Rapid Turnover of the AMP-Adenosine Metabolic Cycle in the Guinea Pig Heart

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The intracellular flux rate through adenosine kinase (adenosine→AMP) in the well-oxygenated heart was investigated, and the relation of the AMP-adenosine metabolic cycle (AMP=adenosine) to transmethylation (S-adenosylhomocysteine [SAH]→adenosine) and coronary flow was determined. Adenosine kinase was blocked in isolated guinea pig hearts by infusion of iodotubercidin in the presence of the adenosine deaminase blocker erythro-9-(2-hydroxy-3-nonyl)adenine (5 μmol/L). Iodotubercidin (1 nmol/L to 4 μmol/L) caused graded increases in venous effluent concentrations of adenosine, from 8±3 to 145±32 nmol/L (mean±SEM, n=3), and in coronary flow, which increased to maximal levels. Flow increases were completely abolished by adenosine deaminase (5 to 10 U/mL). Interstitial adenosine concentrations, estimated using a mathematical model, increased from 22 nmol/L during control conditions to 420 nmol/L during maximal vasodilatation. The possibility that iodotubercidin caused increased venous adenosine by interfering with myocardial energy metabolism was ruled out in separate 3P nuclear magnetic resonance experiments. To estimate total normoxic myocardial production of adenosine (AMP→adenosine→SAH), the time course of coronary venous adenosine release was measured during maximal inhibition of adenosine kinase with 30 μmol/L iodotubercidin. Adenosine release increased more than 15-fold over baseline, reaching a new steady-state value of 3.4±0.3 nmol·min⁻¹·g⁻¹ (n=5) after 4 minutes. In parallel experiments, the relative roles of AMP hydrolysis and transmethylation (SAH hydrolysis) were determined, using adenosine dialdehyde (10 μmol/L) to block SAH hydrolyse. In these experiments, adenosine release increased to similar levels of 3.4±0.5 nmol·min⁻¹·g⁻¹ (n=6) during inhibition of adenosine deaminase and adenosine kinase. It is concluded that (1) maximal increases in coronary flow are elicited by increases in interstitial adenosine concentration to approximately 400 nmol/L, (2) more than 90% of the adenosine produced in the heart is normally rephosphorylated to AMP without escaping into the venous effluent, (3) AMP hydrolysis is the dominant pathway for cardiac adenosine production under normoxic conditions, and (4) the high rate of adenosine salvage is due to rapid turnover of a metabolic cycle between AMP and adenosine. Rapid cycling may serve to amplify the relative importance of AMP hydrolysis over transmethylation in controlling cytosolic adenosine concentrations. (Circ Res. 1993;73:846-856.)

KEY WORDS • coronary flow • S-adenosylhomocysteine • iodotubercidin • adenosine kinase

To further understand the role of adenosine as a local humoral agent in the heart, it is necessary to improve the knowledge of the pathways for adenosine metabolism and transport and to define more precisely the coronary sensitivity for endogenous adenosine. In particular, there are questions concerning the mechanisms of myocardial adenosine production during normoxia and the relative rates of venous adenosine efflux and intracellular adenosine production. There are two pathways for intracellular adenosine production, AMP hydrolysis via the cytosolic form of the enzyme 5'-nucleotidase and transmethylation, which results in adenosine production by the hydrolysis of S-adenosylhomocysteine (SAH) via the reversible cytosolic enzme SAH hydrolyase. Myocardial salvage of adenosine is mediated by adenosine kinase (requiring ATP), which phosphorylates adenosine to form AMP. Adenosine may also be deaminated to form inosine via adenosine deaminase. Thus, four enzymes and a membrane carrier (facilitated diffusion) are involved in controlling the cytosolic concentration of adenosine.

It has been concluded that AMP hydrolysis is the major pathway for myocardial adenosine production during ischemia or hypoxia, based on measurements of coronary venous adenosine concentrations in isolated2-5 and in vivo6-7 hearts and based on the novel SAH technique to assess regional cytosolic adenosine concentrations.8,9 However, during normoxic conditions, there is evidence that the transmethylation pathway, rather than AMP hydrolysis, is the dominant pathway for adenosine production in the guinea pig heart.10,11 This possibility raises questions about the adenosine hypothesis concerning the regulation of myocardial blood flow, since the transmethylation pathway is insensitive to cellular energetics.12

The conclusion regarding the importance of transmethylation was based on measurements showing nearly
equal rates of transmethylation and adenosine salvage via adenosine kinase, suggesting that there is little AMP hydrolysis during normoxia.\textsuperscript{10,11} Adenosine salvage was estimated from the increases in venous release rate of adenosine following pharmacologic blockade of adenosine kinase by 5'-amino-5'-deoxyadenosine in the presence of a blocker of adenosine deaminase. However, 5'-amino-5'-deoxyadenosine proved to be only a weak inhibitor of cardiac adenosine kinase (50% inhibition at 5 μmol/L), so the true rate of salvage might have been underestimated. The possibility of an underestimate was heightened by the finding that blockade of adenosine kinase using a more potent inhibitor (iodotubercidin)\textsuperscript{13} caused greater increases in adenosine release than were observed using 5'-amino-5'-deoxyadenosine. In addition, a mathematical model of adenosine transport and metabolism in the guinea pig heart indicated that adenosine salvage is greater than was estimated using 5'-amino-5'-deoxyadenosine.\textsuperscript{14}

In previous studies, coronary flow sensitivity for increased endogenous adenosine under normoxic conditions was investigated using the adenosine transport blocker dipyridamole\textsuperscript{15} and the adenosine kinase blocker iodotubercidin in the presence of adenosine deaminase inhibition.\textsuperscript{13} Interstitial adenosine concentrations were estimated to increase to 190 nmol/L using a mathematical model in the dipyridamole study and to 1 μmol/L using measurements of epicardial transudate adenosine concentrations in the iodotubercidin study. Although flow was increased in both studies, only single observations were obtained rather than complete dose-response curves. The apparent difference in sensitivity may be due to the different pharmacologic agents used, the different methods used to estimate interstitial adenosine, or the different regions of the dose-response relation that were studied. In neither study were non-specific effects of the pharmacologic agents assessed.

