A2 Adenosine Receptors Inhibit Calcium Influx Through L-Type Calcium Channels in Rod Photoreceptors of the Salamander Retina

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Received 8 January 2001; accepted in final form 23 August 2001

Stella, Salvatore L. Jr., Eric J. Bryson, and Wallace B. Thoreson. A2 adenosine receptors inhibit calcium influx through L-type calcium channels in rod photoreceptors of the salamander retina. J Neurophysiol 87: 351–360, 2002; 10.1152/jn.00010.2001. Presynaptic inhibition is a major mechanism for regulating synaptic transmission in the CNS and adenosine inhibits Ca2+ currents (ICa) to reduce transmitter release at several synapses. Rod photoreceptors possess L-type Ca2+ channels that regulate the release of L-glutamate. In the retina, adenosine is released in the dark when L-glutamate release is maximal. We tested whether adenosine inhibits ICa and intracellular Ca2+ increases in rod photoreceptors in retinal slice and isolated cell preparations. Adenosine inhibited both ICa and the [Ca2+]i increase evoked by depolarization in a dose-dependent manner with ~25% inhibition at 50 μM. An A2-selective agonist, (N6-[2-(3,5-dimethoxyphenyl)-2-(2-methylphenyl)-ethyl]adenosine) (DPMA), but not the A1- or A3-selective agonists, (R)-N6-[1-methyl-2-phenylethyl]adenosine and N6-2-(4-aminophenyl)ethyladenosine, also inhibited ICa and depolarization-induced [Ca2+]i increases. An inhibitor of protein kinase A (PKA), Rp-cAMPS, blocked the effects of DPMA on both ICa and the depolarization-evoked [Ca2+]i increase in rods. The results suggest that activation of A2 receptors stimulates PKA to inhibit L-type Ca2+ channels in rods resulting in a decreased Ca2+ influx that should suppress glutamate release.

INTRODUCTION

Adenosine is a potent neuromodulatory substance that is widely distributed throughout the CNS. Adenosine (or P1) receptors are divided into four known molecular and pharmacological subtypes: A1, A2A, A2B, and A3 receptors (Ralevic and Burnstock 1996). Adenosine is well characterized as an inhibitory transmitter in the CNS (Ribeiro 1995), but excitatory actions of adenosine have also been observed (Sebastiao and Ribeiro 1996). Inhibitory actions of adenosine are commonly mediated by the A1 receptor that couples negatively to adenyl cyclase via a Gs protein to reduce cAMP levels in the cell (Dolphin et al. 1986; Ralevic and Burnstock 1998; van Calker et al. 1997). A2 receptors have been considered to mediate excitatory effects in the brain because they couple positively to adenyl cyclase through a Gi protein to increase cAMP levels (Ralevic and Burnstock 1998; Sebastiao and Ribeiro 1996; van Calker et al. 1979). However, synaptic activation of A2 receptors is not always associated with an increase in ICa or transmitter release. A2 receptor activation enhances P-type but not other Ca2+ channel subtypes in neurons (Satoh et al. 1997; Umemiya and Berger 1994). Adenosine acting at A2 receptors has been shown to inhibit both N- and L-type Ca2+ channels in PC12 cells via stimulation of cAMP and protein kinase A (PKA) (Kobayashi et al. 1998; Park et al. 1998). A2 receptor activation also inhibits ICa, and thus GABA release in suprachiasmatic and arcuate nuclei neurons (Chen and van den Pol 1997). It has thus been suggested that the increased excitability associated with A2 receptor activation arises from inhibition of ICa and the resulting decrease in release of inhibitory transmitter (reviewed in Edwards and Robertson 1999).

Adenosine is released in darkness from the retina (Blazynski and Perez 1991; Paes de Carvalho et al. 1990; Perez et al. 1986). Adenosine is present in human photoreceptors (Braas et al. 1987), and [3H]adenosine is taken up into photoreceptors and horizontal cells via transporters for adenosine (Paes de Carvalho et al. 1990; Studholme and Yazulla 1997), suggesting that adenosine is removed from the extracellular space following release. The presence of A2 receptors in the outer retina and particularly photoreceptors has been demonstrated by autoradiography and in situ hybridization (Blazynski 1990; Kvanta et al. 1997). Adenosine acting on A2 receptors stimulates melanin synthesis (Valenciano et al. 1998) and myoid elongation in cone photoreceptors (Rey and Burnside 1999), providing evidence for a physiological role for A2 receptors on photoreceptors.

Rod photoreceptors use dihydropyridine (DHP)-sensitive L-type Ca2+ channels to control release of the neurotransmitter, L-glutamate (Ayoub and Copenhagen 1991; Copenhagen and Jahr 1989; Corey et al. 1984; Kounenyi and Barnes 2000; Schmitz and Witkovsky 1997). Photoreceptor ICa can be modulated by various ions (H+, cations, anions) (Barnes et al. 1993; Piccolino et al. 1996; Thoreson et al. 1997, 2000) and neurotransmitters (dopamine, somatostatin, and nitric oxide) (Akopian et al. 2000; Kounenyi et al. 1994; Stella and Thoreson 2000). Barnes and Hille (1989) found that adenosine inhibits the Ca2+-activated chloride current in cones, but the actions of adenosine on photoreceptor ICa have not previously been examined.

