Cellular/Molecular

Adenosine-Evoked Hyperpolarization of Retinal Ganglion Cells Is Mediated by G-Protein-Coupled Inwardly Rectifying K^+ and Small Conductance Ca^{2+}-Activated K^+ Channel Activation

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Adenosine is a neuromodulator that activates presynaptic receptors to regulate synaptic transmission and postsynaptic receptors to hyperpolarize neurons. Here, we report that adenosine-induced hyperpolarization of retinal ganglion cells is produced by the activation of A_1 receptors, which initiates a signaling cascade that activates G-protein-coupled inwardly rectifying K^+ (GIRK) channels and small conductance Ca^{2+}-activated K^+ (SK) channels. Rat retinal ganglion cells were stimulated by focal ejection of the adenosine receptor agonist 5′-*N*-ethylcarboxamidoadenosine (NECA) while cell activity was monitored with whole-cell patch recordings and Ca^{2+} imaging. Focal ejections of NECA evoked outward currents in all cells tested and reduced light- and depolarization-induced spiking. The NECA-evoked current was abolished by the A_1 antagonist 1,3-dipropyl-8-cyclopentylxanthine (DPCPX) but was unaffected by A_{2a}, A_{2b}, and A_3 antagonists, indicating that the response was mediated entirely by A_1 receptors. The GIRK channel blocker rTertiapin-Q diminished the NECA-evoked inhibitory current by 56 ± 12%, whereas the SK channel blocker apamin decreased the NECA-induced current by 42 ± 7%. The SK component of the NECA-evoked current coincided with an increase in intracellular Ca^{2+} and was blocked by IP_3 receptor antagonists and depletion of internal Ca^{2+} stores, suggesting that A_1 receptor activation leads to an increase in IP_3, which then elevates intracellular Ca^{2+} and activates SK channels. This A_1-mediated, prolonged SK channel activation has not been described previously. The coactivation of GIRK and SK channels represents a novel mechanism of adenosine-mediated neuromodulation that could contribute to the regulation of retinal ganglion cell activity.

Introduction

Adenosine activation of G-protein-coupled receptors plays an important role in neuromodulation and neuroprotection throughout the CNS. In the brain, adenosine acts both presynaptically and postsynaptically and can increase or decrease neuronal excitability through several mechanisms. Presynaptically, adenosine alters neurotransmission by activating either A_1 receptors to decrease neurotransmitter release or A_{2A} receptors to potentiate neurotransmitter release. The inhibitory presynaptic effects of A_1 receptors are attributed to A_1-mediated inhibition of N-type Ca^{2+} channels and are observed in the laterodorsal tegmentum, hippocampus, hypothalamus, nucleus accumbens, and barrel cortex (Arrigoni et al., 2001; Cunha, 2001; Solinas et al., 2002; Quarta et al., 2004a; Fontanez and Porter, 2006; Borycz et al., 2007; Liu and Gao, 2007). Adenosine-evoked increases in neurotransmitter release are primarily mediated by the A_{2A} receptor-induced activation of P-type Ca^{2+} channels and have also been described in several CNS regions, including the nucleus accumbens, hippocampus, superior colliculus, and habenula (Cunha, 2001; Solinas et al., 2002; Quarta et al., 2004b). Postsynaptically, adenosine modulates cellular excitability through activation of A_1 receptors that are linked to G-protein-coupled inwardly rectifying K^+ (GIRK) channels. Adenosine-evoked hyperpolarizing currents have been demonstrated in the hippocampus, the laterodorsal tegmentum, and the supraoptic nucleus (Luscher et al., 1997; Cunha, 2001; Ponzio and Hatton, 2005).

In the retina, adenosine contributes to the modulation of retinal ganglion cell activity. In ganglion cells of the rat and salamander retinas, A_1 receptor activation inhibits voltage-gated Ca^{2+} channels (Sun et al., 2002; Hartwick et al., 2004). In addition, we have reported that stimulating retinal glial cells of the rat results in the generation of an adenosine-mediated inhibitory current in ganglion cells (Newman, 2003). Stimulated glial cells release ATP that is rapidly converted to adenosine by ectoenzymes. The adenosine activates receptors on the ganglion cells, resulting in cell hyperpolarization mediated by the opening of K^+ channels. However, the intracellular signaling mechanisms responsible for this adenosine-evoked inhibition of retinal ganglion cells have not been elucidated.

In this study, we examine adenosine modulation of retinal ganglion cells in the rat retina. We have investigated which sub-

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types of adenosine receptors and which ion channels mediate the adenosine-evoked hyperpolarization of ganglion cells. We find that the adenosine response is mediated by A_1 receptor activation of two distinct K^+ channels: GIRK channels and small conductance Ca^{2+}-activated K^+ (SK) channels. We also find that adenosine receptor activation results in altered spiking dynamics in the cells. The prolonged SK channel activation we observe, mediated by the A_1 receptor-induced release of Ca^{2+} from intracellular stores, has not been described previously.

Materials and Methods

Isolated retina preparation. Long–Evans rats (150–350 g) were killed by overdose of isoflurane, followed by opening of the chest cavity. The eyes were enucleated and halved, and the retina was peeled from the sclera, as described previously (Newman and Zahs, 1998). The vitreous humor was removed from pieces of retina with forceps. The retina was then incubated in collagenase (120 U/ml) and hyaluronidase (500 U/ml) for 7 min and placed in a recording chamber. The tissue was held in place with a platinum ring bridged by nylon threads and superfused at 2–3 ml/min with either bicarbonate-buffered or HEPES-buffered Ringer's solution. Except where noted otherwise, experiments were conducted at 24°C. The animals used in this study were treated in accordance with the guidelines of the Institutional Animal Care and Use Committee of the University of Minnesota.

Electrophysiology. Whole-cell current-clamp and voltage-clamp recordings were obtained from the somata of retinal ganglion cells using borosilicate patch pipettes. The retina was imaged with differential interference contrast optics (BX60 microscope and a 40×, 0.8 numerical aperture objective; Olympus). Patch pipettes were lowered into the ganglion cell layer, and positive pressure was applied to remove glial processes from the retinal ganglion cell soma. Negative pressure was then applied to create a high resistance seal, followed by an increase in the pressure to break through the cell membrane. Current pulses (600 ms, 100 pA) were administered to the cell to monitor the cell's spiking behavior and ensure that the cell was healthy. Cell voltage was corrected for the pipette junction potential, which equaled 10 mV.

In some experiments, a second patch pipette was used to introduce GTPγS or GDPβS into a cell already being recorded. The cell's resting membrane potential and spiking behavior were monitored after addition of the second patch pipette to ensure that the cell remained healthy. The cell was discarded if there was a large change in the resting membrane potential.

All recordings were made with an Axoclamp-2A amplifier (Molecular Devices). Voltage-clamp holding potentials were set to the cell's initial resting membrane potential. The adenosine agonist 5′-*N*-ethylcarboxamidoadenosine (NECA) was focally ejected onto retinal ganglion cells from a pipette at 5–15 psi using a Picospritzer (General Valve).

