Retinal Ischemic Preconditioning in the Rat: Requirement for Adenosine and Repetitive Induction

Bing Li and Steven Roth

PURPOSE. A brief period of ischemia can induce a remarkably complete state of ischemic tolerance in the retina, a phenomenon known as ischemic preconditioning (IPC). The mechanisms of IPC were studied in the rat retina by examining the role of adenosine as a possible mediator and determining whether IPC protection could be induced more than once in the same rat.

METHODS. Retinal ischemia was produced for 60 minutes in ketamine-xylazine-anesthetized Sprague-Dawley rats, and recovery was measured using electroretinography. Twenty-four hours earlier, the IPC stimulus of 5 minutes of ischemia was applied. To test the role of adenosine as a mediator of IPC, the selective adenosine A1 receptor antagonist 8-cyclopentyl-1,3-dipropylxanthine (DPCPX; 0.45 mg/kg, 2.25 mg/kg, or 4.5 mg/kg), the A2a antagonist 8-(3-chlorostyryl)caffeine (CSC; 0.1 mg/kg or 1.0 mg/kg), or their cyclodextrin vehicle were administered 15 minutes before IPC. To examine whether exogenous adenosine administration could mimic IPC, animals received intravitreal injections of the adenosine A1 receptor stimulant adenosine amine congener (ADAC) or the A2a stimulant CGS21680, followed by ischemia 24 hours later. To test the hypothesis that IPC could be induced repeatedly without loss of protection, rats were divided to receive IPC or sham IPC, followed 10 days later by IPC or a sham procedure, and 24 hours later by 60 minutes of ischemia.

RESULTS. Adenosine A1 receptor blockade with 4.5 mg/kg DPCPX administered intraperitoneally (IP) before or immediately after 5 minutes of ischemia completely blocked IPC protection, whereas lower doses resulted in partial blockade. CSC at the lowest dose (0.1 mg/kg) had no significant effect on IPC’s protective effect, whereas partial blockade was found with 1.0 mg/kg CSC. A1 or A2a receptor stimulation produced partial but significant mimicking of IPC protection, effects that were antagonized by DPCPX or CSC. Ischemic preconditioning applied twice, separated by 10 days, and followed by 60 minutes of ischemia 24 hours after the second IPC stimulus, resulted in nearly identical recovery of function after ischemia compared with IPC performed one time.

CONCLUSIONS. Adenosine, acting through the A1 and A2a receptors, is a critical component in the induction of ischemic tolerance after preconditioning in the retina. The neuroprotective effects of IPC in the retina are lost over time but may be reinduced by subsequent application of the IPC stimulus. (Invest Ophthalmol Vis Sci. 1999;40:1200-1216)

Ischemic tolerance is the induction of a state of resistance to ischemic injury and is believed to result from a stimulus-induced change in expression of as yet incompletely defined “protective” factors.

1. The phenomenon of ischemic preconditioning (IPC), previously documented in the myocardium and by histologic criteria in the brain, has recently been shown to result in dramatic functional and structural protection against ischemic damage in the rat retina. Ischemic preconditioning refers to the production of ischemic tolerance by a previous, brief, nondamaging period of ischemia. In the rat retina, 5 minutes of ischemia either 24 or 72 hours before 60 minutes of ischemia completely prevented functional and histologic evidence of injury. This remarkable protective effect was stronger than that shown previously using any exogenous administered agent. Shortening the window between IPC and ischemia to 1 hour or lengthening the period to 168 hours abolishes the protective effect of IPC, indicating the crucial influence of the amount of time between IPC and a damaging episode of ischemia. At present, the mechanisms and limitations of IPC protection in the retina have not been elucidated.

Ischemic preconditioning obviously cannot be instituted to protect against damage once ischemia has occurred. Nonetheless, because IPC harnesses the endogenous protective potential of a cell, it is of practical significance in the search for effective methods of preventing or treating ischemic injury and for elucidating mechanisms of ischemic damage and protection. Ischemic preconditioning has already been shown to be clinically applicable in the human myocardium. To begin to examine the mechanisms and limitations of IPC protection in the retina, we studied the role of the endogenously formed purine nucleoside, adenosine, as an important mediator of IPC.

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and we then tested whether IPC protection could be induced repeatedly in the same subject.

In myocardium, some of the proposed mechanisms of IPC include formation of adenosine or nitric oxide, opening of K+-adenosine triphosphate channels, activation of protein kinase C, and the production of heat shock proteins. The role of adenosine in IPC may be complex. For example, more than one adenosine receptor subtype is probably critical for IPC; recent studies in cultured myocytes suggest involvement of adenosine A1 and A3 receptors in myocardial IPC. Results in one study in rat cerebral cortex, using histologic criteria, suggested that the adenosine A1 receptor may be involved in ischemic preconditioning. Electrical recovery of hippocampal slices after anoxia in vitro was enhanced by 2 minutes of anoxic preconditioning, but this protection was prevented by blockade of the adenosine A1 receptor, further supporting a role of adenosine as a critical mediator of IPC in the central nervous system. However, the possibility that activation of other adenosine receptor subtypes is a component of IPC in central nervous system tissue has not been examined.

