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5′-Nucleotidase activity and adenosine formation in stimulated, hypoxic and underperfused rat heart

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Changes in 5′-nucleotidase activity were calculated on the basis of alterations in ATP, ADP, phosphocreatine, P_i, Mg^{2+}, IMP and AMP, determined by using ^{31}P n.m.r. spectroscopy and h.p.l.c., during isoprenaline infusion, graded hypoxia and graded underperfusion in isolated rat heart. Calculated activity changes were compared with the total efflux of purines (adenosine + inosine + hypoxanthine) in order to assess the involvement of various 5′-nucleotidases in formation of adenosine. Purine efflux exhibited an exponential relation with cytosolic [AMP] during isoprenaline infusion and hypoxia ($r = 0.92$ and 0.95 respectively), supporting allosteric activation of 5′-nucleotidase under these conditions. Purine efflux displayed a linear relation with cytosolic [AMP] during graded ischaemia ($r = 0.96$), supporting substrate regulation in the ischaemic heart. The calculated activities of membrane-bound ecto-5′-nucleotidase were similar to the observed relations between purine efflux and cytosolic [AMP] in all hearts. The calculated activities of the ATP-activated cytosolic and lysosomal enzymes and of the ATP-inhibited cytosolic 5′-nucleotidase could not explain the observed release of purines under the conditions examined. These results indicate that the kinetic characteristics of the membrane-bound ecto-enzyme are consistent with an important role in the formation of extracellular adenosine, whereas the characteristics of the other 5′-nucleotidases are inconsistent with roles in adenosine formation under the conditions of the present study.

INTRODUCTION

The formation of adenosine in the heart is linked to the metabolic status of the myocardium [1–3]. This fact, coupled with its physiological activities [4–7], has resulted in adenosine being designated a 'retaliatory agent', increasing supply of substrate and O_2 to the compromised myocardium. The enzyme predominantly responsible for synthesis of adenosine is 5′-nucleotidase. Although it is clear that 5′-nucleotidase is the major site of adenosine synthesis within the myocardium, controversy remains concerning the exact nature, location and mechanism of regulation of the specific 5′-nucleotidase(s) responsible for adenosine formation. Several pools of 5′-nucleotidase have been isolated, all displaying different kinetic characteristics. The plasma-membrane-bound ecto-5′-nucleotidase was the first to be located [8–12]. This enzyme is allosterically activated by free Mg^{2+}, and is inhibited by unchelated ATP, ADP, P_i, and to a lesser extent CrP [11–13]. The membrane-bound ecto-enzyme was proposed as the site of adenosine formation [14–17]; however, other evidence contradicts such a role [18–22]. A cytosolic enzyme which is allosterically activated by ATP, ADP and Mg^{2+}, strongly inhibited by P_i, and displays a preference for IMP as substrate, has been isolated from cardiac tissue and has been extensively characterized [23–28]. It is proposed that this pool is important in the formation of physiologically active extracellular adenosine [23–28]. Although the kinetic characteristics of this enzyme do not lend themselves to a role in adenosine formation [21,23], a model has been formulated predicting adenosine formation via this ATP-activated cytosolic enzyme owing to alterations in the 'adenylate energy charge' [24,26,29]. A second myocardial cytosolic enzyme has recently been isolated which is also activated by ATP; however, this form displays a high affinity for AMP as substrate, as opposed to IMP [30]. Little else is known about the kinetic characteristics of this enzyme pool, although it is possible that an enzyme with these characteristics may play a role in adenosine formation.

A lysosomal 5′-nucleotidase [10] and another cytosolic enzyme [31,32] have also been isolated, both of which are inhibited by ATP and ADP. The ATP-inhibited cytosolic enzyme is inhibited to a small degree by P_i, and is activated by Mg^{2+}. Since this cytosolic enzyme has not been located in myocardial tissue, and it is unclear how a lysosomal enzyme could contribute to adenosine formation, the possible roles of these two enzymes in formation of extracellular adenosine remain undetermined.

With a range of possible sites for adenosine formation, both intra- and extra-cellularly, the site and mode of regulation of adenosine formation remain unclear. Although there is evidence that ecto-5′-nucleotidase is not involved in adenosine formation during hypoxia, the results of recent studies support a role for this enzyme (possibly located on the endothelium), utilizing AMP released from myocytes as substrate [14,17]. In the present paper, we describe the results of experiments utilizing ^{31}P-n.m.r. methodology to determine concentrations of the free cytosolic activators, inhibitors and substrate for 5′-nucleotidases during underperfusion, hypoxia and isoprenaline infusion in isovolumic rat heart. These data

Abbreviation used: CrP, phosphocreatine.

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were used to determine whether the known kinetic characteristics of the various 5′-nucleotidases were consistent with a role in the observed formation of extracellular purines in heart.

MATERIALS AND METHODS

Materials

All materials purchased were of analytical grade or better.

Methods

Mature male Wistar rats (300–350 g) were anaesthetized with sodium pentobarbitone (100 mg/kg, intraperitoneally). Hearts were removed rapidly into ice-cold perfusion fluid, cannulated via the aorta, and perfused in an isovolumic Langendorff mode at a constant hydrostatic pressure of 100 mmHg as described previously [33]. A phosphate-free Krebs–Henseleit buffer was used, gassed with O_2/CO_2 (19:1) to yield pH 7.4 at 37 °C [33]. In all experiments, left-ventricular developed pressure was measured from a latex balloon in the left ventricle connected to a Gould–Statham P23-ID pressure transducer by a water-filled line. Hearts were perfused inside a 20 mm-diam. glass n.m.r. tube modified for perfusion work, and allowed to equilibrate for 20 min before experimentation [33]. The temperature of the heart in the n.m.r. apparatus was controlled by bathing the heart with perfusion fluid at 37 °C and setting the Bruker variable-temperature unit (B-VT1000) to 310 K.