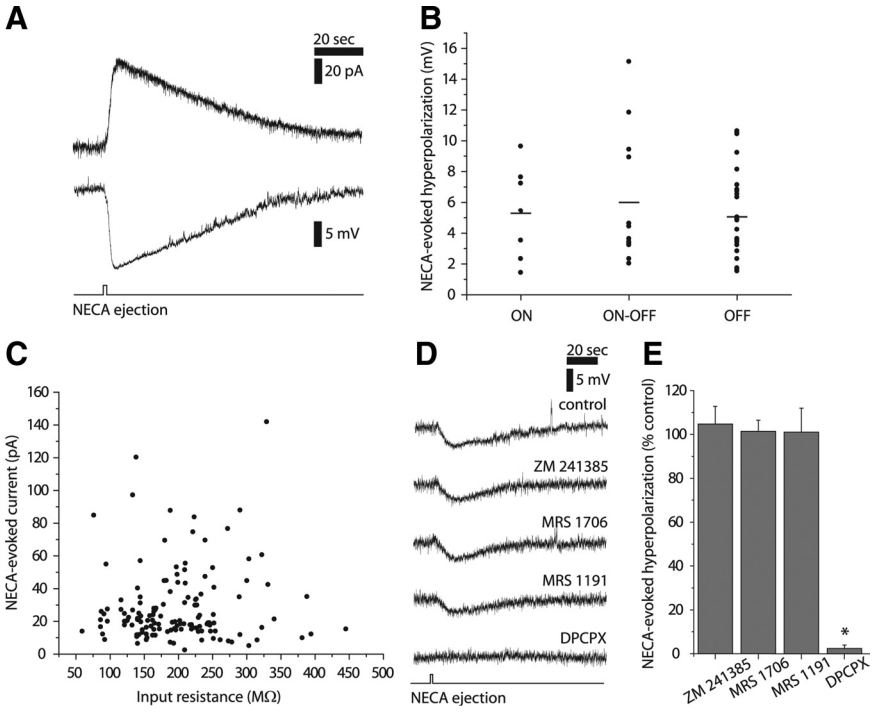

Figure 1. Hyperpolarization of retinal ganglion cells by the adenosine receptor agonist NECA is mediated by activation of A_1 receptors. ***A***, Focal ejection of NECA onto a ganglion cell evokes a prolonged hyperpolarization (recorded in current clamp) and an outward current (recorded in voltage clamp). ***B***, ON, ON-OFF, and OFF classes of retinal ganglion cells all exhibit NECA-evoked hyperpolarization. Mean response amplitudes (horizontal lines) do not differ between ganglion cell classes. ***C***, There is no correlation between the input resistance of retinal ganglion cells and the amplitude of NECA-evoked current. ***D***, ***E***, Antagonists to A_{2A} (ZM 241385), A_{2B} (MRS 1706), and A_3 (MRS 1191) receptors have no effect on the NECA-evoked hyperpolarization recorded from ganglion cells, but the A_1 antagonist DPCPX decreases the NECA-evoked response to $2 \pm 2\%$ of control. Antagonists were applied sequentially for 15 min each, with DPCPX applied last. The current-clamp traces in ***D*** were all recorded from the same ganglion cell. $^*p < 0.001$.

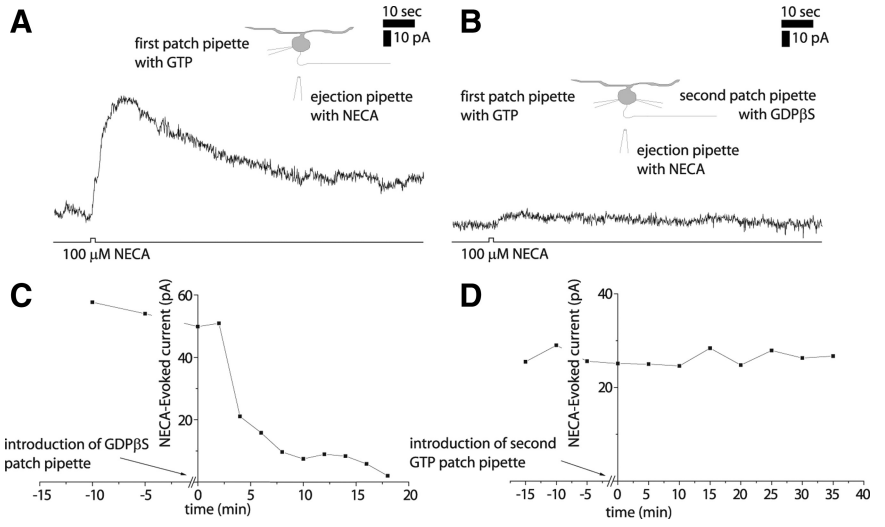

Figure 2. GDPβS, an antagonist of G-protein signaling, decreases NECA-evoked current in retinal ganglion cells. ***A***, NECA ejection evokes an outward current in a ganglion cell. ***B***, The NECA-evoked current is reduced substantially 15 min after GDPβS is introduced into the cell through a second patch pipette. ***C***, The amplitude of NECA-evoked currents as a function of time. At time 0, GDPβS was introduced into the cell through the second patch pipette. As GDPβS diffuses into the cell, the NECA-evoked response decreases. Responses in ***A–C*** are from the same cell. ***D***, Control experiment in which the second patch pipette contains the same intracellular solution as the first pipette. There is no change in the amplitude of the NECA-evoked current, demonstrating that introduction of a second pipette per se does not decrease the cell's NECA response.

Calcium imaging. Fluo-4 K^+ salt (100 μM) was included in the intracellular pipette solution to image Ca^{2+} in retinal ganglion cells. Calcium indicator dye fluorescence was monitored with 488 nm excitation, a 500 nm long-pass barrier filter, and confocal microscopy (Odyssey scanner;

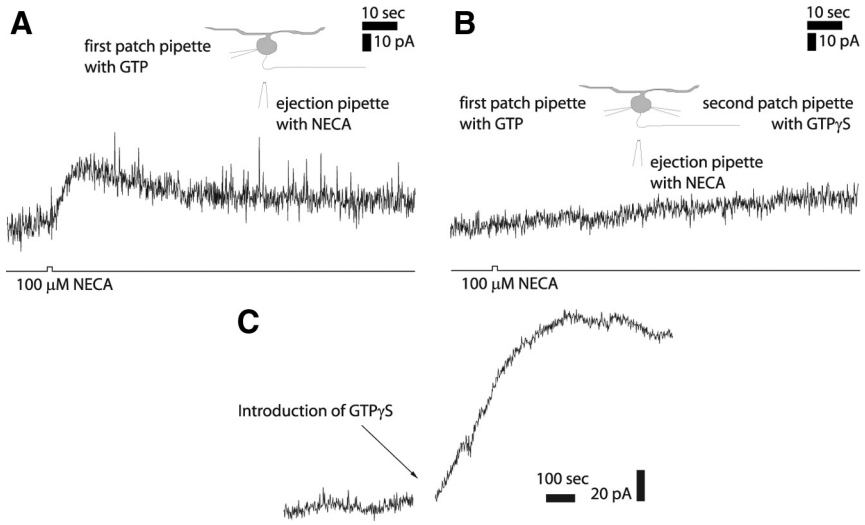

Figure 3. Constitutive activation of G-protein signaling by GTPγS leads to the generation of a prolonged outward current and to a decrease in the NECA response. ***A***, NECA ejection evokes an outward current in a ganglion cell. ***B***, The NECA-evoked current is reduced substantially 15 min after GTPγS is introduced into the cell through a second patch pipette. ***C***, Introduction of GTPγS leads to a prolonged outward current that is substantially larger than the initial NECA-evoked response.