Adenosine A1 and A2a receptors are present in the mammalian retina. We theorized that adenosine is involved in IPC in the retina based on our previous studies. Specifically, we found significant increases in adenosine concentration in the retina, even after only 5 minutes of ischemia, and during reperfusion after brief or prolonged ischemia. Moreover, adenosine is the key mediator of changes in retinal blood flow during or immediately after ischemia and hypoxemia and may provide protective effects during retinal ischemia. To test the hypothesis that adenosine is an integral component of the protective effects of IPC in the retina, we assessed the effects of adenosine receptor blockade on IPC-induced protection against ischemic damage and the possibility that IPC could be pharmacologically "mimicked" in vivo by adenosine receptor stimulation. Specific adenosine receptor antagonists and agonists were studied to elucidate the adenosine receptor subtypes involved.

**MATERIALS AND METHODS**

**Ischemia Method**

Procedures used in this investigation conformed to the ARVO Resolution on the Use of Animals in Ophthalmic and Vision Research and were approved by our Animal Care Committee. We studied Sprague-Dawley rats (175-225 g) purchased from Taconic (Germantown, NY). Animals were maintained on a 12-hours-on-12-hours-off light cycle, except for the night before experiments, when they were kept in the dark. Rats were allowed free access to food and water overnight before experiments. Anesthesia was induced by mask inhalation of 5% isoflurane in oxygen. Once unconscious, animals were injected intramuscularly with a mixture of 35 mg/kg ketamine (Parke-Davis, Morris Plains, NJ), and 5 mg/kg xylazine (Miles, Shawnee Mission, KS). Adequacy of anesthesia was tested by tail-clamping with a hemostat, and supplemental doses of ketamine and xylazine were administered as needed. Body temperature was maintained at 36.5°C to 37.0°C by heated blanket.

Procedures used for producing ischemia have been described in detail. White light flashes lasting 10 μsec (luminance, 4.6 × 10^4 candelas/cm^2) were measured by optometer (model 370; United Detector Technology, Hawthorne, CA) equipped with a photoelectric filter (model 211 United Detector Technology). The flashes were produced by a photostimulator (model PS 22, Grass) placed 15 cm in front of the rat’s eyes. The responses were recorded and measured by Neuropack 8 (Nihon-Kohden, Irvine, CA), as described. Amplified signals (200 msec analysis time, 1–1500 Hz) were stored on computer disk, printed, and analyzed. Amplitude of the a-wave was measured from the baseline to the trough of the a-wave, and b-wave amplitude was measured from the trough of the a-wave to the peak of the b-wave. For all groups tested, baseline b-wave amplitudes in both eyes were approximately 900 μV to 1100 μV. Data at each time point represent the mean of at least three light flashes. These flashes were delivered approximately 3 minutes apart. At the end of a 30-minute recovery period after the end of ischemia, animals received antibiotic eye drops, were kept warm during recovery, and were allowed access to water. After dark adaptation overnight, animals were anesthetized as before for ERG examinations 24, 72, and 168 hours later.

**IPC Studies**

**Effects of Adenosine Receptor Blockade on IPC.** To test the role of adenosine in IPC by pharmacologically antagonizing adenosine receptors, animals were divided into separate groups (Table 1) to receive the adenosine A1 receptor antagonists propracaine (Allergan, Hormiguos, Puerto Rico). Next, a sterile 2-0 silk suture was placed around the optic nerve and blood vessels behind the globe of one eye and pulled through a small length of polyethylene tubing (PE-200, Intramedic; Becton-Dickinson, Parsippany, NJ). By pushing the tubing toward the eye while clamping the suture to maximal tightness, we were able to produce complete ocular ischemia. This procedure resulted in complete loss of the electroretinogram (ERG) b-wave and cessation of flow in retinal vessels verified by fundoscopic examination. Periodic measurement of the ERG and fundoscopic examination were performed during ischemia to ensure the presence of complete ischemia. The opposite eye of each animal served as a nonischemic control.

**Electroretinogram**

Procedures used in our laboratory have been described in detail elsewhere. In brief, the scotopic ERG was recorded from animals dark adapted overnight by placing platinum needle electroencephalogram electrodes (Grass, Providence, RI) in contact with the corneal surfaces of both eyes and a reference electrode on the tongue. Needles were bent to provide maximal contact of the metal with the cornea longitudinally, completely avoiding contact of the cornea with the sharp tip of the needle. Electrode wires were embedded in putty shaped specially for each rat, thus enabling recording of responses from the same contact point on the cornea throughout an experiment. A grounding electrode was placed subcutaneously on the animal's back. The cornea was intermittently irrigated with balanced salt solution (Alcon, Fort Worth, TX) to maintain adequate electrical contact and to prevent exposure keratopathy. Pupillary dilatation was maintained using 0.5% tropicamide (Mydriacyl; Alcon, Humacao, Puerto Rico) and 0.2% cyclopentolate HCl + 1% phenylephrine HCl (Cyclomydri; Alcon). All procedures were performed in dim red light.