The purpose of the present study was to examine the effect of adenosine on ICa and Ca2+ influx in rod photoreceptors.
Driven by evidence that adenosine inhibits $I_{Ca}$ in many other preparations, adenosine is released from retinal neurons in the dark, A$_2$ receptors are located on photoreceptors, and an uptake system for adenosine is present at the photoreceptor synapse, we hypothesized that adenosine acting on A$_2$ receptors may inhibit $I_{Ca}$ and thus decrease Ca$^{2+}$ influx into rods, which would in turn reduce transmitter release. Whole cell perforated patch-clamp recordings and Ca$^{2+}$-imaging experiments with fura-2 were performed on rods in retinal slice and isolated cell preparations. Our results indicate that adenosine inhibits both $I_{Ca}$ and the depolarization-evoked Ca$^{2+}$ influx in a dose-dependent manner and that this inhibition is mediated by A$_2$ receptor activation coupled to a PKA pathway. To our knowledge, this is the first report of inhibition of presynaptic $I_{Ca}$ by A$_2$ receptors at a glutamatergic synapse. Abstracts describing some of these results have previously been presented (Stella and Thoreson 1999; Stella et al. 1999).

**METHODS**

**Tissue preparation**

Larval tiger salamanders (*Ambystoma tigrinum*, Kons, Germantown, WI or Charles Sullivan, TN; 7–10 in) were cared for according to institutional guidelines. Retinal slices were prepared according to the methods of Werblin (1978) and Wu (1987); exact procedures are described in further detail by Thoreson et al. (1997). Briefly, salamanders were killed by decapitation and pithed, the eyes were removed. The resulting eyecup was cut into sections and placed vitreal side down onto a piece of filter paper (Millipore 2 × 5 mm, Type GS, 0.2 μM pores). After the retina adhered to the filter paper, the sclera, choroid, and retinal pigment epithelium were removed under chilled amphibian superfusate. The isolated retina was then cut into 100- to 150-μM slices using a razor-blade tissue chopper (Stoelting). Retinal slices were rotated 90° for viewing of the retinal layers when placed under a water immersion objective (×40, 0.7 NA or ×60, 1.0 NA) and viewed on an upright fixed stage microscope (Olympus BHWI or Nikon E600FN). All procedures were performed under dim light or ½ strength of isolated rods averaged 2.4 ± 0.3 μA (n = 5). Access resistance, clamp speed, and membrane capacitance were measured by analyzing capacitive transients evoked by hyperpolarizing steps from a holding potential of −70 mV. The time constant of the capacitative transient indicated a voltage-clamp speed of 0.97 ± 0.07 ms and a membrane capacitance for rods in the slice of 36.5 ± 3.7 pF (n = 22). For isolated rods, the clamp speed and capacitance determined from the capacitative transient averaged 2.4 ± 0.42 ms and 13.5 ± 1.8 pF (n = 5), respectively. Access resistance was typically between 22 and 30 MΩ; recordings were considered acceptable only when the access resistance was <40 MΩ. Estimates of the voltage errors introduced by the access resistance are included in the legends of figures showing examples of $I_{Ca}$.

Once whole cell access was achieved, light responses were obtained to determine the spectral sensitivity of the cell. Light-evoked responses in rods were generated by light from a tungsten light source that passed through a filter wheel containing interference filters of four different wavelengths (380, 480, 580, and 680 nm). The light stimulus was reflected into the microscope condenser using a beam splitter. Light intensity was controlled by neutral density filters (Wratten gel). The intensity of unattenuated light measurement with a laser power meter (Metrologic) was 1.1 × 10$^6$ photons s$^{-1}$ μm$^{-2}$ at 680 nm, 1.3 × 10$^6$ photons s$^{-1}$ μm$^{-2}$ at 580 nm, and 2.1 × 10$^5$ photons s$^{-1}$ μm$^{-2}$ at 480 nm (the power meter was not accurate at 380 nm). Generally, a flash of 1-s duration was used. The protocol for identifying the spectral sensitivity of a single photoreceptor involved successive flashes of increasing intensity (usually encompassing 2 log units of intensity) at the four wavelengths.

**Solutions and perfusion**

Solutions were applied by a single-pass, gravity-fed perfusion system that delivered medium to the slice chamber (chamber volume: 0.5 ml) at a rate of 1.0 ml/min. The normal amphibian superfusate that bathed the slices contained (in mM) 111 NaCl, 2.5 KCl, 1.8 CaCl$_2$, 0.5 MgCl$_2$, 10 N-2-hydroxyethylpiperezine-N’-2-ethanesulfonic acid (HEPES), and 5 glucose. After obtaining a photoreceptor recording, the superfusate was switched to a Ba$^{2+}$ solution to enhance Ca$^{2+}$ currents. The Ba$^{2+}$ solution contained (in mM) 99 NaCl, 2.5 KCl, 10 BaCl$_2$, 0.5 MgCl$_2$, 10 HEPES, 5 glucose, 0.1 picrotoxin, and 0.1 nitric acid. The pH of all solutions was adjusted to 7.8 with NaOH. The osmolality measured with a vapor pressure osmometer (Wescor) was 242 ± 5 mOsm. Solutions were continuously bubbled with 100% O$_2$.