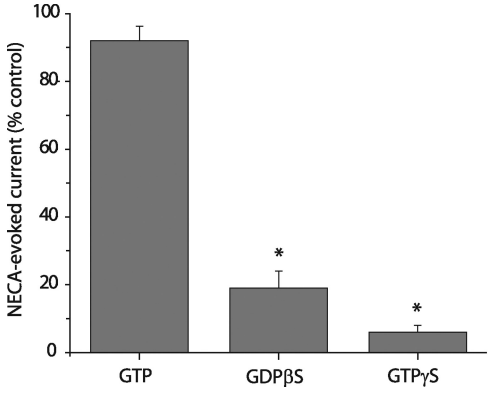

Figure 4. Disruption of G-protein signaling decreases the NECA-evoked response in retinal ganglion cells. Introduction of GDPβS into ganglion cells decreased the NECA-evoked outward current to 19 ± 5% of control. Introduction of GTPγS decreased the amplitude of the NECA response to 6 ± 2% of control. The NECA response was not altered in control experiments in which the second patch pipette contained GTP instead of GTPγS. *$p < 0.05$.

Noran, Thermo Fisher Scientific). Images were averaged for 1 s and were acquired using MetaMorph software (Molecular Devices).

Solutions. The bicarbonate-buffered Ringer's solution contained (in mM) 117.0 NaCl, 3.0 KCl, 2.0 $CaCl_2$, 1.0 $MgSO_4$, 0.5 NaH_2PO_4, 15.0 D-glucose, and 26.0 $NaHCO_3$, bubbled with 5% CO_2 in O_2. In experiments in which Ba^{2+} was included in the perfusate, we used a HEPES-buffered Ringer's solution containing (in mM) 135.5 NaCl, 3.0 KCl, 2.0 $MgCl_2$, 15.0 D-glucose, and 10.0 HEPES, pH 7.4. The intracellular pipette solution contained (in mM) 5.0 Na-methanesulfonate, 128.0 K-methanesulfonate, 2.0 $MgCl_2$, 1.0 glutathione, 2.0 MgATP, 0.2 NaGTP, and 5.0 HEPES, pH 7.4. In some experiments, NaGTP was replaced with either 300 μM GTPγS or 1 mM GDPβS. Lucifer yellow CH (0.005%) was included in the intracellular pipette solution to visualize cells.

All reagents were purchased from Sigma, except NECA, ZM 241385, MRS 1706, 1,3-dipropyl-8-cyclopentylxanthine (DPCPX), and 2-aminoethoxydiphenyl borate (2-APB), which were purchased from Tocris Bioscience; rTertiapin-Q, which was purchased from Alomone Labs; Fluo-4 K^+ salt, which was purchased from Invitrogen; and collagenase and hyaluronidase, which were purchased from Worthington Biochemicals.

Statistics. Numerical values are given as mean ± SEM. Statistical significance was determined with the Student's *t* test for paired and unpaired samples.

Results

NECA-evoked current is mediated by A_1 receptor activation

The response of retinal ganglion cells to adenosine receptor activation was studied in the isolated rat retina. All cells that were monitored had axons (visualized with Lucifer yellow fills), confirming that they were ganglion cells and not displaced amacrine cells. Ganglion cell recordings were made in either voltage-clamp or current-clamp as 100 μM NECA, a nonselective adenosine agonist, was focally ejected onto the cell for 1.5 s. All ganglion cells tested exhibited a slow outward current or hyperpolarization after NECA stimulation (Fig. 1*A*). The outward current ranged from 5 to 113 pA, and the hyperpolarization ranged from 1.5 to 15 mV. In preliminary experiments, when 5 mM EGTA was included in the intracellular pipette solution, ~20% of the ganglion cells tested failed to respond to NECA ejection.

Ganglion cells were classified as ON, ON-OFF, or OFF cells, depending on their spiking response to a 10 s diffuse light flash. All three ganglion cell classes responded to NECA ejections with hyperpolarizations. Response amplitudes were approximately equal for the three ganglion cell classes, with mean hyperpolarization equaling 5.29 ± 1.14 mV ($n = 7$), 6.00 ± 1.23 mV ($n = 12$), and 5.06 ± 0.55 mV ($n = 27$) for ON, ON-OFF, and OFF cells, respectively (Fig. 1*B*). The input resistance of ganglion cells (measured with 100 pA current pulses) was determined as an indicator of cell size. Input resistance ranged from 76 to 445 MΩ ($n = 139$), corresponding to a wide range of ganglion cell sizes (O'Brien et al., 2002). There was no correlation between the amplitude of NECA-evoked outward current and cell input resistance ($r^2 = 0.0063$) (Fig. 1*C*).

Since NECA is a nonselective agonist, we determined which adenosine receptor subtypes mediated the NECA-evoked hyperpolarization. Bath application of selective antagonists for A_{2A} (10 μM ZM 241385), A_{2B} (10 μM MRS 1706), and A_3 (10 μM MRS 1191) receptors had no effect on the NECA-evoked response, although the concentrations used were well above their K_i values. In the presence of these antagonists, responses were 105 ± 8% ($n = 8$; $p > 0.05$), 101 ± 5% ($n = 8$; $p > 0.05$), and 101 ± 11% ($n = 5$; $p > 0.05$) of control, respectively. In contrast, the A_1 receptor antagonist DPCPX decreased the NECA-evoked hyperpolarization to 14 ± 5% of control at 1 nM ($n = 6$; $p < 0.001$) and to 2 ± 2% of control at 10 nM ($n = 7$; $p < 0.001$) (Fig. 1*D*,*E*). These results indicate that the response to focal NECA ejections is mediated solely by A_1 adenosine receptors.