The present study was designed to answer the questions raised above: What is the magnitude of total cellular adenosine production under normoxic conditions in the guinea pig heart compared with the rate of venous adenosine efflux? What is the dominant pathway for cellular adenosine production? What is the coronary flow dose-response relation for increased endogenous adenosine? Total myocardial adenosine production was estimated by measuring venous adenosine release rate during combined selective blockade of adenosine kinase and adenosine deaminase. The role of transmethylation was investigated by observing whether irreversibly blocking SAH hydrolysis caused a decrease in total adenosine production.

Materials and Methods

Isolated Guinea Pig Hearts

Hearts from guinea pigs weighing 300 to 350 g were perfused according to the Langendorff technique with a modified Krebs-Henseleit solution (mmol/L: NaCl, 116; KCl, 4.6; MgSO\textsubscript{4}, 1.1; KH\textsubscript{2}PO\textsubscript{4}, 1.2; NaHCO\textsubscript{3}, 24.9; CaCl\textsubscript{2}, 2.5; glucose, 9.1; and pyruvate, 2) equilibrated with 95% O\textsubscript{2}−5% CO\textsubscript{2} at 37°C. The mitral valve was cut to vent the left ventricle. Hearts were perfused using constant pressure (65 cm H\textsubscript{2}O) during a 20-minute stabilization period. In experiments testing coronary flow responses and mechanical performance during iodotubercidin infusion, constant-pressure perfusion was continued unchanged for the entire experiment. In the other experiments, constant-flow perfusion was initiated by setting the flow rate equal to the level reached at the end of the stabilization period while perfusion pressure was measured. Coronary flow was continuously monitored by an electromagnetic flow probe located just above the aortic canulla. All compounds were infused into the aortic canulla 2 to 3 cm above the level of the aortic valve at rates not exceeding 1% of coronary flow.

Response of Coronary Flow and Cardiac Function to Iodotubercidin

The effect of the adenosine kinase inhibitor iodotubercidin (Research Biochemicals Inc, Natick, Mass) on coronary flow and venous effluent adenosine concentrations was investigated in three hearts during constant-pressure perfusion. Left ventricular pressure and heart frequency were monitored using an intraventricular balloon filled to a volume at which end-diastolic pressure was equal to 4 mm Hg. After the stabilization period of approximately 20 minutes, maximal coronary flow was determined by briefly infusing adenosine at a final concentration of 1 μmol/L. Subsequently, cardiac adenosine deaminase was blocked by erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA, 5 μmol/L), which was infused throughout the remainder of the experiment. Cumulative dose-response curves were measured by infusing iodotubercidin at a series of concentrations between 1 nmol/L and 4 μmol/L. At every iodotubercidin concentration, steady-state coronary flow was measured, and two serial coronary venous effluent samples were collected for analyzing the adenosine concentration.

The effect of iodotubercidin on cardiac contractile performance was tested in a separate series of experiments (n=5) during perfusion using constant pressure. After the stabilization period, heart rate, left ventricular developed pressure, and dP/dt were determined using an intraventricular balloon during blockade of adenosine deaminase (EHNA, 5 μmol/L). Iodotubercidin infusion was initiated at a final concentration of 1 μmol/L, and cardiac function was monitored for 10 minutes.

The effect of adenosine deaminase infusion on the increase in coronary flow caused by iodotubercidin was determined in separate experiments using constant-pressure perfusion (n=3). As in the dose-response experiments, maximal coronary flow was determined by briefly infusing adenosine (1 μmol/L) after the stabilization period. Subsequently, a continuous infusion of iodotubercidin was begun at a final concentration of 1 μmol/L. After coronary flow had increased to a new steady state (3 to 5 minutes), an intracoronary infusion of desalted adenosine deaminase\textsuperscript{16} (Boehringer Mannheim Corp, Mannheim, Germany) was initiated at a final nominal activity of 5 to 10 U/mL, and coronary flow was monitored for the next 15 minutes.

Effect of Enzyme Inhibitors on Venous Adenosine Release Rate

The relative contributions of AMP hydrolysis and SAH hydrolysis to total myocardial adenosine production were investigated in 11 separate constant-flow experiments, which were divided into an adenosine
dialdehyde group (n=6) and a parallel control group (n=5). In both groups, total myocardial adenosine production was estimated by measuring the venous effluent adenosine release rate (venous adenosine concentration×coronary flow rate) during blockade of adenosine deaminase and adenosine kinase. The effect of blocking adenosine production via transmethylly was tested in the adenosine dialdehyde group, whereas transmethylly was left intact in the control group.

After the stabilization period, constant-flow perfusion was initiated. Baseline measurements were obtained, and after 5 minutes adenosine deaminase blockade was begun using a continuous intracoronary infusion of EHNA (5 μmol/L). Ten minutes later, in the adenosine dialdehyde group, SAH hydrolase was irreversibly blocked by infusion of adenosine dialdehyde at a final concentration of 10 μmol/L for 10 minutes. After the blockade was established, adenosine dialdehyde was washed out for 2 minutes. The control hearts were perfused with EHNA for an identical period of time, without any adenosine dialdehyde infusion. Subsequently, adenosine kinase was blocked in both groups by an infusion of iodothubercidin at a supramaximal concentration (30 μmol/L) for 4 minutes.

To measure venous adenosine concentrations, duplicate coronary effluent samples were collected for 90 seconds each at the end of the baseline period and 7 minutes after beginning EHNA infusion. Five serial 60-second samples were also collected immediately before and during iodothubercidin infusion.

Efficacy of Enzyme Inhibition

The extent of in vivo enzyme blockade due to iodothubercidin, EHNA, and adenosine dialdehyde was tested by infusing tracers to assess enzyme activity. To test the inhibition of adenosine kinase and adenosine deaminase, [3-4C]adenosine was infused continuously throughout the experiment at a concentration of approximately 1.5 nmol/L (1000 cpm/mL). Measurements were made of total 1C and [3-4C]adenosine activity in the venous effluent samples (see “Adenosine and SAH Analysis”) and of total 1C activity in arterial samples collected after the heart was removed from the aortic cannula at the end of the experiment. Inhibition of adenosine kinase was assessed by calculating myocardial uptake of infused 1C: 100×(arterial–venous 1C activity+/arterial activity (n=8, four from each group). Inhibition of adenosine deaminase was assessed by comparing venous [3-4C]adenosine with total venous 1C activity (n=4, two from each group).

