DURING MYOCARDIAL HYPOXIA or ischemia, there is a net hydrolysis of ATP to ADP and then AMP, which in turn is hydrolyzed to membrane-permeable adenosine. The release of adenosine during ischemia is beneficial by providing receptor-mediated vasodilatory protection and has also been shown to mediate ischemic preconditioning; i.e., a brief period of ischemia increases myocardial viability during subsequent prolonged ischemia (7, 8, 17). New research also indicates that AMP hydrolysis to adenosine in the myocyte is temporarily beneficial during compromised energy supply through an improvement of the ATP phosphorylation potential (22). During coronary underperfusion the kinetics of phosphocreatine (PCr) and ATP could only be explained by mass action effects of AMP hydrolysis to adenosine, creating a sink for cytosolic ADP (22). AMP hydrolysis, therefore, mediates an open adenylate system. However, AMP hydrolysis also leads to a loss of purines during sustained underperfusion and, therefore, to the depletion of nucleotide pools (15), contributing to the mechanical dysfunction of the myocardium during reperfusion (30). Because a high rate of AMP hydrolysis benefits tissue survival during the onset of underperfusion but will cause tissue injury during sustained underperfusion, the regulation of AMP hydrolysis may be of utmost importance to the survival of the myocardium during compromised flow.

In heart the primary enzymatic pathway responsible for the production of adenosine is the dephosphorylation of AMP by the cytosolic form of 5’-nucleotidase (5’-NT, E.C. 3.1.3.5, AMP → adenosine + Pi) (4). The current literature, however, is unclear regarding the regulation of 5’-NT in normal and ischemic heart. It has been suggested that rat heart 5’-NT is regulated by the adenylate energy charge (14) and that the free concentration of ADP regulates rabbit (33) and dog (4) heart 5’-NT. Pi (27) and pH (2) are also possible regulators of 5’-NT during ischemia. Studies examining ischemic preconditioning have indicated that the hydrolysis of AMP during ischemia is increased or decreased by a preceding brief period of ischemia (10, 19, 25). However, a confounding factor in these studies in the intact heart is that the production of myocardial purines during ischemia depends on the activity of 5’-NT and the cytosolic concentration of AMP, i.e., on the disturbance of the myocardial energy balance. Therefore, the observation of decreased loss of myocardial purines due to ischemic preconditioning may be due to decreased net ATP breakdown rather than decreased 5’-NT activity. Thus the activity and regulation of 5’-NT in the ischemic intact heart remain to be determined.

The purpose of this study was to determine whether the activity of 5’-NT is downregulated during sustained coronary underperfusion. To investigate the regulation of 5’-NT during ischemia in the intact heart, it is necessary to distinguish between the effects of altered 5’-NT activity and effects of energy imbalance (net ATP hydrolysis and cytosolic AMP concentration). We have, therefore, applied a physiologically realistic mathematical model for analyzing simultaneously obtained data of myocardial high-energy phosphates and coronary venous purines. This new, integrated approach allowed us to account for changes in energy metabolism during sustained underperfusion in describing the kinetics of 5’-NT. The results show that, during sustained coronary underperfusion, there is a downregulation of the activity of 5’-NT that may benefit tissue survival by preserving the ATP pool.
MATERIALS AND METHODS

Isolated Perfused Heart Preparation

New Zealand White rabbits (2.2–3.0 kg) were sedated with acetylpromazine (0.8 mg/kg iv) and anesthetized with ketamine (40 mg/kg iv) plus xylazine (5 mg/kg iv). After initial anesthesia the rabbits were treated with heparin (200 U iv) and 8-sulphonylthioepine (10 mg/kg iv) to prevent blood coagulation and adenosine receptor activation during the surgery required for isolation of the heart. After 5 min the heart was rapidly excised, rinsed in ice-cold saline, and immediately mounted by the aorta on a Langendorff apparatus. The hearts were perfused at 37°C at a constant pressure of 80–100 mmHg with a nonrecirculating modified Krebs-Henseleit bicarbonate buffer consisting of (in mM) 118 NaCl, 3.8 KCl, 1.2 KH2PO4, 0.7 MgSO4, 2.1 CaCl2, 0.1 EDTA, 25 NaHCO3, 11 glucose, and 5 pyruvate and 0.1% bovine serum albumin and equilibrated with 95% O2-5% CO2 using a membrane oxygenator, resulting in a pH of 7.35–7.45. A fluid-filled latex balloon was placed in the left ventricle, connected to a pressure transducer, and inflated to yield a systolic pressure between 70 and 90 mmHg, with an end-diastolic pressure <5 mmHg. Pacating electrodes were secured to the epicardial surface, and the hearts were paced at 180 beats/min.