**Electrophysiology**

Patch pipettes were pulled on a Narashige PB-7 vertical puller from borosilicate glass pipettes (1.2 mm OD, 0.95 mm ID, omega dot) and had tips of ~1 μm OD with tip resistances of 10–15 MΩ.

Pipettes were filled with a solution containing (in mM) 54 CsCl, 61.5 CsCH$_2$SO$_4$, 3.5 NaCl$,\text{SO}_4$, and 10 HEPES. The pH was adjusted to 7.2 with CsOH and the osmolarity was adjusted, if necessary, to 242 ± 5 mOsm. To maintain endogenous second-messenger signaling pathways and avoid the rundown that accompanies conventional whole cell recording of $I_{Ca}$, we used the perforated patch method of whole cell recording with the pore forming antibiotic, nystatin (Rae et al. 1991). Nystatin was dissolved in dimethylsulfoxide (DMSO) at a concentration of 120 mg/ml and then diluted into the pipette electrolyte solution to achieve a final concentration of 480 μg/ml. The final working solution was vortexed vigorously for 20–30 s and stored in the refrigerator. Fresh antibiotic solutions were made every 3 h. In successful recordings, seals >1 GΩ were obtained in ≤30 s and cells were usually fully perforated within 5 min of sealing.

Rods were identified by their long, rod-shaped outer segments. For electrophysiology, electrode placement was performed under infrared illumination using either Gen III image intensifiers mounted over the microscope eyepieces or with an infrared-sensitive video camera mounted on the trinocular head of the microscope.

The input resistance ($R_{in}$) of rods in the slice averaged 659.3 ± 48.9 (SE) MΩ (n = 22). $R_{in}$ of isolated rods averaged 2.4 ± 0.3 GΩ (n = 5). Access resistance, clamp speed, and membrane capacitance were measured by analyzing capacitive transients evoked by hyperpolarizing steps from a holding potential of −70 mV. The time constant of the capacitative transient indicated a voltage-clamp speed of 0.97 ± 0.07 ms and a membrane capacitance for rods in the slice of 36.5 ± 3.7 pF (n = 22). For isolated rods, the clamp speed and capacitance determined from the capacitative transient averaged 2.4 ± 0.42 ms and 13.5 ± 1.8 pF (n = 5), respectively. Access resistance was typically between 22 and 30 MΩ; recordings were considered acceptable only when the access resistance was <40 MΩ. Estimates of the voltage errors introduced by the access resistance are included in the legends of figures showing examples of $I_{Ca}$.

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After superfusion with the 10 mM BaCl$_2$ solution was started, voltage ramps (0.5 mV/ms) from −90 to +60 mV were used to assess $I_{Ca}$ every 30 s. Voltage ramps yield a similar current-voltage profile as a step protocol but cause less rundown or inactivation of the current and provide more data points for fitting for analysis (Stella and Thoreson 2000). Drug solutions were applied after $I_{Ca}$ appeared stable for ≥90 s.

Cells were voltage clamped at −70 mV using an Axopatch 200B patch-clamp amplifier (Axon Instruments, Foster City, CA). Currents were acquired and analyzed using Clampex 7.0 software (Axon Instruments). The leak conductance was assumed to be ohmic and equal to the minimum conductance between −75 and −55 mV and then...
dependent inactivation. Images were acquired at 5- to 10-s intervals digitally subtracted. Leak subtraction by blocking \( I_{\text{ca}} \) with 0.1 mM Cd\({}^{2+}\) yields almost identical voltage profiles.

**Measurement of \([\text{Ca}^{2+}]\), transients**

Intracellular \( \text{Ca}^{2+} \) changes were assessed using the ratiometric dye fura-2 (Grynkiewicz et al. 1985). Retinal slices were incubated for 45 min in the dark with 0.5 ml of 10 \( \mu \)M fura-2/AM +0.02% pluronic F-127 (Molecular Probes, Eugene, OR) in a slice chamber at 4 °C. This was followed by an additional incubation in fura-2/AM alone for 1.5 h.

Digital fluorescent images were recorded with a cooled CCD camera (SensiCam, Cooke) on an upright fixed stage microscope (Nikon E600FN) equipped with a \( \times 60 \) (1.0 NA) water-immersion objective. A 150-W Xe bulb (Opti-Quip) was mounted on a Sutter Lambda 10–2 filter wheel with 340- and 380-nm interference filters and coupled to the microscope by a liquid light guide (Sutter). The 380-nm intensity was attenuated with a 0.5-neutral density filter to balance the intensity of emissions evoked by 340 and 380 nm. The fluorescence emitted by the cells on stimulation with 340- or 380-nm light was filtered through a 510 ± 20-nm band-pass emission filter. Axon Imaging Workbench (AIW 2.2) was used to control the camera, filter wheel, and image acquisition. Pixel binning (2 × 2) of the images was used to decrease acquisition rate (acquisition time: 0.5 s). Images were subtracted for background camera noise but no averaging or masking was performed.

To activate voltage-dependent \( \text{Ca}^{2+} \) channels, cells were depolarized by increasing [\( \text{K}^{+} \)] from 2.5 to 50 mM for 1 min. Elevated KCl applications were performed for 15 min intervals to reduce any \( \text{Ca}^{2+} \)-dependent inactivation. Images were acquired at 5- to 10-s intervals during elevated [\( \text{K}^{+} \)] applications. All experiments were performed at room temperature. For analysis, a region of interest was drawn over each experiment was performed on at least three different slice preparations.