Equilibrated hearts were subjected to one of the following procedures.

(*a*) Isoprenaline infusion: isoprenaline was dissolved in perfusion fluid containing 0.4 mM-ascorbate and was infused into the aortic cannula at rates of less than 1% of the coronary flow to give final concentrations of 0.4, 3.0 and 75 nM. Drug infusion was maintained for a period of 20 min.

(*b*) Graded hypoxia: hypoxia was induced by perfusing hearts with perfusate equilibrated with 65%, 50%, 35%, or 5% O_2, the balance being 5% CO_2 in N_2. Control hearts were perfused with 95% O_2. Hypoxic perfusion was maintained for 10 min.

(*c*) Graded underperfusion: underperfusion was controlled via a peristaltic pump as described previously [33]. Hearts were made totally globally ischaemic for 1 min, after which flow was incremented to values of 1.6, 3.4, 5.3, 7.2 and 12 ml/min per g. Flow was maintained contant at each rate for a period of 8 min (3 min equilibration period; 5 min experimental period) before perfusion at the next higher rate.

^{31}P-n.m.r. spectroscopy

^{31}P-n.m.r. spectra of hearts were obtained with a Bruker CXP-3000 spectrometer operating at 121.47 MHz as described previously [33,34]. Spectra were accumulated over 5 min periods. Phosphate metabolites were quantified from spectral peak areas.

Intracellular pH was calculated from the chemical shift of the P_i peak [34]. Absolute metabolite levels were obtained by freeze-clamping hearts at the end of experimentation in tongs cooled in liquid N_2, extracting the frozen wafers by the technique of Chen *et al.* [35], and performing h.p.l.c. analysis [36] as described previously [33]. Briefly, 100 μl of heart extract was injected on to a C-18 reverse-phase column (Waters μ-Bondapak, 10 μm) operating in an isocratic mode. The buffer was 0.30 mM-$NH_4H_2PO_4$, pH 5.8, and was pumped at 1 ml/min. The A_{210} was continuously monitored with a Waters 481-LC variable-wavelength spectrophotometer. Metabolites were quantified by comparison with peak areas for standards. Protein was measured by the method of Lowry *et al.* [37]. Tissue IMP concentration was also determined by the h.p.l.c. method described above [36], and expressed per mg of protein. Concentrations of the other metabolites in the cytosol were calculated by assuming a cytosolic volume of 60% wet weight. ADP was calculated from the creatine kinase equilibrium as follows (Cr, creatine):

$$[ADP] = \frac{[ATP \cdot [Cr]}{[CrP] \cdot [H^+] \cdot K_{ck}}$$

where the creatine kinase equilibrium constant (K_{ck}) was taken to be 10^9 M^{-1} under control conditions of temperature and pH [38]. Cytosolic free AMP concentration was estimated from the myokinase equilibrium equation:

$$[AMP] = K_{mk}\frac{[ADP]^2}{[ATP]}$$

where the myokinase equilibrium constant (K_{mk}) was taken to be 1.12 under the intracellular conditions of temperature and pH [39]. Both the K_{ck} and K_{mk} values are used on the assumption that the creatine kinase and myokinase reactions are close to equilibrium at all times under the conditions of the present study. It is also assumed that CrP, ATP and P_i are unbound and within the cytosol [1,40], and that the ^{31}P-n.m.r. spectra represent cytosolic phosphate compounds, uncontaminated by mitochondrial signals [41]. Both of these assumptions are supported by experimental evidence [1,40,41]. Therefore all metabolite concentrations derived from the spectra represent total cytosolic levels. Since cytosolic Mg^{2+} alters the creatine kinase equilibrium and the myokinase equilibrium, equilibrium constants were recalculated for each new cytosolic $[Mg^{2+}]_{free}$ by the method of Lawson & Veech [39].

Cytosolic $[Mg^{2+}]$ determination

Cytosolic $[Mg^{2+}]_{free}$ was determined by the ^{31}P-n.m.r. technique of Gupta & Moore [42] as outlined below:

$$[Mg^{2+}] = K_D(Mg\text{–}ATP) \cdot (\phi^{-1} - 1) \quad (1)$$

where ϕ is given by:

$$\phi = \frac{[ATP]_f}{[ATP]_t} = \frac{\delta_{\alpha\beta} - \delta_{\alpha\beta}Mg\text{–}ATP}{\delta_{\alpha\beta}ATP - \delta_{\alpha\beta}Mg\text{–}ATP} \quad (2)$$

$[ATP]_f$ is the sum of all unchelated ATP species, and $[ATP]_t$ is the sum of the total cytosolic ATP species. $\delta_{\alpha\beta}$ represents the separation between the α-P and β-P resonances of ATP in the heart, and $\delta_{\alpha\beta}$ATP and $\delta_{\alpha\beta}$Mg–ATP represent the separations when Mg^{2+} is absent or saturating respectively. The value of K_D(Mg–ATP) used is the value calculated by Gupta & Moore [42–44] of 0.045 mM. $\delta_{\alpha\beta}$ATP and $\delta_{\alpha\beta}$Mg–ATP were determined experimentally in solutions containing 5 mM-ATP, 140 mM-KCl, 1 mM-EDTA, 10 mM-Tris/HCl buffer, with or without 20 mM-$MgCl_2$, at 37 °C and pH 7.2.