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The adenosine A1 receptor agonist adenosine amine congener (ADAC) was dissolved in 1 N HCl and diluted with PBS. The pH was adjusted to 7.2 to 7.3 using NaHCO₃, and the solution was injected into the vitreous of both eyes at concentrations of 10 μM or 30 μM. The A2a receptor agonist CGS21680 was dissolved in PBS, the pH was adjusted, and concentrations of 10 μM, 50 μM, or 2000 μM were injected into the vitreous (Fig. 1). Both compounds were purchased from RBI. In control experiments, both eyes were injected with 1 μL PBS (pH 7.2–7.3). These adenosine receptor agonists were chosen because of their previously reported high receptor specificity and because of their relatively significant water solubility compared with all other adenosine receptor acting agents.20–22 The intravitreal route of injection was chosen to avoid the potentially confounding factor of the well-documented effects of adenosine receptor stimulation to produce systemic hypotension, bradycardia, and hypothermia.20 Although it has been reported that ADAC produced no systemic effects after systemic injection in gerbils,23 we found in preliminary experiments, that 1.0 mg/kg ADAC decreased heart rate by approximately 25%. In addition, doses of ADAC of 0.1 mg/kg or 1.0 mg/kg injected IP did not produce a significant mimicking of IPC. Therefore, the systemic route of injection was not used further in these studies.

**Repeatability of IPC.** To assess the repeatability of IPC, animals were divided into four groups (Table 2). Ischemic preconditioning (5 minutes of ischemia) or sham procedure (suture placed for 5 minutes, but not tightened) was followed 10 days later by IPC or sham. A 10-day separation period was chosen because in our earlier study, IPC did not protect the retina if separated from 60 minutes of ischemia by 7 days.5 Twenty-four hours after the second IPC or sham procedure, 60 minutes of ischemia was induced. The ERG was then measured for an additional 7 days, as described.

**Statistics**

Data were analyzed using a computer program (Stata ver. 4.0; Stata, College Station, TX). Electrophoretogram a- and b-wave amplitudes were normalized to baseline values and expressed as a percentage of the baseline. To account for variation in the ERG amplitudes (e.g., day-to-day variation within a subject), values obtained for follow-up examinations after ischemia ended were corrected by dividing the normalized ischemic value by the normalized control value (control ERG amplitude at a given time point divided by the baseline control).4 For each time point after 60 minutes of ischemia (30 minutes, 1 day, 3 days, and 7 days), unpaired t-tests were used to compare results within the various groups to the vehicle-injected animals. In all instances, data are shown as means ± SEM, and P < 0.05 was considered statistically significant.

<table>
<thead>
<tr>
<th>Study Group</th>
<th>Agents Tested</th>
<th>Protocol</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Cyclodextrin vehicle</td>
<td>15 min before IPC</td>
<td>6</td>
</tr>
<tr>
<td>A1 receptor blockade</td>
<td>DPCPX, 0.45, 2.25, or 4.5 mg/kg</td>
<td>15 min before IPC and immediately after (highest dose only)</td>
<td>5-7 per dose</td>
</tr>
<tr>
<td>A2a receptor blockade</td>
<td>CSC, 0.1 or 1.0 mg/kg</td>
<td>15 min before IPC</td>
<td>6 per dose</td>
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Agents administered by IP injection.

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onist 8-cyclopentyl-1,3-dipropoxanthine (DPCPX; 0.45 mg/kg, 2.25 mg/kg, or 4.5 mg/kg), the A2a antagonist 8-(3-chlorostyryl) caffeine (CSC; 0.1 mg/kg or 1.0 mg/kg), or an equal volume of cyclodextrin vehicle used to solubilize the compounds (all from RBI, Natick, MA). The agent or vehicle was administered intraperitoneally (IP) 15 minutes before IPC. Solutions of CSC were kept protected from light exposure. In an additional group, 4.5 mg/kg DPCPX was injected IP immediately after the end of 5 minutes’ IPC. The doses used and the timing of their administration were comparable with those of previous studies in which adenosine antagonists injected IP had significant effects on the brain.17-18 Specifically, experiments with [3H]-labeled DPCPX indicate that doses higher than 1 mg/kg injected IP in the rat would result in the micromolar concentrations necessary to antagonize the A1 receptors in the central nervous system.19 For CSC, previous experiments at comparable doses have shown significant effects in brain after IP injection.17-18 In preliminary experiments, vehicle, DPCPX, or CSC did not alter blood pressure, temperature, or arterial blood gas tension in the rats.