NECA-evoked current is dependent on G-protein signaling

A_1 receptors are coupled to G_i/G_o-proteins that can activate GIRK channels via the βγ subunit of the G-protein heterotrimer (Trussell and Jackson, 1985; Mark and Herlitze, 2000; Cunha, 2001). To determine whether the NECA-evoked current was dependent on this G-protein second-messenger system, we disrupted G-protein signaling within ganglion cells with 100 μM

GDPβS, a nonhydrolyzable guanosine diphosphate that antagonizes G-protein activity. In these experiments, we used a double patch recording protocol with only the second patch pipette containing GDPβS. Using this technique, we ensured that the amplitude of the cell's NECA response was stable by applying multiple NECA ejections to the cell before disrupting G-protein signaling. The technique provided within-cell controls for all of our experiments that disrupted intracellular signaling.

We first patched onto a ganglion cell and ejected NECA onto the cell to determine the magnitude of the cell's response (Fig. 2*A*). NECA ejections were given every 5 min for 15 min before patching onto the cell with a second patch pipette containing GDPβS. After breakthrough of the second pipette, the resting membrane potential of the cell was monitored along with the cell's spiking activity in response to a current pulse to ensure that the cell remained healthy. NECA was ejected onto the cell every 2 min, and the amplitude of the response was recorded (Fig. 2*B*). The amplitude of the NECA-evoked current decreased rapidly as GDPβS diffused into the cell ($n = 7$) (Fig. 2*C*), indicating that the NECA-evoked response is dependent on G-protein signaling. Within 15 min, the NECA-evoked current was reduced to 19 ± 5% of the control response (Fig. 4) ($n = 7$; $p < 0.001$).

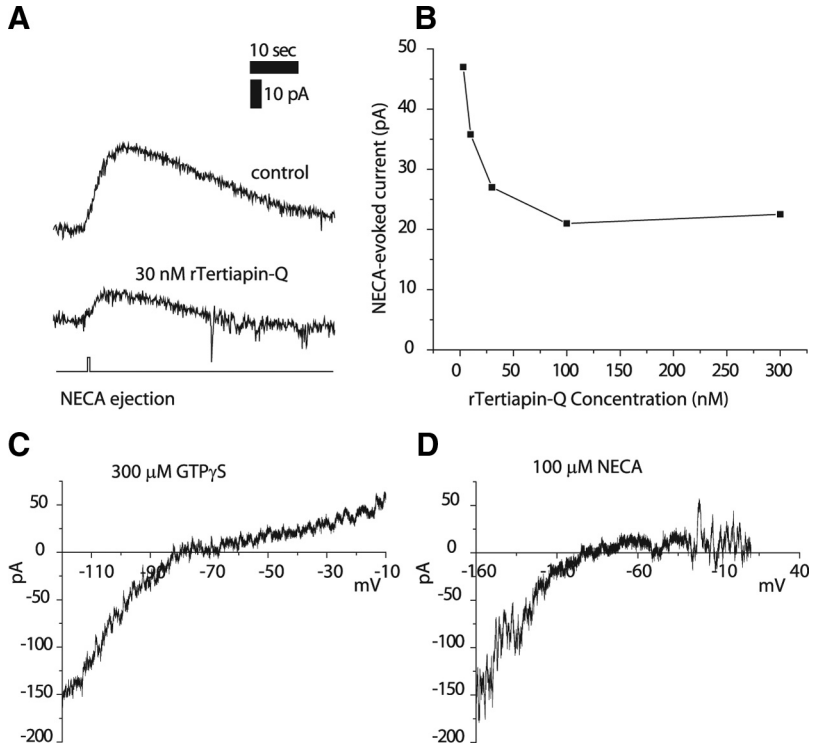

Figure 5. Focal ejection of NECA onto retinal ganglion cells activates GIRK channels. ***A***, NECA-evoked outward currents are decreased by bath application of the GIRK channel blocker rTertiapin-Q. ***B***, Dose–response relationship of rTertiapin-Q inhibition of NECA-evoked current in a single ganglion cell. A substantial fraction of the current remains after GIRK channel block. ***C***, Constitutive activation of G-protein signaling by introduction of GTPγS evokes a current with an inwardly rectifying *I–V* relationship and a reversal potential near the K^+ equilibrium potential. ***D***, NECA ejection evokes a current with a similar inwardly rectifying *I–V* relationship. Recording protocols for the *I–V* plots in ***C*** and ***D*** are given in Results.

Control experiments were conducted with GTP instead of GDPβS in the second pipette to determine whether introduction of the second pipette was responsible for decreasing the cell's response to NECA. After introducing the second pipette, we measured the cell's NECA response every 5 min for over 30 min and saw little change in the amplitude of the NECA-evoked response (Fig. 2*D*). The NECA-evoked outward current was reduced by only 8 ± 4% after 30 min ($n = 6$; $p > 0.05$), indicating that the second pipette did not alter the responsiveness of the retinal ganglion cell (see Fig. 4).

If the NECA-evoked current is dependent on G-protein signaling, then constitutive activation of G-proteins should lead to a saturation of the outward current mediated by G-protein signaling and, consequently, to a diminished NECA response. To test this prediction, we introduced a second patch pipette containing 100 μM GTPγS, a nonhydrolyzable guanosine triphosphate that constitutively activates G-protein signaling. In this experiment, we first patched onto a retinal ganglion cell with a pipette containing GTP and ejected NECA to determine the initial NECA response of the cell (Fig. 3*A*). We then recorded the baseline current of the cell for 5 min as we clamped it at its initial resting membrane potential. A second patch pipette, containing GTPγS, was then patched onto the cell. After breakthrough, the outward current of the cell gradually increased over the next 5 min and reached a plateau level that was greater than the amplitude of the initial NECA response (Fig. 3*C*). A NECA ejection administered during the plateau phase evoked a response that was only 6 ± 2% of the control amplitude ($n = 6$; $p < 0.001$) (Figs. 3*B*, 4), confirming that the NECA-evoked outward current is dependent on G-protein signaling.

GIRK channels contribute to NECA-evoked outward current

Since adenosine is known to activate GIRK channels in other areas of the CNS (Cunha, 2001; Dunwiddie and Masino, 2001) and the outward current recorded from retinal ganglion cells was dependent on G-protein signaling, we hypothesized that GIRK channels mediated the outward current. To test this hypothesis, we determined whether rTertiapin-Q, a synthetic peptide GIRK channel blocker, reduced the NECA-evoked outward current. Bath application of 30 nM rTertiapin-Q diminished the outward current to 44 ± 12% of control ($n = 13$; $p < 0.01$), from 43 ± 8 to 19 ± 4 pA (Fig. 5*A*). Increasing the concentration of rTertiapin-Q to 100 nM or even 300 nM, well above its K_i of 1.25 nM (Jin and Lu, 1999), did not abolish the remaining NECA-evoked current (Fig. 5*B*), indicating that although GIRK channels contribute to the outward current, there is an additional component of the current generated by another channel(s).

Barium, at low concentrations, is a selective blocker of inwardly rectifying K^+ channels, including GIRK channels. We tested whether Ba^{2+} would also block NECA-evoked responses. Bath application of 100 μM Ba^{2+} diminished the NECA response to 45 ± 7% of control ($n = 10$; $p < 0.001$), similar to the reduction produced by rTertiapin-Q.