The cytosolic concentrations of ATP, ADP and AMP

unchelated to Mg^{2+} were determined by using the rearranged form of eqns. (1) and (2):

$$[\text{Nucleotide}]_{\text{unchelated}} = \frac{[\text{Nucleotide}]_{\text{total}}}{\frac{[Mg^{2+}]}{K_D}+1} \quad (3)$$

where the subscript 'unchelated' indicates the free cytosolic concentration of nucleotide unchelated to Mg^{2+} and the subscript 'total' indicates the total free cytosolic concentration of the nucleotide, i.e. bound and unbound to Mg^{2+}. The values of K_D used were 0.045 mM for Mg–ATP [42], 0.3 mM for Mg–ADP [45] and 10 mM for Mg–AMP [46].

Enzyme activities

The activities of the various 5′-nucleotidases were calculated from the changes in the concentrations of unchelated substrate (AMP), the unchelated regulators, ADP and ATP, and P_i and Mg^{2+}, and from changes in the competitive inhibitor IMP. Activity changes were calculated from the equation:

$$v = \frac{V_{max.}}{1+\frac{K_m}{[S]}\left(1+\frac{i}{K_i}\right)}$$

where v is the calculated velocity, $V_{max.}$ the maximum velocity, K_m is the Michaelis constant for substrate utilization (AMP), [S] is the substrate concentration, i is the concentration of inhibitor, and K_i the inhibition constant for the respective inhibitors. The effect of allosteric activation was accounted for by incorporating the term $[1+(K_a/a)]$ as a denominator in the above equation, where a is the concentration of the activator, and K_a represents the activation constant. The K_i K_a and K_m values for ATP, ADP, P_i, Mg^{2+}, IMP and AMP, and the $V_{max.}$ values for the membrane-bound, cytosolic and lysosomal enzymes are as follows.

(1) Ecto-enzyme: ATP K_i, 4.4 μM; ADP K_i, 0.082 μM; P_i K_i, 3 mM; Mg^{2+} K_a, 3 μM; IMP K_i, 14 μM; AMP K_m, 4.7 μM; and $V_{max.}$ 9 nmol/min per mg of protein [11,13,16,22].

(2) Cytosolic enzyme (a): ATP K_a, 150 μM; ADP K_a, 90 μM; P_i K_i, 3 mM; Mg^{2+} K_a, 3.5 mM; IMP K_i, 0.1 mM; AMP K_m, 2400 μM; and $V_{max.}$ 57 nmol/min per mg of protein [23,24,28].

(3) Cytosolic enzyme (b): ATP K_i, 100 μM; ADP K_i, 15 μM; P_i K_i, 19 mM; Mg^{2+} K_a, 6 mM; IMP K_i, 30 μM; AMP K_m, 18 μM; and $V_{max.}$ 12 μmol/min per mg of protein [31,32].

(4) Lysosomal enzyme: ATP K_i, 110 μM; ADP K_i, 79 μM; P_i and Mg^{2+}, not available; IMP K_i, 1.05 mM; AMP K_m, 290 μM; and $V_{max.}$ 500 nmol/min per mg of protein [23].

For ease of interpretation, the calculated activities are shown relative to an arbitrary activity of 1, which is assigned to the activity calculated in hearts under control conditions.

H.p.l.c. of purines

Effluent samples were collected every 1 min from perfused hearts and immediately frozen at −70 °C until assayed for adenosine and its deamination products, inosine and hypoxanthine, by the h.p.l.c. technique of Sellevold *et al.* [47]. Briefly, adenosine, inosine and hypoxanthine were concentrated by injecting 10–20 ml of effluent on to C-18 cartridges (C-18 Sep-Pak; Waters) and subsequently eluting the bound purines with a minimum volume of 80% (v/v) methanol in water. Eluted samples were evaporated at 40 °C and redissolved in 1 ml of distilled water. Concentrated samples (100 μl) were injected on to a C-18 reverse-phase column (Waters μ-Bondapak, 10 μm) operating in an isocratic mode. The elution buffer consisted of 3 mM-tetrabutylammonium hydrogen sulphate and 215 mM-KH_2PO_4 in 3.5% (v/v) acetonitrile in water at pH 5.0 and was pumped at 3 ml/min (Waters 510 pump). Peaks were detected at 245 nm with a variable-wavelength spectrophotometer (Waters 481-LC), and quantified by comparison of the peak areas with those for external standards.

Statistical analysis

All values shown in the text and Figures are means ± S.D. An analysis of variance and/or by Student's t test was used to compare mean values. A value of $P < 0.01$ was accepted as indicating statistical significance. Curve fitting was performed by a least-mean-squares analysis based on single data points. Linear regression was also performed based on single data points.

RESULTS

Metabolic response of hearts to isoprenaline, hypoxia and underperfusion

The concentrations of free cytosolic ATP, ADP, AMP, CrP, P_i and Mg^{2+} during isoprenaline stimulation, hypoxia and underperfusion are shown in Tables 1, 2 and 3 respectively. The relative changes in all cytosolic metabolites were related to the degree of hypoxia, underperfusion or metabolic stimulation. [ATP] decreased significantly on treatment with isoprenaline and during hypoxia, but remained relatively stable during underperfusion. [CrP] decreased significantly under all conditions examined. [ADP] and [AMP] increased in all experiments. [P_i] increased markedly during isoprenaline infusion and during hypoxia, but only increased significantly in underperfused hearts when coronary flow was decreased to below 3.4 ml/min per g. Total tissue IMP was approx. 170–200 nmol/mg of protein in control hearts (Table 4). During isoprenaline infusion and graded hypoxia, tissue IMP increased gradually to maximum values of 324 ± 51 and 346 ± 39 nmol/mg of protein respectively. The increase in tissue IMP was greater in ischaemic hearts, with IMP increasing to a maximum value of 487 ± 88 nmol/mg of protein in hearts perfused at 1.6 ml/min per g (Table 4).