Inhibition of SAH hydrolase was tested at the end of the experiment, after the final effluent sample had been collected, by infusing l-homocysteine thiolactone (1 mmol/L) and [2,8,5-3H]adenosine at a concentration of 10 μmol/L (7000 cpm/mL) for 5 minutes to cause SAH hydrolase–mediated [3H]SAH formation. Immediately afterwards, the hearts were freeze-clamped for analysis of [3H]SAH activity and SAH content (n=11).

31P Nuclear Magnetic Resonance Spectroscopy

The possibility that iodothubercidin interfered with myocardial energy metabolism was tested in separate constant-flow experiments by measuring high-energy phosphate compounds using 31P nuclear magnetic resonance (NMR) spectroscopy.

The 31P NMR spectra were acquired on a Bruker AMX400 WB pulsed Fourier transform NMR spectrometer equipped with a superconducting magnet (Spectrospin and Oxford Instruments) having a field strength of 9.4 T (phosphorus frequency, 161.97 MHz) and a dedicated 20-mm phosphorus probe head. Isolated hearts were perfused inside the magnet at 37°C in a 20-mm NMR sample tube under conditions similar to those used to study hearts outside the magnet. The magnetic field was adjusted using the water proton free induction decay, and a line width at half height of the water proton resonance peak of less than 20 Hz was achieved in every experiment. Partially saturated 31P spectra were accumulated using a radiofrequency pulse width of 62 microseconds, resulting in a 70° tilt angle, 2000 data points in the time domain, and a pulse interval of 3 seconds. Each spectrum was the result of 128 acquisitions. Exponential multiplication of the data (10-Hz line broadening) was followed by Fourier transformation and manual phasing. Creatine phosphate and ATP resonance peaks were integrated using the Bruker UXNMR software after interactive baseline correction. Spectra were acquired during perfusion under baseline conditions and 3 minutes after starting iodothubercidin infusion (30 μmol/L), when perfusion pressure had reached a new steady state.

Adenosine and SAH Analysis

Coronary venous effluent adenosine concentrations were determined by high-performance liquid chromatography (HPLC). Overall analytic recovery of adenosine was determined for each sample using an internal standard of [3H]adenosine added to effluent collection vials before the experiment. To concentrate and desalt the effluent samples (10 to 20 mL), they were passed over Sep-Pak C-18 columns (Waters), rinsed with 2 mL KH2PO4 (10 mmol/L), eluted with 2 mL of 60% methanol, evaporated, and taken up in 200 μL distilled water. Samples were injected on a reverse-phase C-18 column (μBondapak, 10 μm, Waters) using a buffer solution consisting of 95% ammonium acetate (26 mmol/L, pH 5) and 5% methanol, and nucleosides were eluted using a concave methanol (70%) gradient, while monitoring ultraviolet light absorbance at 254 nm. Chromatogram peaks were identified by comparing the retention times of the samples with those of external standards, and peaks were quantified by comparing the integrated peak areas with those of external standards after interactive baseline correction. The HPLC adenosine fractions were collected and analyzed for 3H and 1C activity using a liquid scintillation counter (model PW7400, Philips). Total activity of 1C was also determined in aliquots of venous and arterial perfusate samples without any separation. Venous adenosine concentrations were calculated by accounting for known dilutions and the adenosine recovery determined in every sample (see above). Adenosine recovery averaged 78±4% (mean±SD, n=57).

Myocardial [3H]SAH activity and SAH content were determined in weighed, lyophilized, freeze-clamped heart samples, which were homogenized in ice-cold perchloric acid (0.5 mol/L) and centrifuged at 10 000g. The supernatant was neutralized using 1N KOH and centrifuged, and the resulting supernatant was lyophilized and taken up in distilled water. Samples were
Fig 1. Dose-response curves show the effect of intracoronary iodotubercidin on increases in coronary flow (top) and venous adenosine concentrations (bottom) in identical hearts. Hearts were studied during perfusion at a constant pressure of 48 mm Hg. Coronary flow under control conditions averaged 7.0±1.2 mL·min⁻¹·g⁻¹. Values are mean±SEM (n=3).

Partitioned on the same HPLC C-18 column described above, and while monitoring absorbance at 254 nm, SAH was eluted using the same buffer and gradient described above for adenosine. The SAH peak on the chromatogram was identified by comparison with external standards and quantified by peak area integration using external SAH calibration standards. The HPLC SAH fraction was collected and analyzed for ³H activity.

Statistics

Differences between sample means were judged to be significant at P<.05 by Student’s t test. The 95% confidence interval (two tailed) was determined for the difference between adenosine release rates in the control and adenosine dialdehyde groups by pooling the last three adenosine measurements during iodotubercidin infusion in each experiment. Data are reported as mean±SEM.

Results

Coronary Sensitivity to Increased Endogenous Adenosine

Cumulative dose-response curves were measured for the effects of intracoronary infusion of iodotubercidin on coronary flow (Fig 1, top) and venous adenosine concentrations (Fig 1, bottom) during normoxic perfusion in the presence of EHNA. Coronary flow and venous adenosine concentrations showed a threshold response at an iodotubercidin concentration of 4 nmol/L and increased in a dose-dependent manner to maximal values at iodotubercidin concentrations of approximately 1 μmol/L. Flow increased to the same level when adenosine was infused at a maximally effective concentration of 1 μmol/L. The relation between the measured coronary venous concentrations of endogenous adenosine and coronary flow elicited by iodotubercidin is shown in Fig 2 (solid circles). Half-maximal increases in coronary flow were observed at a venous effluent adenosine concentration of approximately 35 nmol/L, and maximal flow increases were observed at a concentration of 140 nmol/L. Fig 2 also shows estimates of interstitial adenosine concentrations and flow (open circles) associated with each of the venous measurements, calculated using a mathematical model (see "Appendix") and described more fully in "Discussion."

Iodotubercidin had no significant effect on cardiac contractile performance when infused at a maximally effective concentration of 1 μmol/L. When compared with the preceding baseline period, left ventricular developed pressure remained unchanged (92±11 and 91±12 mm Hg, respectively; mean±SEM; n=5), as did dP/dt. Heart rate decreased from 268±10 to 216±16 beats per minute.

To verify that the increases in coronary flow induced by iodotubercidin were mediated by endogenous adenosine, adenosine deaminase was infused during the

Fig 2. Graph shows the relation of endogenous adenosine concentrations and increases in coronary flow elicited by intracoronary iodotubercidin infusion. The data on venous adenosine concentrations (■) were replotted from Fig 1 by pooling the individual measurements made at each iodotubercidin concentration. Estimates of the associated interstitial adenosine concentrations (○) were obtained by modeling the venous measurements. Values are mean±SEM (n=3).