Statistical analysis was performed using paired and unpaired Student’s \( t \)-test (GraphPad Prism 3.0). Significance was chosen as \( P < 0.05 \), and variance is reported as ±SE.

**Drug solutions**

Adenosine, ATP, \((R)-N^\circ-(1\text{-methyl-2-phenylethyl})\text{adenosine} (\text{R-Pia})\), \(N^\circ-2\text{-}(3.5\text{-dimethoxyphenyl})-2\text{-}(2\text{-methylphenyl})\text{-ethyl} \text{adenosine} (\text{DPMA})\), \(N^\circ-2\text{-}(4\text{-aminophenyl})\text{ethyl} \text{adenosine} (\text{APNEA})\), and Rp-adenosine \(3\text{'},5\text{'}-\text{cyclic monophosphothioate triethylamine (Rp-cAMPS) were obtained from Sigma Chemical (St. Louis, MO). Solutions containing (−)Bay K 8644 (Research Biochemicals International) or nisoldipine (Zeneca Pharmaceuticals) were prepared by diluting 10,000× DMSO stock solutions into the superfusate. R-Pia, DPMA, and APNEA stocks were dissolved in DMSO as 50 mM stock solutions. All other stock solutions (1,000×) were prepared in distilled water or superfusate. Superfusion with 0.1% DMSO alone did not alter the \( I_{\text{ca}} \) amplitude.**

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**FIG. 1.** Rod photoreceptors contain L-type \( I_{\text{ca}} \). A rod photoreceptor in a retinal slice was held at −70 mV and ramped from −90 to +60 mV at 0.5 mV/ms. The graph shows overlaid current-voltage relationships of voltage ramps in control superfusate (thin trace) followed by perfusion with Bay K 8644 (1 \( \mu \)M, thick trace) and then nisoldipine (5 \( \mu \)M, dashed trace). Bay 8644 (1 \( \mu \)M) enhanced the peak \( I_{\text{ca}} \) without shifting the I-V relationship along the voltage axis. Nisoldipine (5 \( \mu \)M) suppressed \( I_{\text{ca}} \) and shifted the I-V relationship to more positive potentials. Voltage error at the peak of the current due to uncompensated access resistance was estimated to be −5.4 mV for control, −8 mV for Bay K 8644, and −2.4 mV for nisoldipine.

**FIG. 2.** Adenosine (50 \( \mu \)M) inhibited rod \( I_{\text{ca}} \). A: the graph shows I-V relationships of voltage ramps obtained in control superfusate (thin trace) and in the presence of adenosine (50 \( \mu \)M, thick trace). Voltage error at the peak of the current due to uncompensated access resistance was estimated to be −14 mV in control and −11.5 mV with adenosine. B: time course of adenosine-induced changes in the amplitude of \( I_{\text{ca}} \) in a rod. Voltage ramps in A were obtained at time points 1 (control) and 2 (adenosine). The voltage was ramped from −90 to +60 mV at 0.5 mV/ms.

**FIG. 3.** Effect of adenosine on rod \( I_{\text{ca}} \). Data values were obtained from peak amplitude measurements of \( I_{\text{ca}} \) from voltage ramps. Amplitudes of the currents obtained in test solutions were normalized to currents obtained in the control solution (\( I_{\text{ca}}/I_{\text{ca}} \) control). Asterisks denote significant changes with respect to control (\( P < 0.05 \)). Each point represents the mean ± SE. Number of experiments is shown in parentheses. * \( P < 0.05 \) compared with control. Adenosine concentrations: 1 \( \mu \)M: −11.3 ± 3.8 %, \( n = 3, P = 0.098; 10 \mu M: -13.5 ± 5.4 %, n = 4, P = 0.089; 25 \mu M: -20.6 ± 3.7%, n = 5, P = 0.005; 50 \mu M: -22.8 ± 3.3%, n = 9, P = 0.001.
not affect any of the properties of $I_{Ca}$ or Ca$^{2+}$-imaging responses that we studied.

RESULTS

Characterization of L-type calcium currents in rod photoreceptors

We will refer to the inward current evoked by a depolarizing voltage ramp in rods in the presence of 10 mM Ba$^{2+}$ as $I_{Ca}$. In the presence of 10 mM Ba$^{2+}$, a slowly developing U-shaped current-voltage relationship appeared above −50 mV that peaked between −25 and −10 mV and then diminished at more positive potentials. The current typically reversed between +50 and +60 mV. The peak amplitude of $I_{Ca}$ in rods averaged −371.7 ± 31.7 pA (n = 23) and the voltage at which the current was half-maximal ($V_{50}$) averaged −31.0 ± 1.6 mV (n = 23).