In all animals, ischemia for 5 minutes constituted the preconditioning stimulus. Sixty minutes of ischemia was induced 24 hours after IPC. This time sequence was selected because of an earlier study in our laboratory in which a 24-hour time sequence was selected because in our earlier study, IPC did not protect the retina if separated from 60 minutes of ischemia by 7 days.24

**Pharmacologic Mimicking of IPC.** To examine whether the administration of exogenous adenosine mimics IPC, adenosine receptor stimulants (or their saline control agents) were injected into the vitreous of both eyes 24 hours before 60 minutes of ischemia. Simultaneous IP injection of the adenosine receptor antagonists DPCPX (2.25 mg/kg or 4.5 mg/kg), or CSC (0.1 mg/kg or 1.0 mg/kg) was performed in a subset of the experiments to determine the specific receptor subtypes involved (Fig. 1). Under direct vision and with care taken to avoid contact with the lens, the vitreous was entered through the sclera with a 30-gauge needle that was attached to a 5-μL Hamilton syringe directed posteriorly. One microliter solution was injected into the vitreous (Fig. 1). Both compounds were purchased from RBI. In control experiments, both eyes were injected with 1 μL PBS (pH 7.2–7.3). These adenosine receptor agonists were chosen because of their previously reported high receptor specificity and because of their relatively significant water solubility compared with all other adenosine receptor acting agents.20–22 The intravitreal route of injection was chosen to avoid the potentially confounding factor of the well-documented effects of adenosine receptor stimulation to produce systemic hypotension, bradycardia, and hypothermia.20 Although it has been reported that ADAC produced no systemic effects after systemic injection in gerbils,23 we found in preliminary experiments, that 1.0 mg/kg ADAC decreased heart rate by approximately 25%. In addition, doses of ADAC of 0.1 mg/kg or 1.0 mg/kg injected IP did not produce a significant mimicking of IPC. Therefore, the systemic route of injection was not used further in these studies.

**Repeatability of IPC.** To assess the repeatability of IPC, animals were divided into four groups (Table 2). Ischemic preconditioning (5 minutes of ischemia) or sham procedure (suture placed for 5 minutes, but not tightened) was followed 10 days later by IPC or sham. A 10-day separation period was chosen because in our earlier study, IPC did not protect the retina if separated from 60 minutes of ischemia by 7 days. Twenty-four hours after the second IPC or sham procedure, 60 minutes of ischemia was induced. The ERG was then measured for an additional 7 days, as described.

**Statistics**

Data were analyzed using a computer program (Stata ver. 4.0; Stata, College Station, TX). Electrophoretogram a- and b-wave amplitudes were normalized to baseline values and expressed as a percentage of the baseline. To account for variation in the ERG amplitudes (e.g., day-to-day variation within a subject), values obtained for follow-up examinations after ischemia ended were corrected by dividing the normalized ischemic value by the normalized control value (control ERG amplitude at a given time point divided by the baseline control).4 For each time point after 60 minutes of ischemia (30 minutes, 1 day, 3 days, and 7 days), unpaired t-tests were used to compare results within the various groups to the vehicle-injected animals. In all instances, data are shown as means ± SEM, and P < 0.05 was considered statistically significant.
FIGURE 1. Experimental design for testing the efficacy of adenosine A1 or A2a receptor agonists in the mimicking of IPC. In the first set of experiments (top), both eyes of the rats received intravitreal injections of 1 μl PBS, the adenosine A1 receptor agonist ADAC, or the adenosine A2a receptor agonist CGS21680. Twenty-four hours after the injections, ischemia was induced for 60 minutes in one eye of each rat by temporary ligation of the vessels behind the eye. In the second set of experiments (bottom), rats received intravitreal injections of ADAC (30 μM) or CGS21680 (2000 μM) and simultaneously received DPCPX (A1 receptor antagonist) or CSC (A2a receptor antagonist) by IP injection. The doses of each agent and the numbers of rats in each group are shown within the boxes.

RESULTS

Effects of Adenosine Receptor Blockade on IPC Protection

With injection of vehicle alone before IPC, followed by 60 minutes of ischemia 24 hours later (n = 6), the a-wave recovery compared with baseline 30 minutes, 1, 3, and 7 days later was 18% ± 5%, 78% ± 6%, 99% ± 9%, and 100% ± 7%, respectively. For the b-wave, recovery was 14% ± 3%, 89% ± 5%, 105% ± 9%, and 97% ± 5%, respectively. There were no significant changes in the control eye ERG values over the time course of the experiment. Also, in the eye subjected to IPC, recovery of the a- and b-waves the next day, immediately before the onset of 60 minutes of ischemia, was 100% ± 6% and 106% ± 7%, respectively, not significantly different from baseline. These results are similar to those in our previous study in which we initially showed the profound protective effect of IPC against ischemic damage in the rat retina.

Pretreatment with the adenosine A1 receptor antagonist DPCPX before the IPC stimulus significantly attenuated recovery of the a- and b-waves after 60 minutes of ischemia compared with the effect of vehicle (Fig. 2). The effect of DPCPX was most prominent at a dose of 4.5 mg/kg. At a dose of 2.25 mg/kg, recovery was also suppressed, but not consistently at each postischemia time point, as occurred with the highest dose. At this dose, recovery of the a-wave was suppressed on days 1 and 7 after ischemia, with a trend toward suppression on the third postischemia day that was not statistically significant because of a relatively large SE. After the lowest dose of DPCPX, 0.45 mg/kg, recovery was suppressed only at 30 minutes and 1 day after ischemia for the a-wave, and for the b-wave, only at 1 day after ischemia.

When treated with DPCPX (4.5 mg/kg) immediately after IPC (n = 4), recovery of the a- and b-waves was completely suppressed for up to 7 days after 60 minutes of ischemia (data not shown graphically). Nonetheless, this suppression of recovery was not caused by an effect on the recovery after IPC before the onset of 60 minutes of ischemia, was 100% ± 6% and 106% ± 7%, respectively, not significantly different from baseline. These results are similar to those in our previous study in which we initially showed the profound protective effect of IPC against ischemic damage in the rat retina.