Fig 3. Graph shows the effect of intracoronary infusion of adenosine deaminase (5 to 10 U/mL) on coronary vasodilatation due to iodotubercidin infusion in a representative experiment using constant-pressure perfusion (48 mm Hg).
administration of iodotubercidin to inactivate adenosine. In a representative experiment (Fig 3), continuous infusion of iodotubercidin (1 μmol/L) increased coronary flow, and the effect was fully reversed by adenosine deaminase at a final concentration of 5 to 10 U/mL. Taking baseline flow in individual experiments, averaging 4.7±0.6 (n=3) mL·min⁻¹·g⁻¹, as 100%, iodotubercidin increased flow to 180±10%, and adenosine deaminase decreased flow to 101±7%. Flow during iodotubercidin infusion (8.4±0.8 mL·min⁻¹·g⁻¹) attained the same level as during intracoronary infusion of 1 μmol/L adenosine (8.0±0.5 mL·min⁻¹·g⁻¹) in the same experiments.

**Contribution of SAH Hydrolase to Total Myocardial Adenosine Production**

*Baseline conditions.* In separate experiments, the contribution of transmethylation to total myocardial adenosine production was assessed. Under baseline conditions before any enzyme inhibitors were administered, the constant coronary flow rate and perfusion pressure were similar in the control group and the group to be treated with adenosine dialdehyde to block transmethylation. Coronary flow averaged 14.2±1.3 (n=5) and 12.7±1.1 (n=6) mL·min⁻¹·g⁻¹ in the control group and adenosine dialdehyde group, respectively, and coronary perfusion pressure averaged 54±2 and 51±2 mm Hg, respectively. Venous adenosine release rate (venous concentration×flow) averaged 196±45 and 106±28 pmol·min⁻¹·g⁻¹ in the control group and adenosine dialdehyde group, respectively (Fig 4, top).

*Control group.* In the control group, total normoxic myocardial adenosine production was estimated by measuring venous adenosine release during blockade of adenosine deaminase and adenosine kinase. Blockade of adenosine deaminase using a continuous infusion of EHNA (5 μmol/L) caused an increase in venous adenosine release of 2.3±0.5-fold (P<0.0025, paired comparison with baseline) (Fig 4, top). Subsequent blockade of adenosine kinase by an intracoronary infusion of iodotubercidin (30 μmol/L) caused a rapid 10-fold increase in venous adenosine release to a value of 3360±322 pmol·min⁻¹·g⁻¹ after 4 minutes. Coronary perfusion pressure decreased by the end of the first minute of iodotubercidin infusion to a value of 34±2 mm Hg and reached a final value of 31±1 mm Hg. Iodotubercidin was infused at a concentration nearly 10 times higher than the maximally effective concentration used in the dose-response experiments described above.

*Adenosine dialdehyde group.* In the adenosine dialdehyde group, the contribution of transmethylation to total myocardial adenosine production was estimated by measuring venous adenosine release after infusion of an irreversible blocker of SAH hydrolase, adenosine dialdehyde (10 μmol/L). Blockade of adenosine deaminase and adenosine kinase was identical to that in the control group. EHNA caused a 2.9±1.1-fold increase (P<0.0025) in venous adenosine release, similar to that in the control group. There were no changes in perfusion pressure due to EHNA in either group. Infusion of adenosine dialdehyde had no effect on venous adenosine release or perfusion pressure. Iodotubercidin (30 μmol/L) increased adenosine release to a value of 3370±536 pmol·min⁻¹·g⁻¹ at the end of 4 minutes (Fig 4, top), a value not significantly different from that in the control group.

**Fig 4.** Top, Graph shows the effect of enzyme blockers on venous adenosine release rate. In both control (n=5) and adenosine dialdehyde (n=6) groups, erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA, 5 μmol/L) and iodotubercidin (30 μmol/L) infusions were begun and continued as indicated. The adenosine dialdehyde group was treated with intracoronary adenosine dialdehyde (10 μmol/L) for 10 minutes, beginning at 14 minutes, followed by washout. The last five serial 1-minute venous samples were collected without interruption. There was no significant difference between groups at any point. Values are mean±SEM (one direction only). Bottom, Graph shows the effect of enzyme blockers on myocardial uptake of intracoronary [³⁰C]adenosine, infused continuously in experiments shown in the graph at the top. Uptake was defined as 100×(arterial–venous [³⁰C] activity)×arterial activity and was measured in the same effluent samples used for analyzing unlabeled adenosine. Results were pooled from both control and adenosine dialdehyde groups. Values are mean±SEM (n=8).

Perfusion pressure followed a similar time course as in the control group, decreasing to 32±2 mm Hg at the end of the first minute of iodotubercidin infusion and reaching a final value of 28±1 mm Hg.

Although there was no significant difference between the adenosine release measurements in the control group and adenosine dialdehyde group, variability between hearts might have obscured a true difference. An estimate of the largest difference that might have been overlooked is provided by the 95% confidence interval for the difference between mean release rates, which had a value of 1.0 nmoL·min⁻¹·g⁻¹.

*In vivo tests of enzyme inhibitors.* Inhibition of adenosine deaminase was tested in four experiments (two from each group) by measuring the fraction of total venous [³⁰C] activity that was in the form of [³⁰C] adenosine during intracoronary infusion of [³⁰C] adenosine at a nonsasasas constant concentration of approximately 1.5 μmol/L throughout the experiment. Under baseline conditions, 85±3% of the venous [³⁰C] activity was in the form of [³⁰C] adenosine, and the amount was increased by EHNA infusion to 97±1%. During iodo-
tubercidin infusion, there was sustained blockade of adenosine deaminase, since 101±2% of venous ¹⁴C activity was in the form of [²⁴⁰]adenosine. Inhibition of adenosine kinase was demonstrated by the finding that iodotubercidin decreased myocardial uptake of infused [²⁴⁰]adenosine from a baseline value of 50±6% to undetectable levels (Fig 4, bottom). Inhibition of SAH hydrolase was demonstrated by the finding that myocardial production of [²¹]SAH due to intracoronary infusion of [²¹]adenosine (10 μmol/L) and L-homocysteine thiolactone (1 mmol/L) for 5 minutes at the end of each experiment was abolished in the adenosine dialdehyde group (Fig 5, left graph). In this group, [²¹]SAH activity could not be distinguished from zero. On the basis of the unlabeled SAH measurements (Fig 5, right graph), it appears that inhibition was approximately 92%, since SAH content was 12-fold higher in the control group than in the adenosine dialdehyde group.

