted from a subpopulation of amacrine cells contributes to a state that is adaptive for high illumination (Manglapus et al., 1999; Ko et al., 2003; Zawilska et al., 2003). Other signals secreted from the inner retina have received less attention as potential modulators of photoreceptors.

ENSLI amacrine cells in the avian retina secrete two biologically active forms of somatostatin, SS14 and SS28 (Ishimoto et al., 1986; Dowton et al., 1994; Watt and Florack, 1994; Yang et al., 1997). Here, we have shown that both isoforms modulate cone CNGCs by altering their sensitivity to activation by cGMP. These effects depend on the time of day, the immediate history of illumination, and which somatostatin isoform is considered. Thus, SS14 and SS28 decrease the sensitivity of CNGCs to activation by cGMP during the subjective night. This response is similar to the sustained effects of D_2 dopamine receptor agonists (Ko et al., 2003), as it can be observed in LD or DD, it is resistant to PTX, and it does not desensitize. Somatostatin and dopamine are secreted from the inner retina at higher levels during the day and during the night in response to light. These sustained responses may serve to increase the robustness of the endogenous circadian rhythms in cones (Ko et al., 2001, 2003), thereby contributing to a slow daily change in the baseline from which phototransduction and adaptation cascades operate.

The more surprising result of this study is that SS28 evokes a second qualitatively different response, namely a transient increase in the sensitivity of CNGCs to cGMP that can be detected during the early part of the day. This effect can only be observed in cones that had been exposed to light for at least 1–2 h before peptide application, and it is mediated by receptors coupled to PLC through a PTX-sensitive G-protein. This effect is not observed in response to application of SS14. The possible physiological significance of the transient response to SS28 is a matter of greater speculation given the limited information on precise isoform-specific temporal patterns of somatostatin secretion in response to light. Indeed, this response appears paradoxical because increases in the sensitivity of CNGCs to activation by cGMP allow photoreceptors to respond over a broader range of light stimulation intensities (Pugh et al., 1999). It is possible that the transient effect of SS28 is a signal that becomes relevant when freely behaving diurnal animals are experiencing rapid changes in ambient illumination. Relatively rapid desensitization of the response to SS28 may reduce the likelihood of photoreceptor light damage (Vallazza-Deschamps et al., 2005; Sharma and Rohrer, 2007). It bears noting that SS28 and SS14 almost certainly produce additional effects in photoreceptors and possibly in other cell types (Akopian et al., 2000). It is also possible that other neurohormones from the inner retina play a role in modulating photoreceptors at other times of day.

CNGCs were originally identified as components of sensory transduction cascades in retinal (Fesenko et al., 1985; Koch and Kaupp, 1985) and pineal (Dryer and Henderson, 1991; Bonigk et al., 1996) photoreceptors, olfactory neurons (Nakamura and Gold, 1987; Goulding et al., 1992), and taste cells (Misaka et al., 1997). More recently they have been observed in several populations of central neurons (Strijbos et al., 1999), as well as in a variety of nonexcitable peripheral tissues, including endothelial cells, liver, skeletal muscle and sperm (Biel et al., 1994; Weyand et al., 1994; Feng et al., 1996). Biophysical studies, in many cases performed on cloned channels have shown that CNGCs can be modulated by a variety of intracellular signals. The most extensively studied of these is Ca^{2+}/calmodulin, which decreases the sensitivity of several types of CNGCs to cyclic nucleotides (Hsu and Molday, 1994; Bonigk et al., 1996). This is thought to be a physiologically important mechanism for desensitization of odorant responses in olfactory neurons (Munger et al., 2001), and a related soluble Ca^{2+}-binding protein may contribute to light-adaptation in teleost cones (Rebrik and Korenbrot, 1998). CNGCs can also be modulated by tyrosine phosphorylation (Molokanova et al., 1997, 1999), and these types of pathways may play a role in circadian control mechanisms in cones (Chae et al., 2007). In addition, phosphorylation on serine or threonine residues can produce multiple effects on rod CNGC gating properties (Gordon et al., 1992; Molokanova et al., 1999).