To collect coronary venous effluent samples in the nuclear magnetic resonance (NMR) experiments, the azygous vein and inferior vena cava were ligated. Nested outer (Tygon, ½th-in. OD) and inner (PE-90) tubes were threaded through the superior vena cava into the right ventricle and out the pulmonary artery. Coronary venous effluent was withdrawn from the right ventricle through side holes in the inner tube using a peristaltic pump. When coronary flow was subsequently decreased, ~90% of the coronary arterial inflow volume could be collected in this manner. The hearts were then submerged in 37°C perfusate in a 3-cm-diameter plastic, temperature-controlled, water-tight cylinder that was encircled with a solenoid-style radio-frequency NMR coil. The heart setup was placed inside the magnet, the radiofrequency coil was tuned to 81 MHz and matched, the gradient coils were shimmed, and a fully relaxed NMR spectrum was acquired. On completion of each experiment, wet ventricular weight was determined.

Experimental Protocols

Protocol 1. 5'-NT activity during two identical sequential periods of underperfusion. Seven rabbits were used to investigate 5'-NT activity during two sequential and identical periods of underperfusion, each lasting 45 min. After baseline measurements, coronary perfusion (pump flow) was decreased to ~0.24 ml·g⁻¹·min⁻¹ (~4–5% baseline) and held constant for 45 min, then flow was restored to baseline for 20 min. The flow was then again reduced to the same level as during the first period of underperfusion for another 45 min, and after a 20-min reperfusion period the experiment was terminated. NMR spectra and venous samples were acquired identically during both periods of underperfusion.

Protocol 2. Time course experiments: effect of different lengths of underperfusion on 5'-NT activity. Fifteen rabbits were used in non-NMR experiments to investigate the effect of a 0-, 5-, 10-, 20-, or 40-min period of underperfusion on 5'-NT activity during a subsequent 40-min test period of underperfusion. The two sequential periods of underperfusion were separated by a 20-min period of reperfusion. The duration of the first period of underperfusion was 5 min (5/40, n = 3 hearts), 10 min (10/40, n = 3), 20 min (20/40, n = 3), or 40 min (40/40, n = 6). In all hearts the duration of the second (test) period of underperfusion was 40 min. The effect of 0 min of underperfusion in the first period (0/40) was used as a time period of 40 min in the 40/40 group as the test period.

Protocol 3. Time control experiments. Four rabbits were used in NMR experiments to study the effect of time on coronary venous purine release and myocardial high-energy phosphates. Protocol 1 was followed, except that during the first 45-min period, perfusion was maintained at constant baseline levels. Thus a single 45-min period of underperfusion was studied after a 65-min (45 min + 20 min of reperfusion) period of baseline perfusion.

Protocol 4. Indicator-dilution experiments to determine venous sampling delays. Three rabbits were used for separate indicator-dilution experiments to derive a transfer function that would accurately describe the delay and dispersion of the venous, veins, right atrium, and the canula on the kinetics of the venous purine measurements. Briefly, a bolus of 50 µl of indocyanine green dye (2.5 mg/ml), an impermeant tracer, was injected into the aorta during low flow (~0.25 ml·g⁻¹·min⁻¹). Controls were performed to test for the influence of the bolus volume on flow. The dye-dilution curve was detected with a densitometer that had been calibrated as described previously (6). A mathematical model that describes dispersive vascular flow (VASCOP, BTEX40) (16) was used to derive an empirical transfer function from the dye curves with the assumption of a spike input function. This transfer function was then used to derive capillary purine concentrations for model analysis by deconvoluting the venous effluent purine concentrations. This transformation of the data was quite minor, because the mean transit time of the vasculatry system was ~45 s, whereas the mean time of the venous purine curves was much greater (~15 min).

Protocol 5. Effect of adenosine kinase and adenosine deaminase inhibitors. Three rabbits were used to examine the influence of adenosine kinase (adenosine → AMP) and adenosine deaminase (adenosine → inosine) on venous purine efflux during two sequential and identical periods of severe coronary underperfusion. Protocol 1 was followed, except 5 µM erythro-9-[2-hydroxy-3-nonyl]adenine (EHNA) and 2 µM idotubercidin were present in the perfusate during the entire protocol to inhibit adenosine deaminase and adenosine kinase, respectively (21).