However, measurements of unlabeled SAH are less selective than those of [²¹]SAH and underestimate the inhibition, since SAH hydrolase blockade causes continuous increases in SAH content as a result of ongoing transmethylation reactions.² Although the present test of SAH hydrolase activity was for the synthetic direction, adenosine dialdehyde causes equivalent blockade in both the synthetic and hydrolytic directions.²

Selectivity of Iodotubercidin: ³¹P NMR Spectroscopy

The possibility existed that iodotubercidin caused increases in venous adenosine by interfering with myocardial energy metabolism, which would result in an overestimate of the normal rate of adenosine salvage. To test for this possibility, ³¹P NMR spectroscopy was used to assess the effects of iodotubercidin on high-energy phosphate levels, under experimental conditions similar to those used in the control group and adenosine dialdehyde group. During perfusion at a constant flow rate of 5.8±0.2 mL min⁻¹ g⁻¹ (n=3), iodotubercidin at the same concentration as in the above experiments (30 μmol/L) caused a prompt decrease in perfusion pressure from a control value of 53±3 to 43±2 mm Hg. Fig 6 shows phosphorus spectra obtained in a representative experiment during the control period and during iodotubercidin infusion. Because the radiofrequency pulse interval (3 seconds) was in the same range as the relaxation time constant for the creatine phosphate peak, the ratio of creatine phosphate to ATP (both peak area and peak height) was lower than would be obtained for fully relaxed spectra. The inorganic phosphate peak was dominated by the inorganic phosphate present at a physiological concentration (1.2 mmol/L) in the perfusion medium. Most important, however, iodotubercidin did not cause any significant change in the ratio of creatine phosphate to ATP nor in the peak areas themselves (see difference spectrum). Taking peak areas in the control period as 100%, creatine phosphate was 102±2% and ATP was 101±1% in the presence of iodotubercidin.

Discussion

Magnitude of Intracellular Adenosine Salvage

When adenosine salvage was abolished in the well-oxygenated heart by the adenosine kinase blocker iodotubercidin, the rate of coronary venous adenosine release increased more than 15-fold over baseline. This
finding indicates that more than 90% of myocardial adenosine produced under normoxic conditions is normally salvaged intracellularly by rephosphorylation, while only 4% to 5% escapes into the venous outflow, and the remainder is deaminated. By blocking adenosine salvage and deamination, the venous release rate increased to a level normally only observed during severe hypoxia, indicating that intracellular salvage by adenosine kinase is important in controlling myocardial adenosine levels. Thus, the myocardial adenosine production rate in the normoxic perfused guinea pig heart is approximately 3.3 nmol·min⁻¹·g⁻¹, in good agreement with results obtained by Ely et al (3.5 nmol·min⁻¹·g⁻¹) using a similar approach. The NMR results rule out the possibility that iodotubercidin caused increases in adenosine production by interfering with myocardial energy metabolism.

**Mechanism of Adenosine Production**

We found that inhibition of adenosine production via the transmethylation pathway with adenosine dialedhyde did not significantly decrease total myocardial adenosine production in iodotubercidin-treated hearts. This indicates that AMP hydrolysis is the dominant pathway for myocardial adenosine production under normoxic conditions. The normal pathways for myocardial adenosine production include intracellular hydrolysis of AMP via cytosolic 5'-nucleotidase, transmethylation via SAH hydrolase, AMP hydrolysis via alkaline phosphatase, and extracellular AMP hydrolysis via ecto-5'-nucleotidase. Although the present results do not distinguish the activity of alkaline phosphatase and ecto-5'-nucleotidase, their roles were most likely minor in relation to overall adenosine production.

In light of the above finding, the conclusion reached in an earlier study from this laboratory in which transmethylation was suggested to represent the major pathway for myocardial adenosine production in the normoxic heart must be reassessed. In that study, 5'-amino-5'-deoxoadenosine (5 μmol/L), which inhibited adenosine kinase by approximately 50%, was used. In the present study, iodotubercidin (30 μmol/L) inhibited this enzyme completely (Fig 4). Both 5'-amino-5'-deoxoadenosine and iodotubercidin are competitive inhibitors of adenosine kinase, and it is thus likely that we have previously underestimated the in vivo flux through adenosine kinase. As evident in the present study, more complete and probably more specific blockade of adenosine kinase by iodotubercidin demonstrated that AMP hydrolysis, rather than transmethylation, is the major pathway for adenosine production under normoxic conditions.

In the present study, variability in adenosine release between animals limited the accuracy for estimating transmethylation. The 95% confidence interval for the difference between the mean release rates in the two groups of 1.0 nmol·min⁻¹·g⁻¹ provided an upper limit for the transmethylation rate of 1.2 nmol·min⁻¹·g⁻¹. The true rate of transmethylation was probably lower, since transmethylation was estimated to be 160 (Reference 12) and 750 (Reference 11) pmol·min⁻¹·g⁻¹ in previous studies using the same experimental model.

**Normal Adenosine Flux Rates**

Estimates of steady-state adenosine flux rates in the normoxic guinea pig heart are shown in Fig 7. The sum of AMP hydrolysis and transmethylation is equal to 3.3 nmol·min⁻¹·g⁻¹, based on the present results. The transmethylation rate of 0.75 nmol·min⁻¹·g⁻¹ was taken from a previous estimate, since it was not possible to estimate transmethylation directly in the present study. The rates of adenosine phosphorylation, deamination, and membrane efflux are based on reasonable estimates (see “Appendix”) of kinetic parameters for adenosine kinase, adenosine deaminase, and membrane permeability–surface area product, assuming a steady-state cytosolic adenosine concentration of 90 nmol/L. Although the rate of AMP hydrolysis exceeds the rate of transmethylation, the net flux rate through the AMP–adenosine cycle is in the direction of adenosine phosphorylation. This is because adenosine kinase is a sink for both adenosine sources (AMP hydrolysis and transmethylation). Thus, it is the unidirectional flux rates of 5'-nucleotidase and SAH hydrolase that determine which pathway dominates adenosine production, not the net flux through the cycle.