The effects of adenosine receptor blockade on IPC protection are illustrated in Table 2. Study Design to Examine the Repeatability of IPC Protection in the Rat Retina

<table>
<thead>
<tr>
<th>Group</th>
<th>Day 0</th>
<th>Day 10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Twice IPC</td>
<td>IPC</td>
<td>IPC</td>
</tr>
<tr>
<td>Sham twice</td>
<td>Sham IPC</td>
<td>Sham IPC</td>
</tr>
<tr>
<td>Sham + IPC</td>
<td>Sham IPC</td>
<td>IPC</td>
</tr>
<tr>
<td>IPC + sham</td>
<td>IPC</td>
<td>Sham IPC</td>
</tr>
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FIGURE 2. Treatment of rats with the adenosine A1 receptor antagonist DPCPX before IPC attenuated the protective effect on recovery after 60 minutes of ischemia in a dose-dependent manner. Recovery of the a-wave (top) and b-wave (bottom) of the ERG is shown up to 7 days after 60 minutes of ischemia preceded by preconditioning in four groups. Left to right in each graph: vehicle (n = 6), 0.45 mg/kg DPCPX (n = 6), 2.25 mg/kg DPCPX (n = 7), and 4.5 mg/kg DPCPX (n = 5). The a- and b-waves were normalized to preischemia baseline and corrected for day-to-day variation in the ERG, by using values from the nonischemic control eye. Recovery as a percentage of baseline amplitude is plotted on the y-axis and time on the x-axis. The values on the far left are those obtained 24 hours after 5 minutes' IPC but before the onset of 60 minutes of ischemia. All values are mean ± SEM. Statistical significance: *P < 0.05, †P < 0.002, and $P < 0.00002 for DPCPX-treated rats versus vehicle-treated rats.
(i.e., 5 minutes of ischemia itself), because 24 hours after IPC and DPCPX and before 60 minutes of ischemia, the a- and b-wave recovery both measured 89% ± 19% (nonsignificant versus baseline).

Pretreatment with the adenosine A2a receptor antagonist CSC at a dose of 0.1 mg/kg IP before the IPC stimulus did not affect recovery after 60 minutes of ischemia. However, at a dose of 1.0 mg/kg, recovery of the a- and b-waves was significantly suppressed (Fig. 3). This dose of CSC also produced a nonsignificant trend toward suppression of recovery of the a-wave 24 hours after IPC but before 60 minutes of ischemia (76% ± 10%). Representative ERG waveforms for the rats treated with DPCPX or CSC before IPC appear in Figure 4.

Mimicking of IPC by Adenosine Receptor Agonists

ADAC or CGS21680, 30 μM and 2000 μM, respectively, produced partial but significant mimicking of IPC protection when injected 24 hours before 60 minutes of ischemia (Figs. 5 and 6). The aqueous solubility of the two compounds limited the testing of further increases in injected concentration. ADAC (30 μM) caused significant recovery of the a- and b-waves that was evident within the first 30 minutes after ischemia, in comparison with the effect of saline injection alone (Fig. 5). The recovery after ADAC injection was more robust for the a-wave than for the b-wave. Recovery of the a- and b-waves after 30 μM ADAC was significantly greater than that in the saline group on days 1, 3, and 7 after ischemia. Injection of 2000 μM CGS21680 also produced significant recovery of the a- and b-waves, in comparison with the effect of saline injection alone (Fig. 6).

The effects of A1 and A2a receptor blockade on the mimicking of IPC by ADAC are shown in Figures 7 and 8. The maximally effective dose of ADAC (30 μM) was injected into the vitreous simultaneously with 2.25 mg/kg or 4.5 mg/kg DPCPX or 0.1 mg/kg or 1.0 mg/kg CSC injected IP. The effect of DPCPX in blocking IPC mimicking after ADAC was somewhat greater for the a-wave than for the b-wave (Fig. 7). Although 2.25 mg/kg DPCPX partially suppressed the IPC-mimicking effect of ADAC (for the a-wave, at days 3 and 7; for the b-wave at day 7 alone), IPC mimicking was almost completely abolished by 4.5 mg/kg DPCPX. However, this effect of high-dose DPCPX was complicated by a significant decline in the a- and b-wave amplitudes 24 hours after injection of the agents, before the induction of ischemia. But there was no significant decrease in the a- or b-wave amplitude in nonischemic (control) eyes compared with baseline for this group by day 1 after ischemia (a-wave, 105% ± 28%; b-wave, 101% ± 24%), indicating that this effect was only transient.

The effect of A2a receptor blockade on IPC mimicking by ADAC appears in Figure 8. CSC (0.1 mg/kg) was highly effective in blocking the IPC mimicking effect on the a-wave, whereas it significantly blocked the b-wave recovery only on day 7 after ischemia. Increasing the dose of CSC to 1.0 mg/kg resulted in significant, although not complete, reductions in recovery of the a- and b-waves at days 3 and 7 after ischemia.