Figure 1 illustrates an example of the effects of the DHP agonist, Bay K 8644 (1 μM), and the DHP antagonist, nisoldipine (5 μM), on $I_{Ca}$ recorded in a rod. In agreement with other studies on photoreceptors, $I_{Ca}$ was enhanced by Bay K 8644 and incompletely blocked by a high concentration of nisoldipine (Kourennyi and Barnes 2000; Wilkinson and Barnes 1996). In addition to suppressing the peak current, nisoldipine produced a positive shift in the current/voltage relationship for $I_{Ca}$ similar to that shown by Wilkinson and Barnes for cone $I_{Ca}$ (Wilkinson and Barnes 1996). Three millivolts of the rightward shift shown in Fig. 1 can be accounted for by a decrease in $I_{Ca}$ flowing across the access resistance. The remainder of this positive shift may arise from the more potent block of L-type $I_{Ca}$ by nisoldipine at more negative potentials (Albillos et al. 1994). The residual $I_{Ca}$ in photoreceptors recorded in the presence of nisoldipine has been shown to arise largely from unblocked L-type Ca$^{2+}$ channels and not from a second channel type (Kourennyi and Barnes 2000; Wilkinson and Barnes 1996).

Adenosine inhibits rod $I_{Ca}$

The effect of adenosine on rod $I_{Ca}$ is shown in Fig. 2. Figure 2A illustrates current-voltage profiles of $I_{Ca}$ in control conditions (Fig. 2B, 1) and in the presence of adenosine (Fig. 2B, 2). Figure 2B plots the amplitude of $I_{Ca}$ measured with voltage ramps every 30 s. As illustrated in Fig. 2A, adenosine (50 μM) inhibited $I_{Ca}$ in rods without shifting the current-voltage relationship along the voltage axis. The mean reduction in $I_{Ca}$ by 50 μM adenosine was 22.8 ± 3.3% (n = 9, P = 0.0001). All of the tested at this concentration displayed a similar degree of inhibition in the presence of adenosine. Typically, inhibition of the rod $I_{Ca}$ began within 30 to 60 s, the time required for complete solution exchange in the recording chamber. Full inhibition was observed after 2 min, and currents recovered after ~5 min washout of adenosine (Fig. 2B). As the concentration of adenosine was increased from 1 to 50 μM, a significant inhibition of rod $I_{Ca}$ was seen at concentrations 10 μM, implying that there is a concentration-dependent effect of adenosine on rod $I_{Ca}$ (Fig. 3).

To test whether an intermediary cell type might be involved in adenosine modulation of rod $I_{Ca}$, solitary rods were mechanically isolated by gentle trituration and tested with 50 μM adenosine. As in retinal slices, adenosine significantly inhibited $I_{Ca}$ in isolated rods by 19.0 ± 5.5% (n = 5, P = 0.026), suggesting that adenosine acts directly on rod photoreceptors to modulate $I_{Ca}$.

Adenosine reduces depolarization-evoked Ca$^{2+}$ influx in rods

Ca$^{2+}$-imaging experiments were performed on retinal slices to test whether adenosine reduced depolarization-evoked [Ca$^{2+}$]i increases in rod photoreceptors. Slices were incubated with fura-2/AM. Rod were depolarized by elevating [K+]o from 2.5 to 50 mM for 1 min. Figure 4A shows a brightfield Nomarski image of rod photoreceptors in the retinal slice. Corresponding paired pseudocolor images of fluorescence from the same cells are displayed in Fig. 4B (340, 380, and 340/380 nm), showing cells in both normal (2.5 mM) and elevated (50 mM) [K+]o amphibian superfusate. Figure 4C shows a plot of the [Ca$^{2+}$]i, response measured in a rod photoreceptor before, during, and after the application of adenosine (50 μM). Adenosine produced a reversible inhibition of the depolarization-evoked [Ca$^{2+}$]i increase. On average, adenosine (50 μM) caused a 25.9 ± 3.4% (n = 14, P < 0.0001) reduction in the K$^{+}$-evoked 340/380 ratio change in rod photoreceptors in the slice. Significant inhibition of the K$^{+}$-evoked [Ca$^{2+}$]i increase was seen at concentrations as low as 1 μM (4.9 ± 1.4%, n = 11, P = 0.005), and inhibition increased in a dose-dependent fashion with concentrations ≤50 μM (Fig. 5).

$A_2$ receptor agonists inhibit rod $I_{Ca}$ and Ca$^{2+}$ influx

Adenosine can interact with P2 (purinergic) receptors (Ralevic and Burnstock 1998) that have been shown to modulate Ca$^{2+}$ channels in various neurons (Brown et al. 2000; Dave and Mogul 1996). To test whether a P2 receptor may be involved in the modulation of voltage-gated Ca$^{2+}$ channels in rods, ATP was bath applied to rod photoreceptors in the retinal slice preparation and examined with perforated patch recording of rod $I_{Ca}$ and [Ca$^{2+}$]i imaging using fura 2. ATP did not significantly inhibit $I_{Ca}$ (50 μM ATP: +7.1 ± 11.3%, n = 7, P = 0.549) or the K$^{+}$-evoked Ca$^{2+}$ influx in rod photoreceptors (75 μM ATP: +11.9 ± 7.3%, n = 15, P = 0.1296), suggesting that P2 receptors do not mediate the observed inhibition by adenosine.