The current–voltage (*I–V*) relation of the NECA-evoked outward current provided additional evidence that the response was generated, in part, by GIRK channels. In isolated retinal ganglion cells, inwardly rectifying currents have been recorded after the diffusion of GTPγS into cells (Chen et al., 2004). We recorded a

similar current from retinal ganglion cells in the isolated retina. We patched onto retinal ganglion cells with a pipette containing 300 μM GTPγS and determined the *I–V* relation of the GTPγS-induced current using a voltage ramp sweeping from −110 to 0 mV in 1 s. *I–V* relations were determined immediately after breakthrough and again after, allowing GTPγS to diffuse into the cell for 5 min. Subtracting the two traces yielded an *I–V* relation with pronounced inward rectification and a reversal potential of −80 ± 10 mV ($n = 4$), near the calculated K^+ equilibrium potential of −97 mV (Fig. 5*C*).

We recorded similar inwardly rectifying *I–V* relations for currents evoked by NECA ejections. Retinal ganglion cell *I–V* relations were determined with a voltage ramp (−150 to 20 mV in 1 s) at the peak of the NECA-evoked outward current and subtracted from *I–V* relations determined before the NECA ejection. The resulting NECA-evoked *I–V* relations displayed pronounced inward rectification with a reversal potential at −93 ± 9 mV ($n = 5$) (Fig. 5*D*). NECA-evoked rectification was more pronounced after bath application of apamin to block SK channels.

SK channels mediate an additional component of the NECA-evoked response

In hippocampal organotypic slice cultures, adenosine receptors mediate an increase in the afterhyperpolarization current (I_{AHP}) after action potentials (Gerber and Gahwiler, 1994). One of the channels that contributes to I_{AHP} is the SK channel (Sah and Faber, 2002; Stocker, 2004). Immunolabeling has demonstrated that SK channels are expressed on both the soma and dendrites of retinal ganglion cells (Klocker et al., 2001).

We bath applied apamin (100 nM), a selective SK channel toxin, to determine whether a portion of the NECA-evoked outward current is mediated by SK channels. In the presence of apamin, the outward current evoked by a NECA ejection was diminished to 58 ± 7% of control ($n = 11$; $p < 0.005$) (Fig. 6*A*). In the presence of both apamin and the GIRK channel blocker rTertiapin-Q (30 nM), the NECA-evoked current was further diminished to 18 ± 1% of control ($n = 4$; $p < 0.005$), indicating that activation of GIRK and SK channels are responsible for most, if not all, of the NECA-evoked response. Similar results were obtained when SK and GIRK channels were blocked with apamin (100 nM) and Ba^{2+} (100 μM), respectively (Fig. 6*B*). In the presence of both apamin and Ba^{2+}, the NECA-evoked current was reduced to 15 ± 4% of control ($n = 7$; $p < 0.01$).

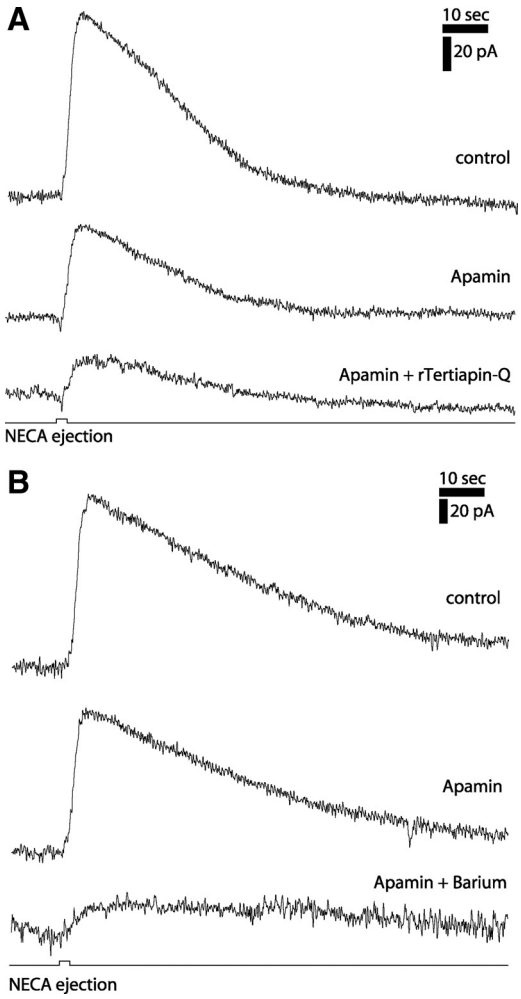

Figure 6. A non-GIRK component of the NECA-evoked response in retinal ganglion cells is generated by SK channels. ***A***, The NECA-evoked outward current is partially blocked by bath application of the SK channel antagonist apamin. Bath application of apamin together with the GIRK channel blocker rTertiapin-Q further diminishes the current. ***B***, Similarly, bath application of apamin partially blocks the NECA-evoked current, and application of apamin together with the GIRK channel blocker barium further diminishes the current.

NECA-evoked SK current is dependent on Ca^{2+} release from IP_3 receptors

A_1 receptor activation can lead to inositol 1,4,5-trisphosphate receptor (IP_3R)-mediated release of Ca^{2+} from internal stores (Basheer et al., 2002). Since the gating of SK channels is Ca^{2+} dependent, we hypothesized that in retinal ganglion cells, the activation of A_1 receptors leads to an IP_3R-mediated increase in intracellular Ca^{2+} and subsequent opening of SK channels. To test this, we depleted intracellular Ca^{2+} stores with cyclopiazonic acid (CPA), which blocks the endoplasmic reticulum Ca^{2+}-ATPase. Bath application of 20 μM CPA for 15 min reduced the NECA-evoked current to 59 ± 10% of control ($n = 6$; $p < 0.005$) (Fig. 7*A*,*D*), indicating that Ca^{2+} release from internal stores mediates a component of the NECA response.

SK channels could be activated by a NECA-evoked Ca^{2+} influx across the plasma membrane as well as by release of Ca^{2+} from internal stores. To test this, retinas were superfused with a Ca^{2+}-free bath solution (containing 10 μM EGTA). NECA-evoked currents, recorded after 10 min in Ca^{2+}-free solution, were not diminished (101 ± 1% of control; $n = 6$; $p > 0.05$). The addition of apamin to the Ca^{2+}-free solution, however, reduced the NECA-evoked current to 63 ± 4% of control ($n = 5$; $p < 0.001$) (Fig. 7*B*,*D*). These results demonstrate that Ca^{2+} influx does not contribute significantly to NECA-activation of SK channels.

If NECA is activating SK channels through the release of Ca^{2+} from internal stores, it is likely that the Ca^{2+} release is through IP_3Rs. We tested this by blocking IP_3Rs with 2-APB. Bath application of 2-APB (20 μM) for 10 min decreased the NECA-evoked outward current to 50 ± 7% of control ($n = 8$; $p < 0.001$) (Fig. 7*C*,*D*). The addition of apamin to the 2-APB solution did not reduce the NECA-evoked current further, demonstrating that block of IP_3Rs completely eliminates the SK-mediated current.