The impact of blockade of the A1 and A2a receptors on the mimicking of IPC by CGS21680 can be seen in Figures 9 and 10. DPCPX (2.25 mg/kg) almost completely abolished the IPC-mimicking effect of CGS21680 (Fig. 9) on the a- and b-waves. This effect was similarly evident after DPCPX at 4.5 mg/kg. CSC (0.1 mg/kg) did not alter the recovery of the a-wave after CGS21680 administration (Fig. 10), and partially blocked recovery of the b-wave at days 1 and 3. At day 7, the trend for attenuation of the b-wave recovery was not significant (P < 0.1). However, at a dose of 1.0 mg/kg, CSC was highly effective in attenuating recovery of the a- and b-waves at days 1, 3, and 7.

The Repeatability of Ischemic Preconditioning

Results of experiments examining the repeatability of IPC are shown in Figure 11. There was no significant difference between results in rats in which sham IPC was administered twice 10 days apart and results in those in which IPC was followed 10 days later by sham IPC (Fig. 11; leftmost two bars of each data set). In both of these groups, minimal recovery was evident, indicating that the protective effect of prior IPC was lost with time. Conversely, there was no significant difference between groups in which IPC was performed 10 days apart ("twice-IPC" experiment) compared with those subjected to sham followed by IPC 10 days later, indicating that IPC may be induced and its protective effect be allowed to dissipate, then be reinduced by later repeated application. Recovery was significantly different between twice-IPC and the sham-followed-by-IPC groups compared with the effect of sham IPC performed twice, further supporting the repeatability of IPC protection.

**DISCUSSION**

The retinal function-sparing effects of ischemic preconditioning in this model were remarkable. Retinal ischemia is a final common pathway for injury in many diseases that result in blindness. Therefore, studies of ischemic preconditioning in animals are of clinical importance in developing treatments to enhance retinal cell survival in ischemic vascular diseases of the retina. Because the effects of IPC on ischemia are so robust, deciphering the mechanisms of IPC may yield important insights into possible treatment strategies for retinal vascular disease. Accordingly, the role of adenosine in IPC was studied. This substance seemed a likely candidate as a critical mediator of IPC based on studies in brain and myocardium and on our earlier studies in the rat retina subjected to ischemic insult. These studies included findings of significant increases in adenosine concentration in the retina, even after only 5 minutes of ischemia, evidence that adenosine regulates retinal blood flow during and immediately after ischemia and hypoxemia. Other recent studies have shown that adenosine may provide protective effects during retinal ischemia.

Profound attenuation of the protective effect of IPC by adenosine receptor blockade confirmed our hypothesis that adenosine is a critical signal for the production of IPC protection in the retina. DPCPX is a selectively adenosine A1 receptor antagonist that penetrates the central nervous system after systemic administration and is 700 times more selective for the A1 than the A2 receptor. It is the most commonly chosen A1 antagonist for in vivo experiments. This compound eliminated the protective effect of IPC in a dose-dependent manner in our experiments, clearly indicating that IPC protection requires the binding of endogenously formed adenosine to retinal adenosine A1 receptors.
FIGURE 3. Treatment of rats with the adenosine A2 receptor antagonist CSC before IPC partially attenuated the protective effect on recovery after 60 minutes of ischemia in a dose-dependent manner. Recovery of the a-wave (top) and b-wave (bottom) of the ERG is shown up to 7 days after 60 minutes of ischemia preceded by preconditioning in three groups. Left to right in each graph: vehicle (n = 6), 0.1 mg/kg CSC (n = 6), and 1.0 mg/kg CSC (n = 6). The a- and b-waves were normalized to pres ischemia baseline and corrected for day-to-day variations in the ERG, by using values from the nonischemic control eye. Recovery as a percentage of baseline amplitude is plotted on the y-axis and time on the x-axis. The values on the far left are those obtained 24 hours after 5 minutes' IPC but before the onset of 60 minutes of ischemia. All values are means ± SEM. Statistical significance: *P < 0.05, †P < 0.009, and ‡P < 0.0007, for CSC-treated rats versus vehicle-treated rats.
Figure 4. Representative ERG traces, from vehicle-, DPCPX-, and CSC-treated rats. ERG was recorded from corneal electrodes in dark-adapted Sprague-Dawley rats. The response to 10-μsec flashes was recorded. The time scale (x-axis) was 200 msec. Scale bar (in microvolts) is displayed at the top right corner of each set of tracings. From top to bottom of each set of traces are shown baseline before IPC; 24 hours after IPC and just before 60 minutes of ischemia; during 60 minutes of ischemia; and 30 minutes, 1, 3, and 7 days after the end of 60 minutes of ischemia.
FIGURE 5. The mimicking of IPC by injection of the specific adenosine A1 receptor agonist ADAC. Doses of 10 μM (n = 3), 30 μM (n = 7), or PBS vehicle (n = 5) were injected (1 μl solution into the midvitreous of both eyes). Twenty-four hours later, ischemia was induced for 60 minutes. The recovery of the ERG a-wave (top) and b-wave (bottom) is displayed as described in the legend to Figure 2. Statistical significance: *P < 0.02 and †P < 0.006, for ADAC-treated rats versus vehicle-treated rats.