The turnover time of the cytosolic adenosine pool can be calculated to be

\[ \frac{(90 \text{ pmol/mL}) \times (0.6 \text{ mL/g})}{3300 \text{ pmol·min}^{-1} \cdot \text{g}^{-1}} = 0.016 \text{ minute} = 1.0 \text{ second} \]

taking a cytosolic volume of 0.6 mL/g. The turnover time of 1 second may be an overestimate, if mitochondria are not permeable for adenosine. In that case, a better estimate of 0.5 second may be obtained using an extramitochondrial cytosolic volume of distribution of 0.33 mL/g measured in buffer-perfused hearts. Although mitochondrial transport of adenosine has not been studied directly, a recent report suggests that the mitochondrial inner membrane is permeable to adenosine. The high turnover rate of adenosine explains the rapid increase in cytosolic adenosine in response to an increase in free cytosolic AMP (see below).

**Coronary Sensitivity for Endogenous Adenosine**

Blocking adenosine salvage caused dose-dependent increases in coronary flow up to maximal levels that were entirely mediated by endogenous adenosine, as indicated by the results with adenosine deaminase. Had an adenosine receptor antagonist been used instead of adenosine deaminase, one could not rule out a possible agonist action of iodotubercidin at adenosine receptors. The venous adenosine concentrations associated with half-maximal increases in coronary flow (35 nmol/L)
during iodotubercidin infusion are in the range observed during hypoxia in isolated guinea pig hearts.17 The increased flow was most likely caused by increased interstitial adenosine acting on vascular smooth muscle receptors; however, endothelial cell receptors might have played a role as well.21,22 The interstitial adenosine concentrations causing increased flow were estimated by analyzing the measurements of venous concentrations and flow, using a mathematical model4 to account for the effects of flow and capillary endothelial cells on the transport and metabolism of adenosine (see “Appendix” for details). At a half-maximal increase in flow, the modeled interstitial adenosine concentration was approximately 90 nmol/L, and at maximal flow, it was 420 nmol/L. As evident from Fig 2, the modeled interstitial adenosine concentration is about three times higher compared with the measured venous effluent adenosine. In a previous study, the dose-response curve for coronary flow increases caused by intracoronary infusion of adenosine revealed that a half-maximal increase in coronary flow was obtained at 200 nmol/L and maximal flow at 2 μmol/L, respectively.17 This is approximately two to four times higher than the modeled interstitial adenosine concentrations of the present study to which the vascular smooth muscle is exposed. These findings are in support of the hypothesis23 that the dose-response curve for exogenously applied adenosine underestimates the true sensitivity of the vascular smooth muscle.

The venous adenosine concentration observed at maximal flow in the present study (140 nmol/L) was greater than the maximal concentration observed during dipyridamole treatment (44 nmol/L) by Wangler et al15 but less than that observed during treatment with iodotubercidin and EHNA (260 nmol/L) by Ely et al.13 The estimate of 420 nmol/L for interstitial adenosine at maximal flow in the present study is again intermediate between the estimates of 190 nmol/L, based on model analysis of dipyridamole results,15 and 1 μmol/L, based on epicardial transudate measurements.13 It is possible that the flow effects of dipyridamole were submaximal, whereas the effects of iodotubercidin and EHNA in the study of Ely et al were supramaximal. The estimate of interstitial adenosine under control conditions (22 nmol/L) in the present study was also intermediate between the model estimate of Wangler et al (7 nmol/L) and the transudate estimates of Ely et al (250 nmol/L) and one of the present authors (180 nmol/L).24 The reason that the model estimates are lower than the transudate estimates is unclear. There is evidence that adenosine concentrations in epicardial transudate are elevated above those in bulk interstitial fluid because of the effect of superficial nerve endings.25 On the other hand, the models may not properly account for the transendothelial transport of adenosine, since they are based on the assumption that the density of membrane adenosine transporters is uniform on the endothelial cell surface.26 If the transporters were highly concentrated within the interendothelial cleft regions, then the models would underestimate interstitial concentrations.14 Nevertheless, all data on endogenous adenosine indicate that there is a steep concentration gradient between the interstitial and the intravascular space.

**AMP-Adenosine Metabolic Cycle**

Since intracellular adenosine production and salvage were proceeding at rates more than 10-fold higher than the venous release rate of adenosine, there is evidence for a high rate of turnover in the AMP-adenosine metabolic cycle mediated by cytosolic 5'-nucleotidase and adenosine kinase. An “AMP-adenosine substrate cycle” in the heart was first described by Arch and Newsholme27 and has also been observed in the liver by Bontemps et al,28 who termed it a “futile cycle.” Arch and Newsholme proposed that the AMP-adenosine cycle serves to increase the sensitivity of cytosolic adenosine concentrations for increases in the rate of AMP hydrolysis. It was emphasized that there would be increased sensitivity only if adenosine kinase were normally saturated. Because other investigators observed that adenosine kinase is not normally saturated in the heart,29,30 the function of the cycle remains in doubt.

To assess the interaction of the AMP-adenosine cycle and transmethylation, a mathematical model of adenosine in the guinea pig heart44 was used to analyze the effect of increasing the rate of AMP hydrolysis in the metabolic system shown schematically in Fig 7. Doubling AMP hydrolysis to a rate of 5.1 nmol·min⁻¹·g⁻¹ in the model caused an increase in the cytosolic adenosine concentration to a new steady-state level shown by the middle curve (normal cycling) in Fig 8 (see “Appendix” for details). The transmethylation rate (taken from a previous study13) remained unchanged, whereas the other flux rates increased as the adenosine concentration rose. To assess the role of AMP-adenosine cycling in the response, hypothetical conditions of 10-fold–increased and 10-fold–decreased cycling were simulated, with the basal cytosolic adenosine concentration remaining unchanged. During reduced cycling, doubling the rate of AMP hydrolysis resulted in a slower and smaller increase in cytosolic adenosine (Fig 8, bottom curve). The response was diminished because the rate of transmethylation was larger than that of AMP hydrolysis.
lysis, diluting the effect of the increase. During increased cycling, doubling AMP hydrolysis resulted in a faster and larger increase in cytosolic adenosine (Fig 8, top curve). This was because the influence of transmethylation was overwhelmed. Further increases in cycling were without additional effect.