2-APB is a nonselective drug. In addition to blocking IP_3Rs, it can block transient receptor potential channels (Zhou et al., 2007) and interfere with mitochondrial Ca^{2+} regulation (Nicoud et al., 2007). To insure that NECA-evoked Ca^{2+} release from internal stores is through IP_3Rs, we blocked the receptors with a second drug, heparin (5 mg/ml), which was added to the intracellular pipette solution. NECA-evoked currents were recorded

immediately after patch pipette breakthrough and again after, allowing heparin to diffuse into the cell for 15 min. Heparin reduced the NECA-evoked current to 53 ± 6% of control ($n = 6$; $p < 0.001$) (Fig. 7*D*), nearly the same reduction as with 2-APB. Bath application of apamin to cells treated with heparin produced no further decrease in NECA-evoked current (56 ± 13% of control; $n = 3$), indicating that heparin block of IP_3Rs, like 2-APB block, completely eliminated the SK mediated current.

Adenosine receptor activation should evoke intracellular Ca^{2+} increases in retinal ganglion cells if a component of the NECA-evoked current is mediated by SK channels. We monitored intracellular Ca^{2+} levels with Fluo-4, a Ca^{2+} indicator dye, to test this prediction (Fig. 8*A*). NECA ejections evoked a 6 ± 1% increase in Ca^{2+} indicator dye fluorescence in the soma of retinal ganglion cells ($n = 9$; $p < 0.01$). The time course of the NECA-evoked Ca^{2+} increase and the simultaneously recorded outward current corresponded closely (Fig. 8*B*). Calcium was also monitored in the dendrites of ganglion cells, revealing dendritic NECA-evoked Ca^{2+} transients in three of the four cells monitored (Fig. 9*C*).

We also tested whether the NECA-evoked Ca^{2+} increases were mediated by IP_3Rs. Blocking IP_3Rs with 2-APB led to an 83 ± 1% decrease in the NECA-evoked Ca^{2+} response measured in the cell soma (Fig. 8*D*,*E*) ($n = 4$; $p < 0.001$). 2-APB also resulted in a 73 ± 9% decrease in the NECA-evoked outward current recorded from these cells ($n = 4$; $p < 0.001$) (Fig. 8*D*,*E*).

NECA-evoked hyperpolarization reduces ganglion cell spiking

Because adenosine receptor activation of GIRK and SK channels evokes ganglion cell hyperpolarization, it should result in a decrease in cell spiking. We tested this by recording ganglion cell action potentials evoked by 500 ms diffuse light flashes. Flashes were repeated at 10 s intervals and reliably evoked bursts of action potentials in ON (Fig. 9*A*), ON-OFF, and OFF ganglion cells. Immediately after activation of adenosine receptors by NECA ejection, the total number of spikes evoked by a light flash decreased significantly (Fig. 9*A*,*C*). The decrease in spiking was correlated with cell hyperpolarization produced by the NECA ejection. On average, the number of light-evoked spikes produced at the peak of the NECA response was reduced to 31% of the pre-NECA spike count ($n = 5$; $p < 0.05$), from 5.9 ± 0.6 to 1.8 ± 0.3 spikes. NECA reduced spiking in ON, ON-OFF, and OFF classes of ganglion cells.

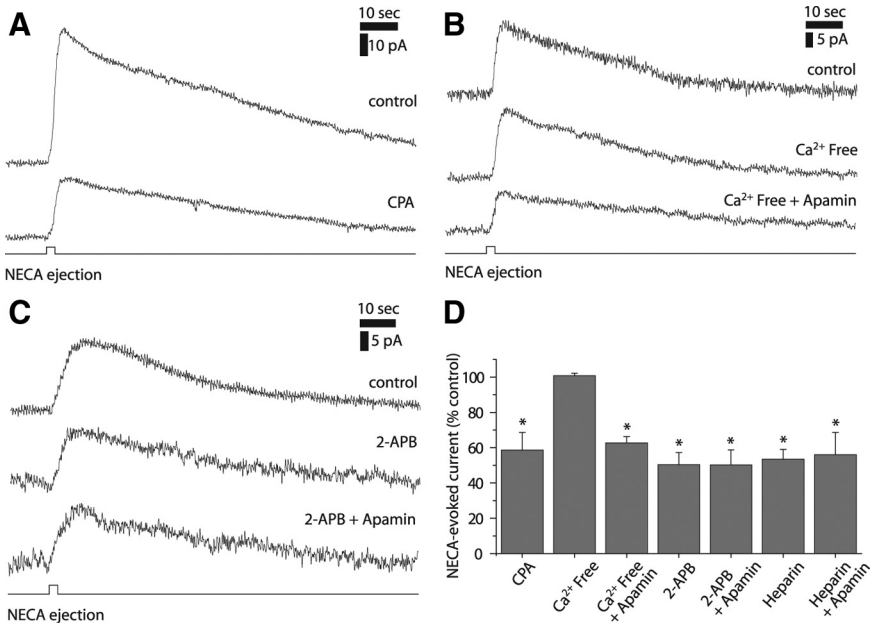

Figure 7. A component of the NECA-evoked response is generated by SK channels activated by Ca^{2+} release from internal stores. ***A***, Bath application of CPA partially reduces the NECA-evoked current. ***B***, Removal of Ca^{2+} from the bath solution does not reduce the NECA-evoked current. The addition of apamin to the Ca^{2+}-free solution partially reduces the current. ***C***, Bath application of 2-APB, an IP_3R antagonist, partially blocks the NECA-evoked current. The addition of apamin to the 2-APB solution does not reduce the current further. ***D***, Summary of the results. Heparin, a second IP_3R antagonist, reduces the NECA-evoked response to a similar degree as 2-APB. *$p < 0.005$.