The relatively selective A2a antagonist CSC partially attenuated IPC but only at the highest dose tested. This compound is a potent A2a antagonist and the most selective agent currently available for systemic administration. Because of its relative insolubility and the requirement to maintain the same diluent and volume relative to that of the control group, the dose range available with this agent is somewhat limited. The results with CSC suggest two possibilities: Either activation of the A2a receptor is also required to produce IPC, or the highest dose of CSC resulted in a partial A1 receptor blockade. Although the relative receptor binding of these compounds in the rat retina in vivo is not yet known, CSC had 25 times more selectivity for the A2a than for the A1 receptor in assays in vitro. Others have reported, however, that CSC has nearly 500 times more selectivity for the A2a than for the A1 receptor. Therefore, to further clarify which adenosine receptor subtypes may be involved in IPC, we performed experiments to mimic IPC by injecting specific adenosine receptor stimulants.

The results of the mimicking experiments indicated that the A1 receptor stimulant ADAC and the A2a stimulant
FIGURE 6. The mimicking of IPC by injection of the specific adenosine A2a receptor agonist adenosine CGS21680. Doses of 10 μM (n = 3), 50 μM (n = 5), and 2000 μM (n = 8), or PBS vehicle (n = 5) were injected (1 μl solution into the midvitreous of both eyes). Twenty-four hours later, ischemia was induced for 60 minutes. The recovery of the ERG a-wave (top) and b-wave (bottom) is displayed as described in the legend for Figure 2. Statistical significance: *P < 0.02 and †P < 0.01, for CGS21680-treated rats versus vehicle-treated rats.

CGS21680 both produced partial but significant mimicking of IPC. Considering the potent inhibition of IPC by adenosine receptor blockade, the partial mimicking by these agents requires explanation. Although the estimated vitreal concentrations of ADAC and CGS21680 far exceed the $K_i$ values (1 nM and 15 nM, respectively), the actual concentration to which retinal cells were exposed cannot be determined from these types of in vivo experiments. It is possible that the vitreous concentrations achieved (estimated at 1 μM and 64 μM for ADAC and CGS21680, respectively) were not actually representative of the degree of adenosine receptor stimulation achieved by IPC in vivo. It is also likely that other mediators are involved in IPC protection—for example, substances such as nitric oxide or excitatory amino acids. Blockade of the effects of ADAC and CGS21680 was thus used to determine receptor-specific effects. DPCPX was highly effective in blocking the effects of ADAC or CGS21680, adding further proof of adenosine A1 receptor involvement in IPC. CSC was less effective as an antagonist at its lower dose, but highly effective at a higher dose, particularly as an antagonist of the mimicking produced by CGS21680. The findings that antagonists of either the A1 or A2a receptor could reverse an adenosine-mimicking effect are
consistent with experiments showing that DPCPX and CSC attenuate IPC and suggest that the A1 and A2a receptors are both involved in IPC in the retina.

In an earlier, considerably more limited study of adenosine and IPC, a single dose of DPCPX (1.0 mg/kg) was used, with histologic criteria of injury, with results suggesting that the adenosine A1 receptor mediates IPC protection in rat hippocampal neurons in vivo. In other experiments in this same study, only 15 minutes after systemic injection of an adenosine A1 receptor stimulant, ischemia was induced. Evidence of protection from ischemic injury was interpreted to indicate an IPC-mimicking effect. However, this time sequence did not replicate the time course of IPC in that study or in ours. Moreover, the specific adenosine receptor subtypes responsible were not positively identified. In perfused hippocampal slices in vitro, adenosine was shown to be a component of
Ischemic Preconditioning: Adenosine and Repeatability

FIGURE 8. The impact of adenosine receptor blockade with CSC on IPC mimicking by ADAC (30 μM). One microliter ADAC was injected into the midvitreous of both eyes. Simultaneously, 0.1 mg/kg (n = 3) or 1.0 mg/kg CSC (n = 3) was injected IP. Twenty-four hours later, ischemia was induced for 60 minutes. The recovery of the ERG a-wave (top) and b-wave (bottom) is displayed as described in the legend for Figure 2. Statistical significance: *P < 0.05, †P < 0.008, for CSC-ADAC-treated rats versus rats treated with ADAC only.

anoxic preconditioning, but similarly, the specific receptor subtypes involved were not definitively established.9

Results in the present study clarify this issue and provide three novel findings regarding the involvement of adenosine in IPC. First, a functional role of adenosine in IPC was established for the first time in the intact central nervous system in vivo. Second, the A1 and A2a receptors were both shown to be involved in this phenomenon. Third, we showed that IPC protection resulted from the release of adenosine during the reperfusion period after the brief IPC stimulus, because DPCPX administered immediately after IPC was highly effective in attenuating the protective effect of IPC. The latter finding is consistent with our previous experiments in the same rat model that showed that a brief ischemic insult was followed by an up to sevenfold postischemia increase in adenosine concentration during reperfusion.12