The model results indicate that AMP-adenosine cycling increases the sensitivity of cytosolic adenosine concentrations to increases in the rate of AMP hydrolysis in the presence of a second source of adenosine production, ie, transmethylation. Saturation of adenosine kinase plays no role in the increased sensitivity. At the same time, cycling serves to keep the rate of adenosine rephosphorylation high, minimizing purine losses. The physiological function of AMP-adenosine cycling appears to be that of keeping cytosolic adenosine concentrations under the control of myocardial energetic status, even during normoxic conditions when the net flux rate through the AMP-adenosine cycle is less than the rate of transmethylation. The importance of the AMP-adenosine cycle would be increased if the true rate of transmethylation were greater than the assumed rate of 0.75 nmol · min\(^{-1}\) · g\(^{-1}\) but would be less if the transmethylation were lower.

The normal adenosine response in the model shown by the middle curve in Fig 8 was 82% complete in 2 seconds, based on a volume of distribution for adenosine of 0.6 mL/g. If the extramitochondrial volume of distribution of 0.33 mL/g was used for modeling, then the response was 95% complete in 2 seconds, in line with the faster turnover time of the adenosine pool noted above. The different volumes of distribution changed steady-state concentrations by less than 1%.

Other studies have shown that myocardial adenosine production increases monotonically with cytosolic AMP concentrations over a wide range of phosphorylation potentials.\(^{5,5,31}\) The explanation may be that the \(K_m\) of cytosolic 5'-nucleotidase for AMP is in the millimolar range,\(^{32}\) whereas normal cytosolic AMP concentrations are in the nanomolar range,\(^3\) and the adenylyl kinase reaction is near equilibrium.\(^{32}\) Further increases in the sensitivity of adenosine production to phosphorylation potential may result from the allosteric activation of cytosolic 5'-nucleotidase by ADP.\(^{32}\) The present results demonstrate that the mechanistic coupling between adenosine production and phosphorylation potential is not obligatory, since iodotubercidin produced a complete pharmacologic dissociation. In addition, our results indicate that adenosine production could be sensitively controlled if adenosine kinase were subject to allosteric inhibition. However, there are no studies on the possible regulation of adenosine kinase by phosphorylation potential.

The metabolic cost of the normal level of cycling (Fig 7) is approximately 5 nmol ATP hydrolyzed per minute per gram, assuming that hydrolysis of one AMP and phosphorylation of one adenosine each cost one ATP equivalent in the long term. Relatively large increases in the cost would be required to achieve relatively modest further increases in sensitivity (Fig 8, increased cycling). Although this suggests that normal cycling may represent an optimized balance between the benefits of cycling versus the energetic cost, even increased cycling would constitute less than 1% of total myocardial ATP hydrolysis.

**Effect of Adenosine Deaminase on Interstitial Adenosine**

Intracoronary infusion of adenosine deaminase has been used in a number of studies to test the role of endogenous adenosine in the regulation of coronary flow. However, concerns were expressed whether the enzyme was effective in substantially reducing interstitial adenosine concentrations.\(^{34}\) Adenosine concentrations measured in epicardial and endocardial transudate fluid collected using porous discs were only moderately decreased by intracoronary adenosine deaminase infused at concentrations similar to those used in the present study.\(^{25}\) However, in another study, adenosine could not be detected in epicardial transudate during infusion of adenosine deaminase at lower concentrations than in the present study in normoxic or hypoxic hearts of rats and guinea pigs.\(^{24}\) The cause of the discrepancy is not clear. In the present study, adenosine deaminase decreased interstitial adenosine concentrations below the threshold for vasodilation, even though net adenosine production was elevated to levels causing maximal coronary flow. The finding demonstrates that adenosine deaminase at an arterial concentration of 5 to 10 U/mL in the perfused guinea pig heart is an effective tool for testing the adenosine hypothesis.

**Selectivity and Efficacy of Enzyme Inhibitors**

The selectivity of iodotubercidin was demonstrated in NMR experiments, which showed that iodotubercidin did not alter myocardial energetics, and in the adenosine deaminase experiments, which demonstrated that it did not act as an agonist at coronary adenosine receptors. Further evidence for selectivity is that iodotubercidin, at the concentration used in the present study, did not alter the activity of cytosolic 5'-nucleotidase, purified from canine myocardium (Patricia Metting, Medical College of Ohio, personal communication), and that it did not act as an agonist at adenosine A\(_1\) or A\(_2\) receptors in the brain.\(^{36}\) Selectivity of adenosine dialdehyde was demonstrated in a previous investigation by the finding that it did not alter kinetic parameters of adenosine deaminase, adenosine kinase, or cytosolic 5'-nucleotidase isolated from the guinea pig heart.\(^{12}\) Efficacy of the blockade of adenosine deaminase, adenosine kinase, and SAH hydrolase was demonstrated by the findings that, in the presence of EHNA, all coronary venous \(^{32}\)C activity was in the form of \(^{32}\)Cadenosine, that iodotubercidin abolished myocardial uptake of \(^{32}\)Cadenosine, and that adenosine dialdehyde abolished myocardial formation of \(^3\)HSAH from infused \(^3\)Hadenosine and l-homocysteine thiolactone, respectively.

**Endothelial Cell Adenosine Production**

Normally, capillary endothelial cells contribute approximately 14% of the adenosine in the coronary venous effluent; this value is based on measurements of the specific activity of venous adenosine released from hearts that were prelabeled with an intracoronary infusion of tracer adenosine.\(^{37}\) It has been observed that iodotubercidin caused increased adenosine release from endothelial cells in culture.\(^{38}\) However, in the present study, the endothelial cell contribution most likely de-
creased during iodotubercidin infusion, on the basis of the finding that iodotubercidin caused a decrease in the specific activity of adenosine released from prelabeled hearts (authors' unpublished observations). These results make it likely that venous adenosine in the presence of iodotubercidin and EHNA in the present experiments was mainly, but not entirely, derived from cardiomyocytes.

Appendix

Estimating Interstitial Adenosine Concentrations

To estimate interstitial adenosine concentrations during graded inhibition of adenosine kinase due to iodotubercidin, a model of adenosine transport and metabolism in the guinea pig heart was used.14 The model accounts for the effects of capillary flow and endothelial cell transport and metabolism on capillary and interstitial adenosine concentrations. Interstitial concentrations were estimated by fitting the observed venous concentrations and measured steady-state flow rates in individual experiments. Venous concentrations were fitted by adjusting the \( K_m \) of adenosine kinase in endothelial cells and cardiomyocytes, assuming that iodotubercidin had equal effects in both cells. Increasing the \( K_m \) caused increases in both venous and interstitial concentrations in the model. Other model parameters were kept constant at values reported previously.14 Individual interstitial estimates at each iodotubercidin concentration were pooled to produce the values (mean ± SEM) in Fig 2.