NMR Spectroscopy

High-energy phosphate data were obtained using a 4.7-T magnet (Bruker) and a CSI spectrometer (GE-Omega), as previously described (22). In every experiment, one fully relaxed spectrum was acquired before the protocol began by summing 40 free induction decays obtained every 20 s. The areas of the PCR and ATP peaks in the fully relaxed spectrum were obtained by integration using Omega software and averaging the γ, α, and β peaks of ATP. Partially saturated 31P spectra were acquired every 88 s by summing 32 free induction decays obtained every 2.7 s. The partially saturated spectra were analyzed using an automated fitting routine (12), and peak areas were expressed relative to the average baseline values and to the area of an internal phenylphosphonic acid standard. Intracellular pH was determined, and intracellular pH and the free intracellular Mg²⁺ concentration were calculated as described previously (22). Because of interference by extracellular P, in the perfusion medium, baseline and reperfusion intracellular pH were assumed to equal 7.1 (24). Cytosolic free AMP concentration was calculated using creatine kinase and adenylate kinase equilibrium expressions (Eq. 1) adjusted to the measured H⁺ and Mg²⁺ concentrations (Eq. 2). It was assumed that the total concentration of PCR + creatine (Cr) decreased by 7% during each of
the two periods of underperfusion, as observed previously (22)

\[ [AMP] = \frac{K_{MK}^{eq} \cdot [ATP]}{K_{MK}^{eq} \cdot [Cr]} \]  \tag{1}

\[ K_{MK}^{eq} = 10^{-0.97 \cdot pH + 7.52 + 3.12 \cdot H^+} \]  \tag{2}

where \([PCr]\) and \([Cr]\) are PCr and Cr concentrations and \(K_{MK}^{eq}\) and \(K_{MK}^{eq}\) are the equilibrium dissociation constants for the myokinase and creatine kinase reactions, respectively.

Determination of Venous Purines and Lactate

Baseline samples were collected every 2 min for an 8-min period. During underperfusion, samples of the effluent were initially collected every 2 min and, after 15 min, every 5 min, into ice-cold vials containing 10 µM EHNA to prevent enzymatic degradation of adenosine to inosine (21) during analysis. Because of a detection limit of 100 nM, baseline samples (15–20 ml) were first desalted and concentrated 85 times using Sep-Pak cartridges (Waters). The samples were passed through the cartridges and then eluted first with 2 ml of 70% methanol and then with 2 ml of phosphate buffer (10 mM, pH 5.4). The eluent (4 ml) was then evaporated and reconstituted with water to 200 µl. Effluent samples collected during underperfusion were diluted 1:5 with 10 µM EHNA because of the high purine concentrations. Samples (75 µl) were injected onto a C18 reverse-phase column (Upchurch, 5 µm, underperfusion were diluted 1:5 with 10 µM EHNA because with water to 200 µl. Effluent samples collected during the two periods of underperfusion, as observed previously (22)

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Model Analysis

A mathematical model of myocardial phosphoenergetics and nucleotide metabolism, as depicted in Fig. 1, was used to analyze the data. The model describes the intracellular concentrations of PCr, Cr, ATP, ADP, AMP, Pi, adenosine, and inosine, the enzymes creatine kinase, myokinase, cytosolic 5'-NT, adenosine kinase, and adenosine deaminase, the membrane transport of adenosine and inosine, and Pi and Cr in exchange with an interstitial region. The model consists of a set of ordinary differential equations based on the conservation of mass. The model, which describes parenchymal cell (cardiomyocyte), interstitial space, endothelial cell, and capillary regions, is an extension of a previous model that described only parenchymal cell and extracellular regions (22). Because it is not feasible at this stage of our knowledge of the underlying biology to accurately describe all processes whereby ATP is synthesized (oxidative phosphorylation, glycolysis) and hydrolyzed (e.g., Ca2+ and ion pumps, myofibril contraction, adenosinetriphosphatases, homeostasis), a simplification was used. A continuous function, \(rATP\), was therefore defined in the parenchymal cell region as

\( \Delta rATP = \text{rate of ATP synthesis} - \text{rate of ATP hydrolysis} \)  \tag{3}

having units of moles per liter per minute. Thus, if \(\Delta rATP = 0\), the rates of ATP synthesis and hydrolysis are precisely matched. \(\Delta rATP\) provides a flexible means for describing the time course and extent of the net energy imbalance experienced during underperfusion and reperfusion (22). The parameters describing \(\Delta rATP\) were estimated empirically using optimization procedures described below. It was assumed that, under baseline conditions, \(\Delta rATP = 0\). Thus the integral of \(\Delta rATP\) over time in the intact system is equivalent to the total net hydrolysis (if negative) of high-energy phosphate bonds during the interval of the integration.