Cytosolic $[Mg^{2+}]_{free}$ and unchelated [ATP], [ADP] and [AMP]

From the changes in cytosolic [Mg^{2+}], unchelated concentrations of ATP, ADP and AMP were calculated, and are shown in Table 5. Unchelated cytosolic [ATP] declined significantly under all experimental conditions. Unchelated [ADP] declined significantly during isoprenaline infusion and hypoxia, but increased slightly during underperfusion. The increase in unchelated [ADP] in underperfused hearts was not significant ($P > 0.05$). Unchelated [AMP] increased significantly during all experimental conditions (Table 5).

Table 1. Effect of isoprenaline infusion on cytosolic metabolites

The effect of isoprenaline infusion (60, 3 or 0.4 nM) on free cytosolic metabolites (chelated + unchelated) was determined by ^{31}P-n.m.r. spectroscopy. ATP, CrP and P_i were all directly obtained from ^{31}P-n.m.r. spectra, whereas Mg^{2+}, ADP, AMP and H^+ were calculated from the spectral data. The P_i values are depicted without errors, since spectra were added together to obtain sufficient resolution for the small P_i peak. The P_i values therefore represent an average of the spectra obtained. Since pH was calculated from the shift in the P_i, there are also no errors for this value. All values shown represent free cytosolic concentrations. Values shown are means ± S.D. ($n = 10$). Statistical difference between experimental values and control values is indicated by * ($P < 0.01$).

	Cytosolic metabolites						
Isoprenaline (nM)	ATP (mM)	CrP (mM)	Mg^{2+} (mM)	H^+ (nM)	P_i (mM)	ADP (μM)	AMP (nM)
0	6.9±0.5	10.3±0.9	0.76±0.09	79	1.9	64±5	550±45
0.4	5.7±0.4*	8.4±0.7*	1.65±0.11*	79	2.5	78±6	1005±85*
3.0	4.9±0.4*	7.8±0.6*	1.90±0.16*	80	3.4	80±7*	1250±95*
60.0	4.5±0.4*	7.4±0.8*	2.40±0.21*	80	4.1	84±7*	1450±98*

Table 2. Effect of graded hypoxia on cytosolic metabolites

The effect of graded hypoxic perfusion with buffer equilibrated with 95, 65, 50, 35, or 5% O_2; ^{31}P-n.m.r. spectroscopy was used to determine the changes in the free cytosolic metabolites (chelated + unchelated). ATP, CrP and P_i were all directly obtained from ^{31}P-n.m.r. spectra, whereas Mg^{2+}, ADP, AMP and H^+ were calculated from the spectral data. Values shown are means ± S.D. ($n = 10$). P_i and pH are shown without errors, as discussed in the legend to Table 1. Statistical difference between experimental values and control values is indicated by * ($P < 0.01$).

	Cytosolic metabolites						
$[O_2]$	ATP (mM)	CrP (mM)	Mg^{2+} (mM)	H^+ (nM)	P_i (mM)	ADP (μM)	AMP (nM)
95%	6.8±0.3	10.3±0.7	0.75±0.10	68	2.0	64±4	522±43
65%	5.8±0.4*	9.9±0.6	0.98±0.10*	67	2.5	76±6*	894±65*
50%	5.5±0.4*	8.7±0.5*	1.41±0.08*	71	3.5	87±6*	1244±95*
35%	5.2±0.4*	7.3±0.3*	1.63±0.20*	79	3.9	99±8*	1735±98*
5%	5.1±0.3*	7.1±0.5*	2.74±0.21*	79	5.1	106±9*	1994±98*

Table 3. Effect of graded underperfusion on cytosolic metabolites

The effect of graded underperfusion (12, 7.2, 5.3, 3.4 or 1.6 ml/min per g) on free cytosolic metabolites (chelated + unchelated) was determined by ^{31}P-n.m.r. spectroscopy. ATP, CrP and P_i were all directly obtained from ^{31}P-n.m.r. spectra, whereas Mg^{2+}, ADP, AMP and H^+ were calculated from the spectral data. Values shown are means ± S.D. ($n = 10$). P_i and pH are shown without errors, as discussed in the legend to Table 1. Statistical difference between experimental values and control values is indicated by * ($P < 0.01$).

	Cytosolic metabolites						
Flow (ml/min per g)	ATP (mM)	CrP (mM)	Mg^{2+} (mM)	H^+ (nM)	P_i (mM)	ADP (μM)	AMP (nM)
12	5.6±0.4	9.8±1.0	0.76±0.1	80	2.2	51±4	520±55
7.2	5.7±0.5	9.5±0.8	0.73±0.08	81	2.1	55±6	595±61
5.3	5.8±0.4	9.0±0.8	0.72±0.11	88	2.2	58±5	650±61*
3.4	6.0±0.4	7.2±0.8*	0.69±0.09	106	2.3	75±6*	1050±75*
1.8	6.2±0.5	6.5±0.5*	0.70±0.11	125	3.2	83±7*	1250±85*

Table 4. Changes in total tissue IMP

The contents of tissue IMP were determined during isoprenaline (ISO) infusion, graded hypoxia and graded underperfusion. Tissue IMP is expressed as nmol/mg of total tissue protein. All values shown are means ± S.D. ($n = 10$): * and ** indicate statistical differences from control values, at $P < 0.05$ and $P < 0.001$ respectively.