Modeling AMP-Adenosine Cycling

The model used for assessing the effect of AMP-adenosine cycling was a simplified version of the model described above. For clarity, the model shown in Fig 7 included only those features required to describe the effects of AMP-adenosine cycling: adenosine production from AMP and SAH, adenosine removal via adenosine kinase, adenosine deaminase, and membrane efflux. The complete model gave essentially the same results. The differential equation describing the system in Fig 7 was based on the following expression:

\[
V_p \times \frac{d[A]_{pc}}{dt} = \text{AMP hydrolysis+SAH hydrolysis} - \text{adenosine phosphorylation} - \text{adenosine deamination} - \text{adenosine efflux}
\]

(A1)

where \( V_p \) is the volume of the parenchymal cell (0.6 mL/g), \([A]_{pc}\) is the cytosolic adenosine concentration in the parenchymal cell, and \( t \) is time. By assuming that adenosine kinase and adenosine deaminase followed Michaelis-Menten kinetics and that membrane transport was first order, Equation A1 can be written more explicitly as

\[
V_p \times \frac{d[A]_{pc}}{dt} = S \text{AMP} + S \text{SAH} - \frac{V_{max} \times [A]_{pc}}{K_m + [A]_{pc}}
\]

(A2)

where \( S \text{AMP} \) and \( S \text{SAH} \) are the constant rates of AMP hydrolysis and SAH hydrolysis, respectively, the superscript \( \text{c} \) refers to the 35S measurement (see below) and \( K_m \) (2.5 \muM/L) of adenosine kinase, the superscript \( \text{d} \) refers to the \( V_{max} \) (450 nmol \cdot min^{-1} \cdot g^{-1}) and \( K_m \) (83 \muM/L) of adenosine deaminase, \( P_{sc} \) (2.5 mL \cdot min^{-1} \cdot g^{-1}) is the permeability-surface area product for parenchymal cell membrane transport of adenosine, and \([A]_{pc}\) (23 nmol/L) is the concentration of adenosine in interstitial fluid. The values in parentheses were taken directly from a previous study.14

To model the normal conditions of AMP-adenosine cycling observed in the present study, it was assumed that \( S \text{AMP} = 0.75 \text{nmol} \cdot \text{min}^{-1} \cdot \text{g}^{-1} \), and it was calculated that \( S \text{AMP} = 3.3 \text{nmol} \cdot \text{min}^{-1} \cdot \text{g}^{-1} \), since the total rate of adenosine production was 3.3 nmol \cdot min^{-1} \cdot g^{-1}. It was further assumed that the steady-state cytosolic adenosine concentration \([A]_{pc}\) equaled 90 nmol/L, which constrained \( V_{max} \), for adenosine kinase to 76 nmol \cdot min^{-1} \cdot g^{-1}. The steady-state flux rates for adenosine kinase, adenosine deaminase, and membrane efflux shown in Fig 7 were calculated as follows:

\[
\frac{V_{max} \times [A]_{pc}}{K_m + [A]_{pc}} = \frac{76 \text{nmol} \cdot \text{min}^{-1} \cdot \text{g}^{-1} \times 0.09 \text{nmol/mL}}{2.5 \text{nmol/mL} + 0.09 \text{nmol/mL}}
\]

(A3)

\[
\frac{V_{max} \times [A]_{pc}}{K_m + [A]_{pc}} = \frac{2.6 \text{nmol} \cdot \text{min}^{-1} \cdot \text{g}^{-1}}{2.5 \text{nmol/mL} \times 0.09 \text{nmol/mL}}
\]

(A4)

\[
\frac{V_{max} \times [A]_{pc}}{K_m + [A]_{pc}} = \frac{0.5 \text{nmol} \cdot \text{min}^{-1} \cdot \text{g}^{-1}}{2.5 \text{nmol/mL} \times 0.09 \text{nmol/mL}}
\]

(A5)

Sensitivity of the transport/metabolic system for increases in the rate of AMP hydrolysis was assessed by doubling \( S \text{AMP} \) via a step function and numerically integrating Equation A2 to model the resulting time course for the increase in \([A]_{pc}\). The model accounted for the effect of increased \([A]_{pc}\) on the rates of adenosine kinase, adenosine deaminase, and membrane transport, all of which increased to new steady-state levels. The model solution for \([A]_{pc}\), based on the cycling rate determined in the present study, is shown by the middle curve (normal cycling) in Fig 8, in which the increase in AMP hydrolysis was imposed at a time of 5 seconds. The twofold increase in \( S \text{AMP} \) caused an 80% increase in the steady-state value of \([A]_{pc}\). It was assumed that there were no changes in \( S \text{SAH} \) due to increased \([A]_{pc}\), based on the kinetics of SAH hydrolysis.

To model the hypothetical conditions of increased and reduced rates of AMP-adenosine cycling, the \( V_{max} \) for adenosine kinase was increased and decreased 10-fold, with respect to the normal value of 76 nmol \cdot min^{-1} \cdot g^{-1}, and \( S \text{AMP} \) was increased and decreased approximately 10-fold, with respect to the normal value of 2.55 nmol \cdot min^{-1} \cdot g^{-1}, determined in the present study. The changes in \( V_{max} \) and \( S \text{AMP} \) were made in parallel, so that both were increased or both were decreased. The result was to model increased and decreased rates of turnover of the AMP-adenosine cycle, without altering the steady-state cytosolic adenosine concentration, \([A]_{pc}\). Because \([A]_{pc}\) was the same as during normal cycling, steady-state flux rates for adenosine deaminase and membrane efflux remained unchanged. No other model parameters were changed.

The effect of increased and decreased turnover of the cycle on the sensitivity for increases in the rate of AMP hydrolysis was determined in the same way as for normal cycling. Separate model solutions were obtained for twofold step increases in \( S \text{AMP} \) from the new steady-state starting levels, representing increased and reduced cycling, and the results are shown in Fig 8. The twofold increase in \( S \text{AMP} \) resulted in steady-state increases in \([A]_{pc}\) of 100% and 18% during increased and reduced cycling, respectively. During increased cycling, the response was faster, and during reduced cycling, it was slower.

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