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Involvement of the A1 and A2a receptors in IPC may seem surprising in view of our recent demonstration of opposing effects of these receptor subtypes in a rat model of retinal ischemia-reperfusion injury. However, there is precedence for a role in IPC of multiple adenosine receptor subtypes, because in cultured myocytes, the A1 and A3 receptors are both involved in the induction of ischemic tolerance. A wide array of functional effects result from the activation of the adenosine A1 and A2a receptors. It is commonly held that activation of adenosine A1 receptors decreases excitatory amino acid release and Ca$^{2+}$ entry into cells and hyperpolarizes postsynaptic membranes. Both of these effects may explain the findings that preischemia administration of an adenosine A1 receptor agonist decreases ischemic damage.
whereas A1 receptor blockade worsens injury. 27 Adenosine also stimulates protein kinases and phospholipases and may alter gene expression. 32 Therefore, stimulation of the A1 receptor may lead directly to the expression of protective proteins. Alternately, adenosine A1 stimulation may function in a permissive role, maintaining the retinal cells in a state wherein stress-induced alterations in gene expression and protein synthesis occur more efficiently. 33

In contrast to the effect of stimulating A1 receptors, activation of the A2a receptor enhances release of acetylcholine, the excitatory neurotransmitter glutamate, and γ-aminobutyric acid, 34 effects that could be neurotoxic. 35 Thus, blockade of the adenosine A2a receptor leads to profound protection against ischemic injury in the retina. 27 Stimulation of the A2a receptor decreases leukocyte adhesion and the associated release of oxygen free radicals. 36 It is not yet clear how these effects may be related to IPC protection. One possibility is that adenosine A2a-mediated enhancement of the extracellular release of excitatory amino acids could, paradoxically, stimulate the retina's endogenous protective system. 27 Alternately, A2a
stimulation may prevent ischemic damage by enhancement of postischemia reperfusion.\textsuperscript{13,36,39}

In summary, our experiments show a complex role of adenosine in ischemic preconditioning, not unlike the multi-adenosine-receptor subtype involvement recently shown for IPC in the myocardium.\textsuperscript{7} In our earlier study, non preconditioned ischemic retinae showed relatively less structural damage to the photoreceptors than to the inner retina, consistent with the greater sensitivity of the inner retina to ischemic injury.\textsuperscript{4,40} Yet, recovery of the a- and b-waves of the ERG was severely decreased by ischemia in our previous study and in the present one, and both waves completely recovered from ischemia induced after application of IPC, showing that IPC protected the inner and outer retina. Alteration of a-wave
recovery despite relative histologic preservation of the photoreceptors was explained as a result of either documented damage to the outer nuclear layer or of photoreceptor damage that was not visible using light microscopy. In the present study, adenosine receptor blockade prevented recovery of the a- and b-waves, whereas adenosine receptor stimulation enhanced their recovery, implying an effect of such blockade on inner and outer retinal function.

Apart from one earlier study indicating that infusion of adenosine may alter light responses in the retina and optic nerve of intact, perfused cat retina in vivo, little is known about the functional effects of adenosine in the retina. Adenosine A1 and A2 receptor mRNAs have both been found in the retina of the rat. Adenosine immunoreactivity has been shown in the inner and outer retina, including the photoreceptors. The results of the present experiment showing effects on the a- and b-waves of the ERG directly support a role for adenosine receptors in inner and outer retinal function.

Our experiments showed that IPC could be induced, its protective effect allowed to dissipate, and its protection restored by a subsequent treatment. The amount of time separating IPC and ischemia is vitally important in determining whether protection, or alternately, worsening, of injury occurs. For example, a 1-hour separation between IPC and ischemia in the retina resulted in severe ischemic damage, whereas a 24- to 72-hour separation resulted in profound protection. Allowing 168 hours between IPC and ischemia in this model resulted in no significant protection. Similarly, a brief separation between ischemic insults in rat cerebral cortex led to enhanced injury.

In the only other study of repeated application of IPC, ischemic tolerance (by histologic criteria) could be produced in the gerbil hippocampus by two episodes of 2 minutes of forebrain ischemia 1 day apart. The investigators allowed re-circulation for 4 weeks, repeated the paradigm, and induced 3 minutes of lethal ischemia 3 days later. Animals receiving the second IPC were protected. This latter study is conceptually similar to ours, but the results differ somewhat in time course from our findings of IPC repeatability, suggesting that the mechanisms responsible in the retina may differ from those in the cerebral cortex.

Our findings add further support to the theory that IPC protection in the retina is the result of a transient change in the expression of critical protective proteins. In our earlier report, we theorized that heat shock proteins are responsible. Apparently retinal cells possess the capacity to enhance transiently such protective proteins repeatedly. There are important clinical implications of this result. In transient ischemic attacks, patients have repeated episodes of nondamaging cerebral ischemia, similar to the ischemic insult that induces ischemic tolerance in animal studies. If transient ischemic attacks are protective against damage that follows more severe ischemic injury in the newborn pig retina.

In conclusion, we show that adenosine was a necessary mediator of IPC and that IPC could be induced repeatedly. Deciphering the mechanisms of IPC is significant, because it could lead to the development of clinically relevant strategies for protecting the retina from ischemic damage.

References

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