Permeability-surface area (PS) products were used to describe linear membrane exchange of adenosine, inosine, Pi, and creatine with an interstitial region and transport between capillary and interstitial regions. Enzyme dissociation constants and PS products were taken from literature values (22), with the exception of the Michaelis constant (Km) and maximal reaction velocity (Vmax) of 5'-NT. We chose to use accepted PS values, which are based on normal flow, because...
exploratory modeling indicated that reduced PS values could fit the purine data only when adenosine deaminase activities were unrealistically elevated, whereas the 5'-NT and ΔrATP parameters were minimally influenced. The present model incorporated the recently observed inhibition of adenosine kinase due to increased Pi (5, 9) [i.e., inhibition constant (K_i) of adenosine kinase for Pi = 3 mM].

To fit the model to the NMR and purine data from protocol 1, an automated least-squares optimization routine (SIMPLEX) was used to simultaneously fit the PCr, ATP, inosine, and adenosine curves by adjusting the K_m and V_max of 5'-NT and the parameters of the ΔrATP function. All other model parameters were held constant during fitting or were changed according to direct measurements (flow, intracellular pH, Mg^{2+}). Because the model does not include hypoxanthine, the coronary venous hypoxanthine data were added to the inosine data for fitting with the model inosine data. Means ± SE of the parameter estimates were obtained by fitting the data from each individual experiment. Initial concentrations of ATP (6.08 mM), PCr (10.6 mM), and Cr (13.2 mM) used in the modeling were taken from biochemical measurements of freeze-clamped hearts performed previously in this laboratory (22). The initial intracellular concentration of P_i was assumed to be 0.4 mM (11), and the initial pH was assumed to be 7.1 (24). The equilibrium constant for the creatine kinase reaction was adjusted on the basis of NMR measurements of intracellular pH and Mg^{2+} (23).

To analyze the data from protocol 2, where only purine data and no NMR data were obtained, the venous purine effluent data from the second (test) period of underperfusion were deconvoluted, as described above, to derive capillary concentrations of inosine (-hypoxanthine) and adenosine. The inosine and adenosine curves were then fit by the model by optimizing for the parameters describing 5'-NT kinetics and the ΔrATP function. The robustness of this procedure was confirmed by testing the fit obtained by analyzing the purine data only from the protocol 1 experiments.

Statistical Analysis

Values are means ± SE. Mean values of the model results (i.e., V_max and K_m of 5'-NT and the ΔrATP parameters) were determined from the separate model solutions for each experiment. Statistical significance of differences (P < 0.05) was determined by using the nonparametric Mann-Whitney test for statistical variation.

RESULTS

Attenuation of Purine Efflux During a Second Period of Underperfusion

The NMR, purine, and metabolic data for the two successive and identical periods of underperfusion (5% baseline flow) in protocol 1 experiments are shown in Fig. 2. PCr fell rapidly to 2.3 mM after the onset of the first period of underperfusion and to 2.7 mM in the second period, recovering quickly toward baseline during both periods of reperfusion. The ATP concentration fell from 6.1 to 3.3 mM in the first period but only to 2.6 mM in the second period and did not recover toward baseline values during either reperfusion period. Therefore, the decrease in ATP was much reduced in the second period of underperfusion compared with the first period. During the first period of underperfusion, coronary effluent purine release rate (i.e., venous concentration × flow) peaked within the first 10 min at a rate of 36 nmol·g^{-1}·min^{-1}, resulting in a total purine release of 934 ± 161 nmol/g during the entire 45 min of underperfusion. Purine release was severely attenuated (75% decrease, total purine release = 196 ± 26 nmol/g for 2nd 45-min period).

Fig. 2. Nuclear magnetic resonance, purine, and metabolic data from 2 successive periods of underperfusion (5% baseline flow) in protocol 1 experiments. A: PCr (●) and ATP (○). B: calculated cytosolic ADP (●) and AMP (○). C: intracellular Pi. D: intracellular pH. E: lactate concentration in venous effluent. F: purine concentration in venous effluent; total purine release was 934 ± 161 nmol/g for 1st and 196 ± 26 nmol/g for 2nd 45-min period. G: left ventricular developed pressure.
underperfusion during both periods, accompanied by an incomplete recovery during reperfusion.