The absence of an ATP effect suggests that adenosine acts at an adenosine receptor. There are four major subtypes of adenosine receptors: A1, A2A, A2B, and A3. R-PIA is an A1-selective receptor agonist, DPMA is an A2-selective receptor agonist that interacts with both A2A and A2B receptors, and APNEA is an A3-selective receptor agonist (Bridges et al. 1988; Ralevic and Burnstock 1998). To characterize the aden-
I in 4.7%, same three adenosine receptor agonists on depolarization-evoked increases in rods from retinal slice preparations by adenosine. Fura-2-loaded slices were stimulated with elevated \([K^+]_o\), in the presence of different concentrations of adenosine (1–50 μM). Inhibition of \(K^+\)-evoked ratio change produced by adenosine was normalized to the average of the initial control and the subsequent recovery following the test solution. Each point represents the mean ± SE. Number of experiments is shown in parentheses. *P < 0.05 compared with control. Adenosine concentrations: 1 μM: −4.9 ± 1.3%, n = 11, P = 0.005; 10 μM: −14.1 ± 3.2%, n = 8, P = 0.003; 25 μM: −12.3 ± 3.1%, n = 12, P = 0.002; 50 μM: −25.9 ± 3.4%, n = 14, P < 0.0001.

Adenosine receptor subtype that mediates inhibition of \(I_{Ca}\) we tested the effects of these three agonists on the rod \(I_{Ca}\). As shown in Fig. 6B, only the \(\alpha_2\)-selective agonist, DPMA (2 μM), significantly inhibited the rod \(I_{Ca}\). Like adenosine, DPMA did not cause a significant shift in the current-voltage relationship along the voltage axis (Fig. 6B, mean shift in \(V_{50}\) = +0.8 ± 0.63 mV, n = 6, P = 0.1563). The \(\alpha_1\)-selective agonist, R-PIA (2 μM), and \(\alpha_2\)-selective agonist, APNEA (2 μM), did not significantly inhibit rod \(I_{Ca}\) (Fig. 6A and C). Adenosine receptor agonists were tested at 2 or 10 μM. Figure 6D illustrates the overall results with R-PIA (2 μM), DPMA (2 μM), and APNEA (10 μM) on rod \(I_{Ca}\). DPMA at concentrations of 2 and 10 μM inhibited \(I_{Ca}\) in rods by 15.1 ± 4.4% (n = 12, P < 0.0001) and 25.0 ± 5.8% (n = 3, P = 0.05), respectively.

However, neither R-PIA nor APNEA had any effect on the rod \(I_{Ca}\) at any of the concentrations tested (R-PIA, 2 μM: −0.5 ± 4.7%, n = 6, P = 0.92; APNEA, 2 μM: 0.0 ± 3.5%, n = 5, P = 1.0; APNEA, 10 μM: +2.4 ± 5.8%, n = 7, P = 0.6982). These results suggest that the effects of adenosine on rod \(I_{Ca}\) are mediated by \(\alpha_2\) receptors.

Using \(Ca^{2+}\) imaging techniques, we tested the effects of the same three adenosine receptor agonists on depolarization-evoked \([Ca^{2+}]_i\) increases. Figure 7A displays responses to a series of \(K^+\)-evoked depolarizations measured in the inner segment of a rod photoreceptor in the presence of R-PIA (10 μM), DPMA (10 μM), and APNEA (10 μM). Only the \(\alpha_2\)-selective agonist, DPMA, inhibited the depolarization-evoked \([Ca^{2+}]_i\) increase in rods. Figure 7B shows the overall results of adenosine receptor agonists on the depolarization-induced \([Ca^{2+}]_i\) increases in rods. In agreement with the effects of these agonists on rod \(I_{Ca}\), DPMA (10 μM) reduced the \(K^+\)-evoked \([Ca^{2+}]_i\) increase in rods by 30.9 ± 4.9% (n = 12, P < 0.0001), while R-PIA (10 μM) and APNEA (10 μM) did not significantly alter the \(K^+\)-evoked \([Ca^{2+}]_i\) increase (R-PIA, +3.0 ± 3.3%, n = 12, P = 0.355; APNEA, −4.4 ± 2.7%, n = 12, P = 0.129).

**DISCUSSION**

The present study indicates that activation of \(\alpha_2\) receptors by adenosine stimulates PKA activity, which in turn inhibits voltage-gated \(L\)-type \(Ca^{2+}\) channels in rod photoreceptors. Similar concentrations of adenosine also produced a comparable inhibition of depolarization-evoked \([Ca^{2+}]_i\) increases. Adenosine released in the retina in darkness (Blazynski and Perez 1991; Paes de Carvalho et al. 1990; Perez et al. 1986) may therefore inhibit \(I_{Ca}\) and reduce \(Ca^{2+}\) entry into rods that would likely inhibit their release of glutamate.

**Adenosine receptor pharmacology and signaling pathways**

Purine and pyrimidine receptors are divided into two large families, P1 (or adenosine) receptors and P2 (or purinergic) receptors (for review, Ralevic and Burnstock 1998). ATP (50–75 μM) had no effect on the \(I_{Ca}\) or the depolarization-evoked \([Ca^{2+}]_i\) increase in rods, indicating that P2 receptors are unlikely to contribute to the inhibition produced by adenosine. Adenosine receptor subtypes were classified originally by their effects on adenylyl cyclase activity: \(A_1\) receptors...
inhibit and A2 receptors stimulate adenylyl cyclase (Londos et al. 1980; van Calker et al. 1979). However, the classification has since been broadened to include a new A3 receptor subtype (Zhou et al. 1992) and two subtypes of A2 receptors, A2A and A2B (Furlong et al. 1992; Maenhaut et al. 1990; Pierce et al. 1992). To discriminate among A1, A2, and A3 receptor subtypes, we used selective agonists for each receptor subtype: R-PIA for A1 receptors, DPMA for A2 receptors, and APNEA for A3 receptors. The ability of the A2-selective agonist, DPMA, but not the A1 or A3 receptor agonists to inhibit both the depolarization-evoked Ca2+ increase and Ica in rods (Figs. 6 and 7) is consistent with binding studies that have localized A2 receptors to photoreceptor inner and outer segments (McIntosh and Blazynski 1994). We did not attempt to pharmacologically discriminate between A2A and A2B receptors. However, mRNA for the A2A receptor has been shown to be expressed in the outer nuclear layer and ganglion cell layer, whereas A2B receptor mRNA is absent from the retina (Kvanta et al. 1997), suggesting that A2A receptors are likely to be responsible for the effects observed in the present study. In a