ISO (nM)	IMP (nmol/mg)	Hypoxia (% O_2)	IMP (nmol/mg)	Ischaemia (ml/min)	IMP (nmol/mg)
0	178±61	95%	183±56	12	199±48
0.4	204±62	65%	207±41	7.2	244±39
3.0	252±48*	50%	252±49*	5.3	327±46**
75	324±51**	35%	294±58**	3.4	406±61**
		5%	346±59**	1.6	487±88**

Table 5. Changes in free ATP, ADP and ADP unchelated to Mg^{2+}

Changes in free cytosolic ATP, ADP and AMP were calculated from the cytosolic concentrations of nucleotides and free Mg^{2+} shown in Tables 1, 2 and 3 by using eqn. (3), by using K_D values of 0.045 mM for Mg–ATP [42], 0.3 mM for Mg–ADP [45] and 10 mM for Mg–AMP [46]. All values shown are means ± S.D. ($n = 10$): * and ** indicate statistical differences from control values, at $P < 0.01$ and $P < 0.001$ respectively.

Treatment	ATP (μM)	ADP (μM)	AMP (nM)
Control	322±35	17±2	516±34
Isoprenaline			
0.4 nM	119±32**	11±2*	652±58**
3.0 nM	76±8**	9±1*	869±855**
60 nM	54±6**	8±1*	987±91**
Hypoxia			
65% O_2	271±25*	15±2	713±80**
50% O_2	196±23**	14±1	830±72**
35% O_2	140±16**	12±1*	961±90**
5% O_2	84±10**	9±1*	1078±95**
Underperfusion			
7.2 ml/min	320±41	17±2	551±60
5.3 ml/min	336±39	18±2	611±68
3.4 ml/min	361±45	23±2*	987±100**
1.6 ml/min	331±45	25±3**	1001±110**

Purine release

The release of adenosine, inosine and hypoxanthine (total purines) during stimulation, hypoxia and underperfusion is depicted in Fig, 1, plotted as a function of unchelated cytosolic [AMP] (from Table 5). Purine release is depicted as the total purines released during the experimental periods (20 min during isoprenaline infusion, 10 min during hypoxia, and 5 min during underperfusion). In isoprenaline-treated hearts and during hypoxia, the release of purines increased exponentially as unchelated cytosolic [AMP] increased ($r = 0.92$ and 0.95 respectively) (Figs. 1*a* and 1*b*). Alternatively, production of purines displayed a linear relationship with unchelated [AMP] during underperfusion ($r = 0.96$) (Fig. 1*c*).

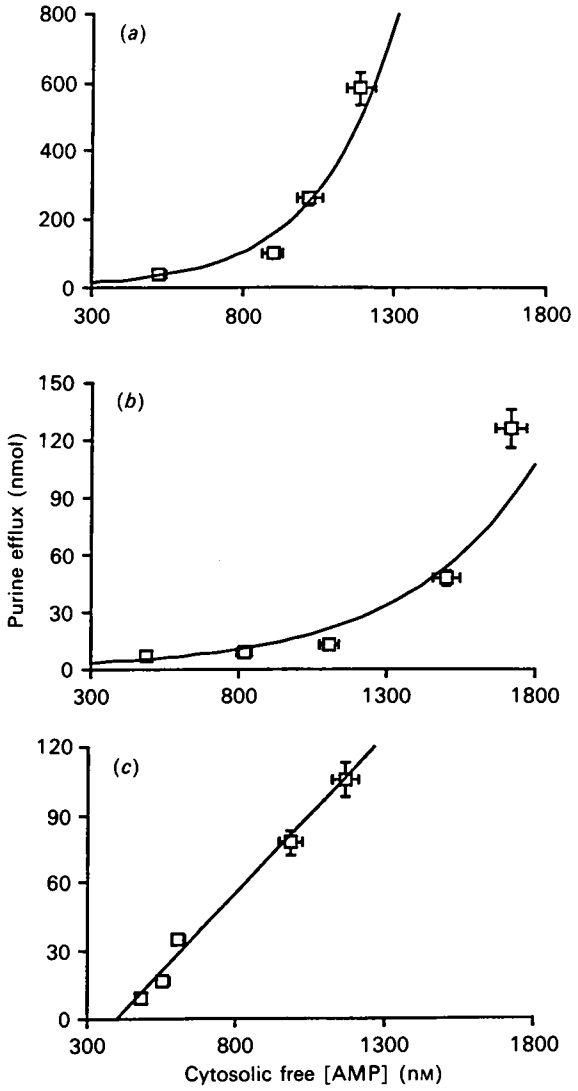

Fig. 1. Release of purines during isoprenaline infusion, hypoxia and underperfusion

The release of purines (adenosine + inosine + hypoxanthine) into the coronary-venous effluent was directly measured during (*a*) isoprenaline infusion, (*b*) graded hypoxia and (*c*) graded underperfusion. Purine release is shown plotted against unchelated cytosolic [AMP] (calculated as shown in Table 5). Curves were fitted by a least-mean-squares analysis based on single experimental values. Exponential fits for the isoprenaline-treated and hypoxic hearts were significant ($r = 0.92$ and 0.95 respectively). The linear fit for underperfused hearts was also significant ($r = 0.96$). Values shown are expressed as means ± S.D. ($n = 10$).

Calculated enzyme activity

Changes in the calculated relative activities of ecto-5'-nucleotidase, ATP-activated cytosolic 5'-nucleotidase, ATP-inhibited 5'-nucleotidase and lysosomal 5'-nucleotidase during isoprenaline infusion, hypoxia and underperfusion are depicted in Figs. 2, 3, 4 and 5 respectively. The values are expressed relative to the calculated activity in control hearts. The ecto-5'-nucleotidase activity appeared to increase exponentially as unchelated [AMP] increased during isoprenaline infusion and hypoxia ($r = 0.91$ and 0.93 respectively) (Fig. 2).