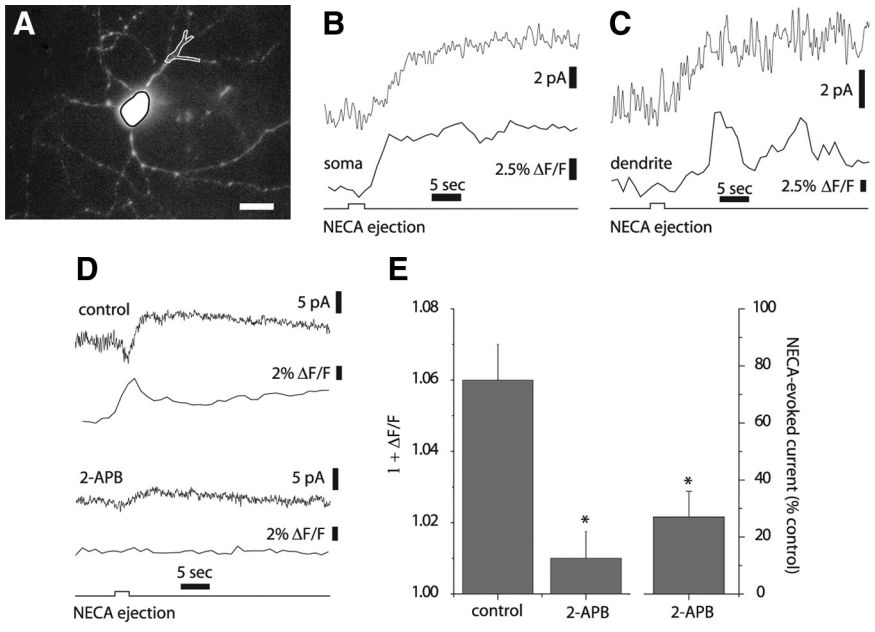

Figure 8. Adenosine receptor activation evokes an IP_3R-mediated intracellular Ca^{2+} increase in retinal ganglion cells. ***A***, NECA-evoked intracellular Ca^{2+} changes were monitored in the soma and dendrite of a ganglion cell filled with the Ca^{2+} indicator dye Fluo-4. Calcium traces, shown in ***B*** and ***C***, were taken from regions delineated in the image. Scale bar, 20 μm. ***B***, NECA evokes a somatic Ca^{2+} increase that has a similar time course as the evoked current. ***C***, NECA evokes transient Ca^{2+} increases in a dendrite of the same cell. ***D***, The NECA-evoked increase in Ca^{2+} within the soma of a ganglion cell is blocked by 2-APB. 2APB also reduces the NECA-evoked outward current. ***E***, Summary of experiments shown in ***D***. The left two bars indicate NECA-evoked Ca^{2+} responses in control and 2-APB-treated retinas. The right bar shows the 2-APB-mediated reduction in NECA-evoked current. *$p < 0.001$.

Ejection of NECA could affect neurons in the retina besides ganglion cells. Thus, modulation of light-evoked spiking in ganglion cells might be caused by a presynaptic mechanism rather than direct NECA modulation of ganglion cells. We tested

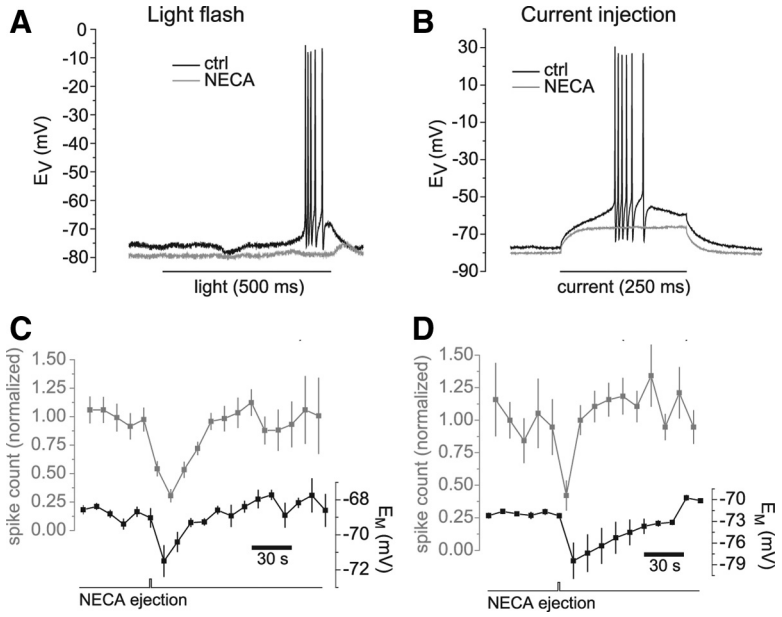

Figure 9. NECA-evoked hyperpolarization reduces ganglion cell spiking. Spiking is evoked by diffuse light flashes (***A***, ***C***) or by depolarizing current injection (***B***, ***D***). Stimuli are repeated every 10 s. ***A***, ***B***, In the absence of NECA, the stimulus reliably evokes action potentials (black traces) in ganglion cells. Five seconds after ejecting NECA (100 μM) onto the ganglion cell, the cell is hyperpolarized, and the same stimulus no longer evokes action potentials (gray traces). ctrl, Control. ***C***, ***D***, The total number of spikes evoked by the stimulus is shown in gray. Membrane potential is shown in black. A transient reduction in spiking after NECA ejection matches the time course of the NECA-evoked hyperpolarization. Responses from ON, ON-OFF, and OFF classes of ganglion cells were pooled.

whether modulation of spiking was a direct NECA affect on ganglion cells by evoking spikes in ganglion cells by injection of 250 ms depolarizing current pulses. Current pulses (20–100 pA) were repeated every 10 s and reliably evoked bursts of action potentials (Fig. 9*B*). Immediately after a NECA ejection, the total number of spikes evoked by a current pulse decreased significantly (Fig. 9*B*,*D*). The decrease in spiking was correlated with cell hyperpolarization produced by the NECA ejection, although spiking recovered more rapidly than did the membrane potential. On average, the number of spikes produced at the peak of the NECA response was reduced to 42% of the pre-NECA spike count ($n = 4$; $p < 0.05$), from 4.8 ± 0.9 to 2.0 ± 0.5 spikes.

In addition to reducing the total number of spikes evoked by a stimulus, NECA also altered the interspike interval and afterhyperpolarization amplitude. For spike trains evoked by depolarizing current pulses, the interspike interval between the first and second spikes of a burst decreased immediately after a NECA ejection ($n = 3$) (Fig. 10*A*,*B*). In addition, NECA reduced the amplitude of the afterhyperpolarization after action potentials ($n = 4$) (Fig. 10*A*,*C*). The decrease in interspike interval and the reduction in afterhyperpolarization were both correlated with the hyperpolarization evoked by NECA ejection (Fig. 10*B*,*C*). The results demonstrate that activation of adenosine receptors can modulate spike timing as well as spike generation in retinal ganglion cells.

Experiments in this study were conducted at 24°C, as the isolated retina remains healthy for a longer time at this temperature than at physiological temperature. However, we conducted a few experiments at 35°C to determine whether adenosine receptor activation evokes the same currents that were observed at 24°C. NECA ejection evoked outward currents ranging in amplitude from 10 to 103 pA at 35°C. Bath application of apamin (100 nM) reduced these currents to 66 ± 8% of control ($n = 6$; $p < 0.01$). Bath application of apamin and Ba^{2+} (100 μM) together further reduced the currents to 25 ± 6% of control ($n = 5$; $p < 0.01$). We conclude that at 35°C, as at 24°C, NECA evokes both GIRK and SK currents in retinal ganglion cells.

Discussion

Our results demonstrate that stimulation of adenosine receptors activates two K^+ currents in retinal ganglion cells, one that is mediated by GIRK channels and the other by SK channels. Although all four subtypes of adenosine receptors (A_1, A_{2A}, A_{2B}, and A_3) are expressed in the retina and both A_1 and A_3 receptors are expressed in ganglion cells (Braas et al., 1987; Blazynski and Perez, 1991; Zhang et al., 2006), the A_1 receptor is responsible for activating both the GIRK and SK components of the K^+ current.