**FIG. 6.** Effect of adenosine receptor agonists on rod photoreceptor Ica. A: the A1-selective adenosine receptor agonist, (R)-N’-(1-methyl-2-phenyl-ethyl)adenosine (R-PIA, 2 μM), did not inhibit rod Ica. Voltage error at the peak of the current due to uncompensated access resistance was estimated to be −9 mV in both records. B: the A2-selective agonist, N’-[2-(3,5-dimethoxy-phenyl)-ethyl]adenosine (DPMA, 2 μM), inhibited rod Ica. Voltage error at the peak of the current due to uncompensated access resistance was estimated to be −2.6 mV in control and −2.1 mV in DPMA. C: the A3-selective adenosine receptor agonist, N’-2-(4-aminophenyl)ethyl)adenosine (APNEA, 10 μM), did not inhibit rod Ica. Voltage error at the peak of the current due to uncompensated access resistance was estimated to be −4 mV in both records. Each graph shows I-V relationships of voltage ramps obtained in control superfusate (—) and test solution (— - -). D: bar graph comparing effects of R-PIA, DPMA, and APNEA on Ica in rods. Amplitude of the currents obtained in test solutions were normalized to currents obtained in the control solution (Ica/control). *, significant changes with respect to control (P < 0.05). R-PIA, −0.5 ± 4.7%, n = 6, P = 0.92; DPMA, −15.1 ± 4.7%, n = 11, P < 0.0001; APNEA, +2.4 ± 5.8%, n = 7, P = 0.69.

**FIG. 7.** Effect of adenosine receptor agonists on [Ca2+]i increases evoked by elevated [K+]o in rods. A: a graphic representation of successive applications of adenosine receptor agonists. Depolarization-evoked [Ca2+]i changes in photoreceptor inner segments produced by 1-min applications of elevated (50 mM) [K+]o were measured in the presence of the A1 receptor agonist, R-PIA (10 μM), the A2 receptor agonist, DPMA (10 μM), and the A3 receptor agonist, APNEA (10 μM). B: bar graphs comparing the effects of R-PIA, DPMA, and APNEA on [Ca2+]i responses in rods. K+ -evoked [Ca2+]i increases in test solutions were normalized to the average of the initial control and subsequent recovery. Each bar graph represents the mean ± SE. *, P < 0.05 compared with control. R-PIA: +3.0 ± 3.3%, n = 12, P = 0.36; DPMA: −30.9 ± 4.9%, n = 12, P < 0.0001; APNEA: −4.4 ± 2.7%, n = 12, P = 0.13.
A

FIG. 8. Rp-cAMPS (10 μM) prevented inhibition of \( I_{\text{cAMP}} \) by DPMA (10 μM) in rods. A: time course of changes in \( I_{\text{cAMP}} \) amplitude during application of Rp-cAMPS and Rp-cAMPS plus DPMA. B: current-voltage relationships of voltage ramps obtained at time points 1 (control), 2 (Rp-cAMPS), and 3 (Rp-cAMPS + DPMA) from A. Control: thick solid trace. Rp-cAMPS (2 μM): thin solid trace. DPMA (2 μM) + Rp-cAMPS (10 μM): thin trace. Voltage error at the peak of the current due to uncompensated access resistance was estimated to be \( -3 \) mV in all 3 records. C: bar graph summarizing relative changes in \( I_{\text{cAMP}} \) amplitude induced by Rp-cAMPS or Rp-cAMPS plus DPMA. Each bar represents the mean ± SE. Asterisk, \( P < 0.05 \) compared with control. *DPMA significantly inhibited \( I_{\text{cAMP}} \) (\( -15.1 \pm 4.7\% \), \( n = 11 \), \( P < 0.0001 \)) but not in the presence of Rp-cAMPS (DPMA + Rp-cAMPS \( I_{\text{cAMP}} \) : +12.2 ± 6.8%, \( n = 4 \), \( P = 0.14 \)). DPMA produced significantly less inhibition in the presence of Rp-cAMPS (DPMA, \( n = 11 \) vs. DPMA + Rp-cAMP, unpaired t-test, \( P = 0.0001 \)).

Inhibitory effect of DPMA in rods (Figs. 8 and 9). Taken together, these results indicate that \( A_2 \) receptor-mediated changes in \( \text{Ca}^{2+} \) influx from depolarization of \( I_{\text{cAMP}} \) in rods are likely established by stimulation of PKA.