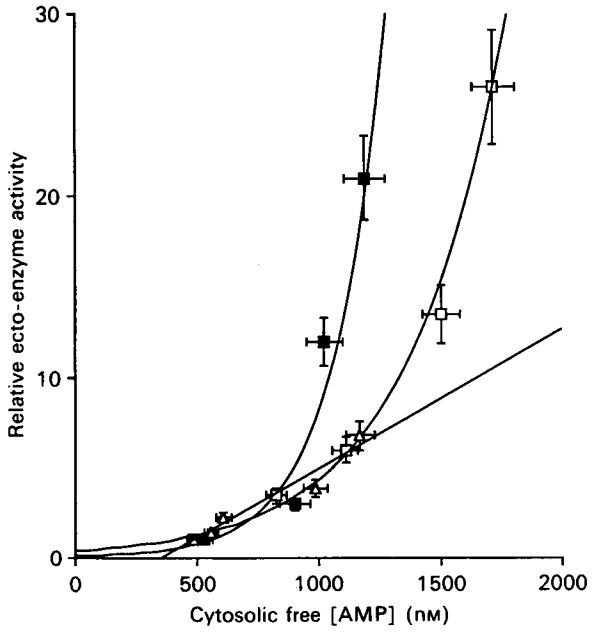

Fig. 2. Calculated activity of ecto-5′-nucleotidase during isoprenaline infusion, graded hypoxia and graded underperfusion

The theoretical activity of ecto-5′-nucleotidase was determined from the changes in unchelated ATP, ADP and AMP, and concentrations of free P_i, IMP and Mg^{2+} as outlined in the Materials and methods section. The activity changes were determined in hearts subjected to isoprenaline infusion (■), hypoxia (□) and underperfusion (△). Curves were fitted by least-mean-squares analysis of single data points: r values for the curves are 0.91, 0.93 and 0.94 respectively.

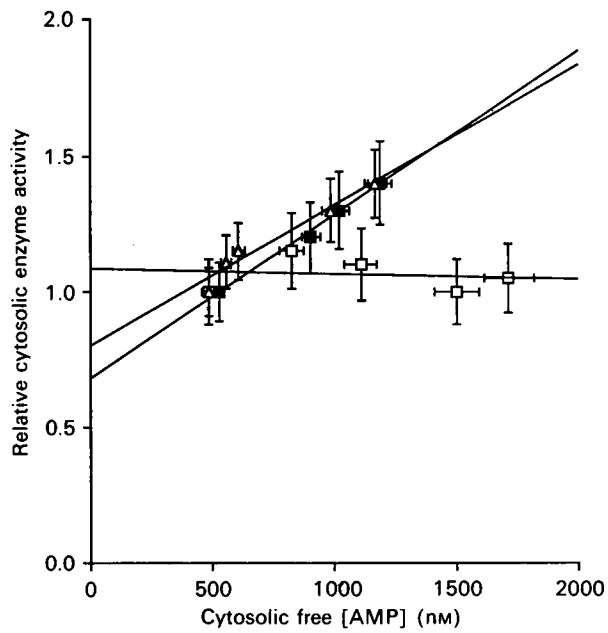

Fig. 3. Calculated activity of ATP-activated cytosolic 5′-nucleotidase during isoprenaline infusion, graded hypoxia and graded underperfusion

The theoretical activity of ATP-activated cytosolic 5′-nucleotidase was determined from the changes in unchelated ATP, ADP and AMP, and concentrations of free P_i, IMP and Mg^{2+} as outlined in the Materials and methods section. The activity changes were determined in hearts subjected to isoprenaline infusion (■), hypoxia (□) and underperfusion (△). Curves were fitted by least-mean-squares analysis of single data points: r values for the curves are 0.91, 0.90 and 0.95 respectively.

Linear curves were not significant ($r < 0.5$). Calculated activity increased by approx. 20-fold during isoprenaline stimulation and hypoxia. During underperfusion, ecto-enzyme activity increased by only approx. 10-fold, increasing linearly as [AMP] rose ($r = 0.94$) (Fig. 2). The calculated activity of ATP-activated cytosolic 5′-nucleotidase only increased approx. 1.5-fold during underperfusion and hypoxia (Fig. 3). During stimulation, the calculated enzyme activity did not increase significantly. Activity displayed a linear relationship with [AMP] during all conditions ($r = 0.91$, 0.90 and 0.91 respectively) (Fig. 3). The calculated activity of the ATP-inhibited cytosolic enzyme increased markedly during stimulation and hypoxia (> 10-fold), but did not increase significantly during underperfusion (Fig. 4). During isprenaline infusion and hypoxia, the enzyme activity displayed an exponential relationship with [AMP] ($r = 0.90$ and 0.93). Linear fits were less significant ($r = 0.57$ and 0.64). Lysosomal 5′-nucleotidase displayed a linear relationship with unchelated [AMP] during all conditions examined ($r = 0.91, 0.90$ and 0.95 respectively) (Fig. 5). The activity of the lysosomal enzyme increased by a maximum of approx. 5-fold during stimulation and hypoxia, and 2-fold during underperfusion.