Model Analysis

The decreased purine efflux in the second period of underperfusion is suggestive of decreased 5'-NT activity. However, because ATP fell to a lesser degree and ADP and AMP concentrations were lower in the second period than in the first period, this would also be expected to cause decreased purine efflux by mass action. Analysis with a mathematical model was, therefore, performed to differentiate between these two effects. Simultaneous model fits to the PCr, ATP, adenosine, and inosine data using the four-region model of myocardial energetics and enzymatic kinetics are given in Fig. 3. Separate fits were obtained for the two periods of underperfusion, resulting in different values for the parameters of the \( \Delta r_{\text{ATP}} \) function (ATP synthesis – ATP hydrolysis) and values for the \( V_{\text{max}} \) and \( K_m \) for the enzyme 5'-NT, whereas all other model parameters were held constant (Table 1). The integral of \( \Delta r_{\text{ATP}} \), reported in Table 1, represents the total net high-energy phosphate breakdown during the period of underperfusion. The analysis indicates that the decreased purine efflux in the second period of underperfusion was caused by decreased 5'-NT activity as well as decreased AMP concentration.

The relation between calculated cytosolic AMP concentration and measured total coronary purine efflux (adenosine + inosine + hypoxanthine) for the two periods of underperfusion is shown in Fig. 4, top. Minutes of underperfusion are indicated by the numbers for the first period of underperfusion. The relations form loops (hysteresis) because of membrane transport, diffusion, and convection of the purines, which cause delay and dispersion. Although AMP concentrations increased to higher levels during the first period of underperfusion, in the range where AMP concentrations were similar (<4 \( \mu \)M), purine release was three- to sevenfold higher in the first than in the second period of underperfusion. The model predictions of the relationship between cytosolic free AMP and the vascular purine release are in good agreement with the data, passing within the measurement uncertainty bounds for almost every point (Fig. 4, middle). The results show that the observed degree of hysteresis is to be expected, given the relatively rapid kinetics of cytosolic AMP and realistic values describing membrane transport, diffusion, and convection of the purines. Because the model solutions in Figs. 3 and 4 are identical, representing the best overall fit to the global data set, the fit is powerfully constrained by the requirement for internal self-consistency.

### Table 1. \( V_{\text{max}} \) and \( K_m \) for 5'-NT and integral of \( \Delta r_{\text{ATP}} \)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>1st Period of Underperfusion</th>
<th>2nd Period of Underperfusion</th>
<th>Time Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>( K_m ), ( \mu \text{M} )</td>
<td>2.3 ± 0.5</td>
<td>1.3 ± 0.9</td>
<td>2.4 ± 0.6</td>
</tr>
<tr>
<td>( V_{\text{max}} ), nmol·min(^{-1}·g^{-1})</td>
<td>140 ± 11</td>
<td>67 ± 12*</td>
<td>149 ± 13</td>
</tr>
<tr>
<td>Integral of ( \Delta r_{\text{ATP}} ), mmol/l</td>
<td>−18.9 ± 1.7</td>
<td>−12.5 ± 0.9*</td>
<td>−15.9 ± 1.6</td>
</tr>
</tbody>
</table>

Values (means ± SE) were obtained from optimized model solutions of individual experiments \((n = 7, n = 4\) for time control experiments). \( K_m \), Michaelis constant; \( V_{\text{max}} \), maximal reaction velocity; 5'-NT, 5'-nucleotidase. Integral of \( \Delta r_{\text{ATP}} \), estimated total net high-energy phosphate breakdown during onset and duration of underperfusion. Negative values indicate a net breakdown of energy. *Significantly different from 1st period of underperfusion.

### Kinetics of Cytosolic 5'-NT

The rate of the cytosolic 5'-NT reaction during the first and second periods of underperfusion, as calculated by the model, is shown in Fig. 4, bottom. The high-energy phosphate and purine data from the first period of underperfusion were best fit with a \( V_{\text{max}} \) of 140 nmol·g\(^{-1}·min^{-1}\) and a \( K_m \) of 2.3 \( \mu \)M for the enzyme 5'-NT. The second period was best fit with a decreased
Vmax of 67 nmol·g⁻¹·min⁻¹ and a Kₘ of 1.3 µM (Table 1). The model solutions shown in Figs. 3 and 4 were obtained by fitting the pooled data set. However, for statistical comparison, the parameter estimates described in the text and Table 1 were based on fits of the individual experiments.