Sources of retinal adenosine

Adenosine has been detected in photoreceptors of human, monkey, guinea pig (Braas et al. 1987), fish (Ehinger and Perez 1984), and chick retinas (Paes de Carvalho et al. 1992). Adenosine has also been localized to ganglion cells (Ehinger and Perez 1984) and cells in the inner nuclear layer (Ehinger and Perez 1984; Paes de Carvalho et al. 1992) including rod horizontal cells (Studholme and Yazulla 1997) and amacrine cells (Blazynski 1989). Adenosine is released tonically from retinas in the dark (Blazynski and Perez 1991; Paes de Carvalho et al. 1990; Perez et al. 1986) and likely results from intracellular turnover of ATP in photoreceptor inner segments. In the dark, photoreceptors are depolarized by cations (e.g., \( \text{Na}^+ \), \( \text{Ca}^{2+} \)) entering through cyclic nucleotide channels in the outer segment (Shimazaki and Oakley 1986; Torre 1982). Increased intracellular adenosine levels are generated by the turnover of ATP from a highly active \( \text{Na}^+/\text{K}^+ \)-ATPase in the inner segment that counteracts the cation influx in the outer segment. The increased ATP turnover and breakdown of adenine nucleotides elevates intracellular adenosine levels. Increased adenosine

B

FIG. 9. Rp-cAMPS (10 μM) prevented the inhibition of depolarization-evoked \( \text{Ca}^{2+} \) increases by DPMA (2 μM) in rod photoreceptors. A: effects of successive elevated \( [\text{K}^+]_o \) applications in control superfuse and in the presence of Rp-cAMPS and DPMA. DPMA reversibly inhibited depolarization-evoked \( [\text{Ca}^{2+}]_o \) responses in the same cell following \(-20\) min washout of Rp-cAMPS + DPMA. B: bar graph summarizing relative changes in depolarization-evoked \( [\text{Ca}^{2+}]_o \) responses induced by Rp-cAMPS (10 μM) and Rp-cAMPS (10 μM) plus DPMA (2 μM) in experiments similar to those illustrated in A. *DPMA (2 μM) significantly inhibited the \( \text{K}^+ \)-evoked \( \text{Ca}^{2+} \) increase in rods after washout of Rp-cAMPS + DPMA (\(-36.1 \pm 3.9\% \), \( n = 8 \), \( P < 0.0001 \)). †DPMA (2 μM) produced significantly less inhibition in the presence of Rp-cAMPS (10 μM; paired t-test, \( n = 8 \), \( P = 0.02 \)).

study on cone motility in teleosts, the rank order of potency for adenosine agonists was found to be consistent with \( A_2 \)-like receptors, but antagonist potencies did not correspond precisely to either \( A_{2A} \) or \( A_{2B} \) receptors (Rey and Burnside 1999). 

\( A_2 \) receptor activation elevates cAMP levels in whole retina (Blazynski et al. 1986; Paes de Carvalho and de Mello 1982). \( A_2 \) receptor-mediated inhibition of \( \text{Ca}^{2+} \) influx and \( I_{\text{cAMP}} \) in rods is similar to the inhibition of rod \( I_{\text{cAMP}} \) produced by stimulation of cAMP production or activation of PKA (Stella and Thoreson 2000) and inhibition of PKA with Rp-cAMPS blocked the inhibitory effect of DPMA in rods (Figs. 8 and 9). Taken together, these results indicate that \( A_2 \) receptor-mediated changes in \( \text{Ca}^{2+} \) influx from depolarization of \( I_{\text{cAMP}} \) in rods are likely established by stimulation of PKA.
levels stimulate the efflux of adenosine down its concentration gradient via symmetrical adenosine transporters (Thorn and Jarvis 1996) which appear to be present on photoreceptors (Ehinger and Perez 1984).

ATP can be stored and released from vesicles (e.g., Corcoran et al. 1986; Jo and Schlichter 1999; Santos et al. 1999; von Kugelgen et al. 1994) and extracellular breakdown of ATP can be an important source of extracellular adenosine (Cunha et al. 1998). It appears unlikely that a significant amount of extracellular ATP is converted to adenosine at the photoreceptor synapse in the retinal slice preparation because application of 50–75 μM ATP did not inhibit \( I_{Ca} \) or the depolarization-evoked \( Ca^{2+} \) response. However, the finding that soluble nucleotidases are co-released with ATP from sympathetic neurons suggests that they may be co-localized in synaptic vesicles (Todorov et al. 1997), and this raises the possibility that ATP might be broken down by nucleotidases in synaptic vesicles to ADP, AMP, or adenosine prior to release. Consistent with such a possibility, Blazynski and Perez (1991) have shown that \( K^+ \)-evoked depolarization stimulates the release of adenosine in the retina.

Physiological significance

Previous studies have shown \( Ca^{2+} \) channels can be inhibited by \( A_2 \) receptors resulting in inhibition of transmitter release. For example, activation of \( A_2 \) receptors from cultured supra-

A2A receptors in rat striatum and cultured chick retinal neurons by A2 receptors resulting in inhibition of transmitter release. activated

- \( A_2 \) receptors in rods can regulate L-type channels.
- \( A_2 \) receptors localized to ganglion cells of the retina.
- Endogenous adenosine and adenosine receptors localized to ganglion cells of the retina.
- ATP receptor activation potentiates a voltage-gated calcium channels and neurotransmitter release.

REFERENCES