In the present study, we observed that treatments that cause activation of PLC increase the sensitivity of native cone CNGCs to cGMP during the day, as is seen with SS28. Activation of PLC cleaves PtdIns[4,5]P_2 into Ins[1,4,5]P_3 and DAG. The liberation of DAG in turn causes activation of PKC. We have observed that application of high concentrations of DAG to the cytoplasmic face of excised patches decreases the current evoked by saturating concentrations of cGMP, as has been reported previously for rod type CNGCs (Crary et al., 2000). That effect is unrelated to PKC activation. However, we observed that applying DAG in this way had no effect on the apparent sensitivity of cone CNGCs to cGMP at any concentration of DAG that we tested, in contrast to what we observe with SS28 or MK-678.

More importantly, we observed an increase in the sensitivity of CNGCs to cGMP after exposing intact cells to DAG during the day, and this effect was blocked by Ro-31–8220, an inhibitor that acts on the kinase domain of PKC. These data are consistent with a model in which the effects of SS28 and MK-678 during the early day are mediated by activation of PLC and cytoplasmic PKC, which in turn leads to changes in channel gating. It also bears noting that PLC has been detected in many vertebrate photoreceptors and undergoes light-dependent translocation to the plasma membrane (Gehm et al., 1991; Ghalayini et al., 1991, 1998; Ferreira and Pak, 1994; Peng et al., 1997), which may explain why we see these responses only in cells maintained in LD cycles. Finally, we should note that physiological responses mediated by PLC have not been described previously in vertebrate rods and cones.

Somatostatin actions are mediated by a family of G-protein-coupled receptors. Five types of somatostatin receptors encoded by different genes have been identified in vertebrates, and these receptors can be divided into two classes on the basis of pharmacology and primary structure (Raynor and Reisine, 1992; Patel, 1999). SRIF1 family receptors (comprised of sst_2, sst_3, and sst_5) are selectively activated by MK-678, whereas the only MK-678-resistant SRIF2 receptor in birds is sst_4. Transcripts encoding all four avian somatostatin receptors could be detected by RT-PCR in our cultured photoreceptor preparations. Therefore, our data are consistent with a model in which the sustained effect of SS14 and SS28 is mediated by cone sst_4 because this is the only MK-678 resistant somatostatin receptor known to occur in chickens. The transient light-dependent effect produced by MK-678 and by SS28 could be mediated by sst_2, sst_3, or sst_5. Among these, sst_5

receptors in teleosts and mammals are reported to have a higher affinity for SS28 than SS14 (O'Carroll et al., 1994; Nunn et al., 2002) and they exhibit rapid agonist-dependent internalization (Stroh et al., 2000), especially in response to SST28 (Roth et al., 1997).

The transient effect of SS28 suggests that coupling of at least some cone somatostatin receptors to their biochemical effectors is regulated by previous light illumination and by the circadian cycle. The reason why transient responses to SS28 require previous illumination is unknown. One possibility has already been mentioned, namely that PLC undergoes a light-dependent translocation to the plasma membrane. A more speculative alternative is that this effect entails light-dependent formation of dimers between a somatostatin receptor and an opsin. In mammals, a subpopulation of intrinsically photosensitive retinal ganglion cells express melanopsin, and play a role in nonvisual behavioral responses to light (Peirson and Foster, 2006). However, the chicken retina expresses melanopsin in rod and cone photoreceptors as well as in the inner retina (Chaurasia et al., 2005). Moreover, melanopsin transcripts in photoreceptors are markedly increased during the early morning (ZT0–ZT4) (Chaurasia et al., 2005), the same portion of the circadian cycle in which we observe light-dependent responses to SS28. Formation of receptor dimers can bias a receptor toward activation of a particular G-protein (Herrick-Davis et al., 2005), possibly in this case toward activation of a PTX-sensitive G-protein coupled to activation of PLC. We are currently investigating these possibilities.

In summary, we have demonstrated complex modulation of a photoreceptor ion channel essential for phototransduction by neuropeptides that are secreted from the inner retina. Extrinsic peptidergic modulation of retinal cones may serve to reinforce circadian processes intrinsic to the photoreceptors, and may also contribute to more rapid adaptive responses to changes in ambient illumination.

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