Time Course of Downregulation of 5'-NT

The observation of decreased 5'-NT activity (Vmax) in the second (test) period of underperfusion (40 min; Fig. 5) indicates that the decrease in 5'-NT activity occurred during the latter portion of the first period of underperfusion and that the decrease in activity persisted through the intervening period of reperfusion. To determine the length of underperfusion necessary to induce a decrease in the Vmax of 5'-NT, special time course experiments were performed (protocol 2). Here, the duration of the first period of underperfusion (95% flow reduction) was varied (0, 5, 10, 20, 40 min), while venous purines were measured in the standardized second (test) period of underperfusion (40 min; Fig. 5). No NMR data were obtained. A 5-min period of underperfusion decreased total purine efflux during the test 40-min period of underperfusion by 8% (no significant difference). A 10-min period significantly (P ≤ 0.05) decreased total purine efflux by 40%, and a 20-min period significantly decreased total purine efflux by 51%. Model analysis of the venous purine data from the time course experiments showed a significantly decreased Vmax of 5'-NT after a 20-min period of underperfusion (Fig. 6). Although substrate AMP concentrations were not measured in these experiments, model analysis suggested that there was decreased AMP after 10 min of underperfusion, accounting for the decreased purine efflux (Fig. 5). However, only after 20 min of underperfusion was there a decrease in the Vmax of 5'-NT (Fig. 6).

To test the unlikely possibility that 1 h of perfusion itself caused a decrease in 5'-NT activity, separate time control experiments (protocol 3) were performed. A single 45-min period of underperfusion following a 65-min period of normal perfusion exhibited a purine release of 782 ± 96 nmol/g, which was not statistically different from that during a single period of underperfusion without an extended period of normal perfusion (i.e., 934 ± 161 nmol/g). Model analysis showed no decrease in the activity of 5'-NT in the time control experiments (Table 1). These experiments clearly indi
ciliate that the decreased activity of 5'-NT observed in the second period of underperfusion, as found in protocol 1, was due to underperfusion and was not simply due to time.

5'-NT Downregulation or Activation of Adenosine Kinase?

Studies of guinea pig heart and rabbit cardiomyocytes have shown a high rate of substrate cycling between AMP and adenosine (Fig. 1) catalyzed by the enzymes 5'-NT and adenosine kinase (1, 21, 32). During normoxia the recycling of adenosine to AMP via adenosine kinase is essential for the maintenance of low, nonvasodilatory concentrations of adenosine. New findings show that endogenous inhibition of adenosine kinase plays an important role in elevating adenosine levels during ATP depletion (5, 9). To test the unlikely possibility that the decrease in venous purines in the second period of underperfusion was unexpectedly due to an increase in the activity of adenosine kinase, we performed identical successive underperfusion experiments (95% flow decrease) in the absence and presence of iodotubercidin and EHNA. The results, shown in Table 2, indicate that, in the absence of iodotubercidin and EHNA, purine release was attenuated by 75% during the second period of underperfusion and by 69% in the presence of iodotubercidin. If there was an unexpected increase in adenosine recycling via activation of adenosine kinase during the second period of underperfusion, then there should have been reduced attenuation of the purine release in the presence of iodotubercidin. However, the results show no detectable difference. These data show that the attenuated purine release during the second period of underperfusion was not due to an upregulation of adenosine kinase.

DISCUSSION

The major finding of the present study was a decreased activity (Vmax) of cytosolic 5'-NT in the second of two identical sequential periods of coronary underperfusion. The downregulation of 5'-NT was slow in onset, requiring 20 min of underperfusion, and persistent during a 20-min period of reperfusion. Although previous studies reported decreased AMP hydrolysis during ischemia, they did not distinguish between decreased activity of 5'-NT and decreased substrate AMP concentrations, which also occurs. Here we report the first quantitative description of the downregulation of 5'-NT in vivo while accounting for the potentially confounding effects of decreased AMP concentration. Downregulation of 5'-NT may be important for preserving myocardin adenine nucleotide stores during sustained coronary underperfusion.

Energy Imbalance During Underperfusion

The metabolic data on PCr, P i, pH, and lactate presented in Fig. 2 were similar for both periods of underperfusion. However, ATP depletion and venous purine concentration, as well as the calculated intracellular ADP and AMP concentrations, were lower in the second than in the first period of underperfusion. These findings indicate that the degree of energy imbalance was less during the second than during the first period. The model estimate was that the total high-energy phosphate depletion (ΔrATP) in the second period of underperfusion was 66% of that of the first period (Table 1).

The estimate of the integral of ΔrATP of -18.9 mM (Table 1) means that there was a net hydrolysis of high-energy phosphate bonds of 18.9 mM during the first period of underperfusion. The total number of high-energy phosphate bonds available is equal to [PCr] + 3 × [ATP] + 2 × [ADP] + [AMP] = 10.6 + 3 × 6.08 + 2 × 0.07 + 0.0007 = 29.0 mM during baseline conditions (22), where [PCr], [ATP], [ADP], and [AMP] represent PCr, ATP, ADP, and AMP concentrations. Thus 65% of the total high-energy phosphate bonds were hydrolyzed by the end of the first period of underperfusion. On reperfusion, the high-energy phosphate bonds were partly restored, and during the second period of underperfusion there was a net hydrolysis of 40% of the phosphate bonds.