Neurons in many CNS regions exhibit an increase in K^+ conductance in response to extracellular adenosine (Trussell and Jackson, 1985; Gerber et al., 1989). Typically, this inhibitory response is attributed to the postsynaptic activation of GIRK channels (Cunha, 2001; Dunwiddie and Masino, 2001). It is not surprising, therefore, that the NECA-evoked outward current that we observed in retinal ganglion cells is mediated, in part, by GIRK channels. A previous study of retinal ganglion cells demonstrated that GIRK channels are expressed on the somata and dendrites of these retinal neurons and that bath application of $GABA_B$ agonists leads to GIRK channel activation in these cells (Chen et al., 2004).

Our results reveal, however, that GIRK channels account for only about half of the NECA-evoked current recorded from retinal ganglion cells. The remainder of the K^+ current is generated by SK channels. Previous studies have demonstrated that SK channels are expressed on retinal ganglion cells (Klocker et al., 2001) and contribute to the regulation of spike generation (Wang et al., 1998). In hippocampal organotypic slice cultures, adenosine increases the I_{AHP} (Gerber and Gahwiler, 1994). In addition, stimulation of G-protein-coupled receptors in human lens (Rhodes et al., 2003) and rat distal colon (Van Crombruggen and Lefebvre, 2004) result in SK channel activation. However, direct evidence linking adenosine and SK channel activation has been lacking. Our results show that in retinal ganglion cells, A_1 receptors initiate an intracellular signaling cascade that activates SK channels.

SK channels are activated by Ca^{2+}, and a NECA-evoked SK current must be preceded by an increase in intracellular Ca^{2+}. In basal forebrain neurons, A_1 receptors stimulate an IP_3R-mediated Ca^{2+} release from internal stores (Basheer et al., 2002), suggesting a mechanism by which adenosine could lead to the opening of SK channels. We now report that A_1 receptor activation in retinal ganglion cells also leads to an IP_3R-dependent increase in intracellular Ca^{2+} and to a subsequent activation of SK channels. Furthermore, in contrast to SK currents, which are activated by Ca^{2+} influx through voltage-gated Ca^{2+} channels and are of brief duration, retinal ganglion cell SK currents mediated by Ca^{2+} release from internal stores are prolonged, lasting

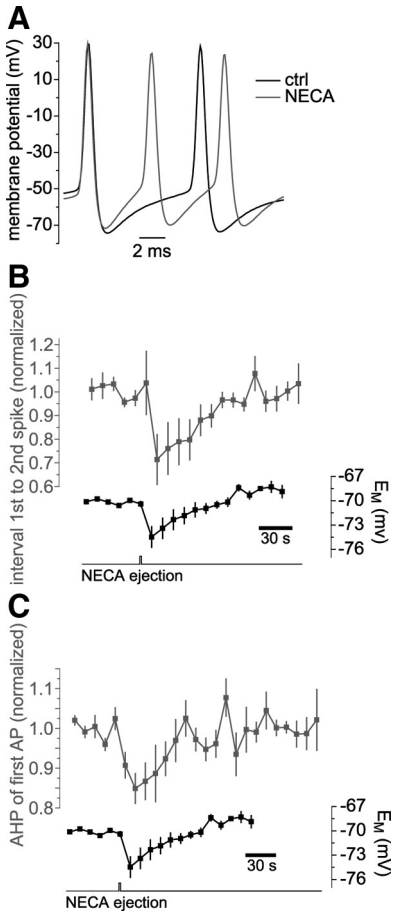

Figure 10. NECA reduces interspike interval (ISI) and afterhyperpolarization (AHP) in ganglion cells. ***A***, Action potentials recorded from a ganglion cell, evoked by a depolarizing current injection, immediately before (black) or after (gray) NECA ejection. Both ISI and AHP are reduced by NECA. ctrl, Control. ***B***, The ISI, measured as the time between the peaks of the first and second action potentials, is reduced by NECA ejection. The reduction in ISI matches the time course of the NECA-evoked hyperpolarization. Depolarizing stimuli are repeated every 10 s. ***C***, The AHP, measured from the spike threshold, is reduced by NECA ejection.

tens of seconds. To our knowledge, this is the first report of adenosine receptor-mediated SK channel activation.

The NECA-induced activation of GIRK and SK currents in retinal ganglion cells suggests that adenosine functions as a neuromodulator of these neurons. We observed that NECA ejection results in a reduction of light-evoked as well as depolarization-evoked spiking. The timing of spike generation is also altered by NECA, which decreases interspike intervals for the initial spikes in a burst. The reduction in interspike interval could be attributable to a number of mechanisms, including a NECA-induced reduction in the amplitude of the afterhyperpolarization. Elucidation of the mechanism(s) responsible for modulation of spike timing in retinal ganglion cells awaits additional studies.

In the retina, both glial cells (Newman, 2003) and amacrine cells (Neal and Cunningham, 1994; Santos et al., 1999) release ATP, which is rapidly metabolized in the extracellular space, providing a source of adenosine to activate retinal ganglion cell A_1 receptors. We have previously shown that ATP released from stimulated glial cells is hydrolyzed to adenosine and hyperpolarizes retinal ganglion cells by activating adenosine receptors (Newman, 2003). Adenosine levels are altered during light/dark adaptation and circadian cycles, with extracellular adenosine elevated in the dark-adapted retina and during the subjective night (Ribelayga and Mangel, 2005). These adenosine changes could alter the firing rate of retinal ganglion cells under different lighting conditions. It remains to be determined how this modulation contributes to information processing in the retina.

The adenosine-mediated inhibitory mechanisms we report here may play a role in neuroprotection as well as neuromodulation. Ischemia raises the concentration of extracellular adenosine in the retina (Ribelayga and Mangel, 2005). Ligation of the central retinal artery causes ocular ischemia and a subsequent increase in adenosine that persists for 30 min after ischemic release (Roth et al., 1997). This ischemic condition is accompanied by significant neuronal loss that is exacerbated by A_1 receptor antagonists, suggesting that A_1 receptor activation is neuroprotective. A_1 receptor antagonists also reduce the recovery of the electroretinogram a- and b-waves after ischemia (Li et al., 1999). As we demonstrate, A_1 receptor activation leads to a prolonged hyperpolarization of ganglion cells by activating both GIRK and SK channels. The hyperpolarization decreases the cell's firing rate and may alleviate ischemia-induced excitotoxicity.

Our results demonstrate that activation of A_1 receptors on retinal ganglion cells leads to the opening of GIRK channels. A_1 receptor activation also leads to the prolonged opening of SK channels, which is mediated by the IP_3R-dependent release of Ca^{2+} from internal stores. This represents a new mechanism by which adenosine can function as an endogenous neuromodulator and neuroprotective agent.

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