DISCUSSION

The nature and location of the 5′-nucleotidase which catalyses the formation of the extracellular signal, adenosine, from AMP remains unclear. The present study was therefore undertaken in order to examine whether the known kinetic characteristics of the various 5′-nucleotidases isolated from heart, kidney and placental tissue [10,23,24,31,32] are consistent with the observed efflux of adenosine from the heart, and therefore consistent with a possible role in the regulation of adenosine release and coronary blood flow [5]. Several assumptions have been made in calculating theoretical activities of the enzymes examined. First it is assumed that the concentrations of nucleotides measured represent cytosolic values. There is sufficient evidence that over 80% of cardiac ATP, ADP and CrP reside within the cytosol and not within the mitochondria [1,40]. Furthermore, it has been demonstrated that mitochondrial metabolites are not visible in the ^{31}P-n.m.r. spectra [41]. This assumption therefore appears valid. Secondly, in incorporating the competitive effects of IMP on the rate of AMP hydrolysis by 5′-nucleotidase, it is assumed that the changes in IMP determined by h.p.l.c. represent an index of cytosolic changes. Although it has been demonstrated that IMP resides predominantly within the mitochondria [48], it has also been shown that changes in IMP during anaerobic conditions or ischaemia occur predominantly within the cytosol, whereas the mitochondrial fraction remains essentially unchanged [48]. As a result, the changes in tissue [IMP] measured in this study (Table 4) should represent changes in cytosolic [IMP], and the activity changes calculated from these values should represent the competitive effect of IMP on AMP hydrolysis. Finally, it is assumed that net efflux of

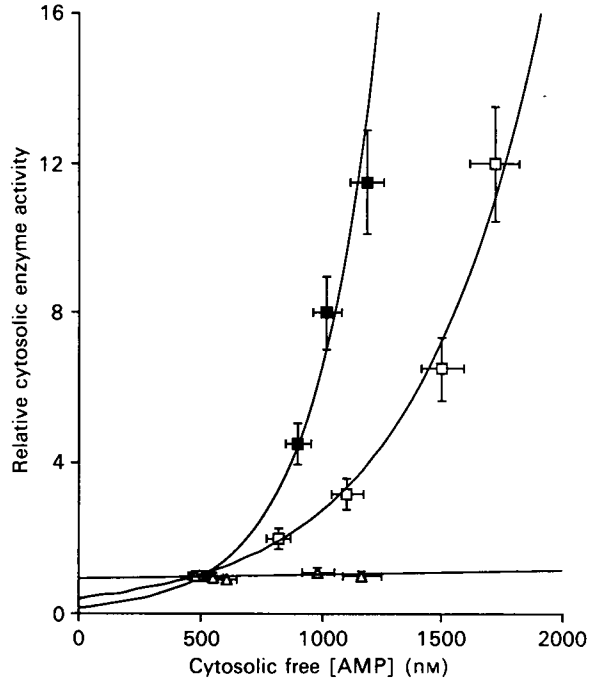

Fig. 4. Calculated activity of ATP-inhibited cytosolic 5′-nucleotidases during isoprenaline infusion, graded hypoxia and graded underperfusion

The theoretical activity of ATP-inhibited cytosolic 5′-nucleotidase was determined from the changes in unchelated ATP, ADP and AMP, and concentrations of free P_i, IMP and Mg^{2+} as outlined in the Materials and methods section. The activity changes were determined in hearts subjected to isoprenaline infusion (■), hypoxia (□) and underperfusion (△). Curves were fitted by least-mean-squares analysis of single data points: r values for the curves are 0.90, 0.93 and 0.98 respectively.

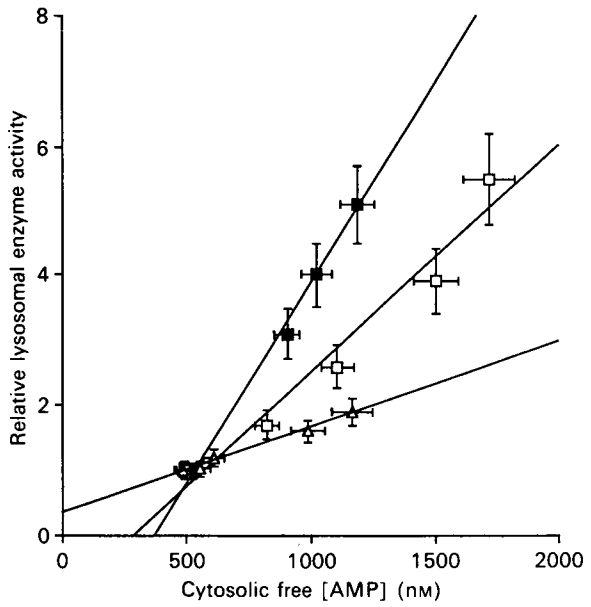

Fig. 5. Calculated activity of lysosomal 5′-nucleotidase during isoprenaline infusion, graded hypoxia and graded underperfusion

The theoretical activity of lysosomal 5′-nucleotidase was determined from the changes in unchelated ATP, ADP and AMP, and concentrations of free P_i, IMP and Mg^{2+} as outlined in the Materials and methods section. The activity changes were determined in hearts subjected to isoprenaline infusion (■), hypoxia (□) and underperfusion (△). Curves were fitted by least-mean-squares analysis of single data points: r values for the curves are 0.91, 0.90 and 0.95 respectively.

adenosine, inosine and hypoxanthine represents an index of adenosine formation in the myocardium. It should be noted that uptake of adenosine by endothelium and deamination of AMP to IMP will effect the release of adenosine, inosine and hypoxanthine. Therefore efflux of purines is only an index of changes in global myocardial adenosine production and not a precise value representing AMP hydrolysis via 5′-nucleotidase. Nevertheless, the efflux of purines should proceed as a function of the metabolic status of the myocardium, and conditions under which there is an elevated efflux of purines from the heart should be accompanied by increased flux of AMP through 5′-nucleotidase [2,3,48,49]. If the theoretical flux of AMP through a particular 5′-nucleotidase pool remains unchanged or decreases in the face of measurable elevations in purine efflux, it is questionable whether that particular enzyme is involved in adenosine formation.