Because the oxygen delivery rate was the same for both periods of underperfusion and the rates of glycolysis, as evidenced by the lactate release rates and the pH

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Table 2. Total purine release with and without iodotubercidin and EHNA

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Iodotubercidin and EHNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>1st period of underperfusion</td>
<td>1,110 ± 138</td>
<td>822 ± 93</td>
</tr>
<tr>
<td>2nd period of underperfusion</td>
<td>272 ± 70</td>
<td>255 ± 17</td>
</tr>
<tr>
<td>%Decrease</td>
<td>75 ± 7</td>
<td>68 ± 6</td>
</tr>
</tbody>
</table>

Values are means ± SE expressed as nmol/g; n = 6 for control and 3 for iodotubercidin and erythro-9-[2-hydroxy-3-nonyl]adenine (EHNA). There were no significant differences between groups.
time courses (Fig. 2), were similar, the rates for ATP synthesis were probably similar for the two periods. Therefore, it is likely that the rate of ATP hydrolysis was lower in the second than in the first period of underperfusion. Because Murry et al. (25) also reported slower utilization of energy as a result of a previous ischemic episode, this may be a general phenomenon. If this is so, then purine production during ischemia will normally be reduced as a result of a preceding period of ischemia because of lower substrate AMP concentrations. This effect may have confounded previous studies of 5'-NT activity during ischemia (see Relation to Other Studies of Ischemia).

Downregulation of 5'-NT

The direct evidence for the downregulation of 5'-NT is the vascular purine efflux-AMP relationship, which is depicted in Fig. 4; it shows that, at similar intracellular concentrations of AMP, coronary purine efflux was reduced in the second period of underperfusion. This qualitative finding is entirely model independent. However, without the model analysis, it would not be possible to arrive at a quantitative estimate of the degree of inhibition of 5'-NT. Because the model assumes Michaelis-Menten kinetics for 5'-NT, enzyme velocity curves of the form shown in Fig. 4, bottom, were expected.

The model assumes constant values for the $V_{\text{max}}$ and $K_m$ of 5'-NT in each period of underperfusion, and on the basis of this assumption, accurate fits of the data were obtained (Figs. 3 and 4). However, the results in Fig. 6 imply that the $V_{\text{max}}$ of 5'-NT was decreased after 20 min of underperfusion in the first period. The probable explanation for this apparent discrepancy is that during the first period of underperfusion, in the protocol 1 experiments, the data provide little sensitivity for changes in the activity of 5'-NT after 20 min, since by that time, AMP concentrations were decreased to low values. Therefore, the decrease in $V_{\text{max}}$ of 5'-NT after 20 min of underperfusion, in the protocol 1 experiments, would be expected to have little effect on the model fit or the data. This was confirmed by modifying the model to impose a linear decrease in the $V_{\text{max}}$ of 5'-NT after 10 min of underperfusion. When this version of the model was used to fit the data, there were no appreciable changes in the estimates of the parameters describing 5'-NT kinetics and the function $\Delta r_{\text{ATP}}$.

The model also assumed that the enzyme adenosine kinase (adenosine $\rightarrow$ AMP) was inhibited during underperfusion, as has been demonstrated during hypoxia (5, 9). The mechanism of inhibition was assumed to be increased P$_i$ concentration on the basis of in vitro enzyme assay studies (9). The absolute values of $V_{\text{max}}$ and $K_m$ for 5'-NT estimated in the present study depend on the degree of inhibition of adenosine kinase. However, even when the modeling assumed no inhibition of adenosine kinase, there was a decrease in 5'-NT activity in the second period of underperfusion similar to that reported in Table 1 (results not shown).

It is interesting to note that the relation between AMP concentration and venous purine release showed such marked hysteresis. That such hysteresis is also predicted by the model indicates that the hysteresis can be explained by membrane and endothelial cell transport delays. This result demonstrates the importance of collecting complete time course data in studies of the regulation of myocardial adenosine formation. It appears that assumptions of a steady-state response to ischemia should be avoided.

Possible Regulatory Mechanisms

The primary pathway for 5'-AMP hydrolysis to adenosine in heart is by cytosolic 5'-NT (27). Darvish et al. (4) report the activity of extracted cytosolic 5'-NT in the presence of 0.1 mM AMP and physiological concentrations of ADP and Mg$^{2+}$ to be $\sim$150 nmol·min$^{-1}$·g$^{-1}$, which is in good agreement with the estimated in situ $V_{\text{max}}$ of 140 nmol·min$^{-1}$·g$^{-1}$ reported here. Other in vitro studies have provided evidence that 5'-NT is inhibited by high concentrations of protons (H$^+$) (4) and P$_i$ (14, 27) and that ADP increases the apparent affinity of 5'-NT for its substrate, AMP (33). An innovative study by Bak and Ingwall (2) showed the effects of acidosis on purine efflux by subjecting isolated hearts to hypoxia or global ischemia and concluded that H$^+$ is a potent inhibitor of 5'-NT. In the present study the activity of 5'-NT was downregulated after 20 min of underperfusion. Because the intracellular concentrations of H$^+$ and P$_i$ were elevated during this period, it is possible that either of these acts as a regulatory effector for cytosolic 5'-NT. It would seem unlikely, however, that inhibition by protonation or elevated P$_i$ levels would be slow in onset, since H$^+$ and P$_i$ rose quickly at the onset of underperfusion. It is also unlikely that the reperfusion period plays a regulatory role in the downregulation of 5'-NT, since the time course experiments (protocol 2) had identical periods of reperfusion. Each group was exposed to a 20-min period of reperfusion, yet 5'-NT remained normally active for the 5/40 and 10/40 groups.

Whereas previous in vitro studies implicated allosteric interactions for the regulation of 5'-NT, it is possible that 5'-NT downregulation during sustained underperfusion is not mediated by the known allosteric regulators, since it is slow in onset and persistent, even through a 20-min period of reperfusion. One may speculate that an inhibitory substance may be binding tightly to the enzyme, thereby decreasing its catalytic availability, or that enhanced turnover/depressed de novo synthesis may be diminishing the amount of reactive 5'-NT.

Relation to Other Studies of Ischemia

Because the production of myocardial purines during ischemia depends on 5'-NT and adenosine kinase activity and the cytosolic AMP concentration, it has been difficult to describe the regulation of myocardial purine production. For example, Reimer et al. (28) observed no cumulative depletion of the adenine nucleotide pool after repeated occlusions and listed four possible explanations for the preservation of ATP. These included a
downregulated utilization of high-energy phosphates and the loss or inhibition of 5'-NT. Their data suggested that both phenomena were involved, yet at that stage it was impossible to distinguish and quantify the two effects.

It has been reported that ischemic preconditioning is associated with an increase as well as a decrease in 5'-NT activity. On the basis of enzyme extraction measurements, Kitakaze et al. (17, 18, 20) found that ischemic preconditioning increases 5'-NT activity during ischemia and, thus, leads to an elevated adenosine release during reperfusion, thereby causing beneficial effects. Others, however, dispute this view and claim rather that 5'-NT is downregulated by preconditioning. For example, a number of studies in the intact heart (13, 28, 29, 31) have reported less ATP breakdown and less AMP and nucleoside accumulation during repetitive ischemic periods than during prolonged ischemic periods. In addition, Murry et al. (25) observed decreased tissue accumulation of ADP, AMP, and adenosine during 40 min of ischemia in preconditioned hearts and reported decreased energy metabolism due to ischemic preconditioning. Furthermore, Bradamante et al. (3) observed stable ATP levels during repetitive short periods of ischemia in rat heart concomitant with steadily decreasing rates of purine efflux. Because all these studies tend to show decreased high-energy phosphate depletion during repetitive periods of ischemia, decreased purines are to be expected because of lower AMP concentrations. This effect would tend to confound studies in which decreased purines in repetitive ischemia were attributed to inhibition of 5'-NT. Therefore, we believe that the present study is the first to rigorously demonstrate downregulation of 5'-NT due to repetitive ischemia, independently of the altered energetic state.

In conclusion, we are able to report the downregulation of 5'-NT during sustained severe coronary underperfusion. Because a potentially confounding factor in the study of the regulation of 5'-NT in the intact heart is the degree of energetic imbalance, it was necessary to obtain high-energy phosphate and purine efflux data and analyze these with an integrative model to clearly demonstrate decreased activity of 5'-NT during underperfusion. Analysis of the data indicates a regulatory mechanism that has a slow 20-min onset and persists, even through a 20-min period of reperfusion. Downregulation of 5'-NT may be advantageous for long-term tissue survival during sustained underperfusion by preserving adenine nucleotide stores.

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