The present results for purine efflux demonstrate that there appears to be an exponential relationship between purine efflux and free cytosolic [AMP] during isoprenaline infusion and graded hypoxia (Figs. 1*a* and 1*b*). This suggests that the activity of the 5′-nucleotidase(s) responsible for adenosine production increases disproportionately, is allosterically activated, as substrate (AMP) increases. During underperfusion, release of purines exhibited a linear relationship with [AMP] (Fig. 1*c*), indicating that adenosine formation in these hearts proceeds as a direct function of the concentrations of available substrate, AMP. This latter observation is in agreement with a recent study in which it is proposed that adenosine formation is substrate-regulated in the ischaemic myocardium [1,2]. The total efflux of purines observed in the present study is similar to the release observed in the study by Bunger & Soboll in guinea-pig hearts [1]. The linear relationship between free cytosolic AMP and purine release in the study by Bunger & Soboll was obtained in hearts made ischaemic, simultaneously stimulated with noradrenaline, and supplied with different substrates. From the present study, in which the relationship was examined under a range of conditions, it is clear that the relation between AMP and purine efflux depends strongly on the nature of the stimulus used (Fig. 1). This may reflect the different metabolic events which occur during the different experimental conditions (Tables 1, 2, 3 and 5). For example, ATP hydrolysis is less marked during the periods of graded ischaemia examined in this study (Table 3) than during hypoxia and stimulation (Tables 1 and 2). Similarly, cytosolic [Mg^{2+}] does not appear to change significantly during ischaemia (Table 3).

As expected, under the conditions examined ATP and/or CrP decrease and ADP, AMP and P_i increase (Tables 1, 2 and 3). These metabolites, together with Mg^{2+}, are known to activate or inhibit 5′-nucleotidases [10–13,24–26]. Tissue IMP increases significantly under

all conditions, and will therefore compete with AMP as substrate for 5′-nucleotidase (Table 4). This effect will be more pronounced in those enzymes which display a preference for IMP as substrate. The metabolic changes associated with the experimental conditions in this study are similar to those observed in earlier studies [1,33,34].

Interestingly, myocardial cytosolic [Mg^{2+}] appears to increase quite significantly during isoprenaline infusion and hypoxia (Tables 1 and 2). The control value of cytosolic [Mg^{2+}] of 0.76 ± 0.1 mM in this study is in good agreement with previous results [42–44,50–52]. The observed changes in [Mg^{2+}] during stimulation and hypoxia (Tables 1 and 2) are significant and appear to correlate inversely with [ATP], in agreement with the results of Murphy *et al.* [52]. Supporting this relationship, [ATP] did not decline significantly during underperfusion, and cytosolic [Mg^{2+}] did not change significantly (Table 3). Changes in cytosolic [Mg^{2+}] have been previously observed, during underperfusion in rat heart [52], and after trauma in the rat brain [53]. The validity of the technique used in the present study to determine cytosolic Mg^{2+} has been verified in studies using alternative methods [43,44]. More recent studies using alternative methodologies also support the values of [Mg^{2+}] found in the present study [50,52]. The factors which may effect the shift in the α and β spectral peaks, and the calculation of Mg^{2+}, are temperature, pH and ionic strength. In this study, temperature is maintained at 37 °C, and the pH, as determined via the shift in the P_i peak relative to the CrP peak [34], does not significantly change during isoprenaline treatment or hypoxia. During underperfusion, the pH changes by a maximum of approx. 0.3 pH unit (Table 3). This change would not be expected to affect significantly the Mg^{2+}-dependent shift in $\delta_{\alpha\beta}$ [53]. It has also been shown that the addition of Na^+, Ca^{2+} or K^+ to mixtures of Mg–ATP does not alter the Mg^{2+}-dependent shift in $\delta_{\alpha\beta}$ [53]. The present results support the involvement of acute fluctuations in intracellular Mg^{2+} in regulation of 5′-nucleotidase activity.

Assuming that the metabolite concentrations determined by ^{31}P-n.m.r. methodology represent cytosolic values, and that cytosolic Mg^{2+} is in equilibrium with these nucleotides, unchelated and chelated nucleotide concentrations can be calculated. Owing to the increases in cytosolic [Mg^{2+}] in the stimulated and hypoxic hearts, unchelated adenine nucleotide concentrations alter. The concentrations of adenine nucleotide measured by ^{31}P-n.m.r. methods represent total free cytosolic values, chelated and unchelated to Mg^{2+} [41]. The concentrations of unchelated cytosolic ATP, ADP and AMP will therefore be lower than the total cytosolic values as [Mg^{2+}] rises. This is of importance, since it has been shown that unchelated nucleotides inhibit or activate 5′-nucleotidase [10,11,19]. Although the unchelated concentrations of ATP and AMP decline and increase respectively, as do the total cytosolic values (Table 5), unchelated [ADP] actually appears to decrease during isoprenaline infusion and hypoxia (Table 5), owing to the disproportionate elevations in free [Mg^{2+}] (Tables 1 and 2). Therefore, previous suggestions [21,23] that increases in inhibitory ADP at the expense of ATP would inhibit ecto-5′-nucleotidase are not supported by these results.

From the above-mentioned metabolic changes, the theoretical activities of four enzymes were calculated. The changes in the calculated activity of the ecto-5′-nucleotidase (Fig. 2) are fully consistent with the observed release of purines and the relations between purine release and [AMP] under all conditions examined (Fig. 1). In the stimulated and hypoxic hearts, the calculated activity increased exponentially, whereas in the underperfused hearts the activity increased linearly. Similarly, the total increases in the activity of the enzyme under these conditions (Fig. 2) closely matches the relative increases in purine efflux (Fig. 1). This close comparison between the nature of the observed correlations of purine release with unchelated [AMP] and the calculated changes in activity of ecto-5′-nucleotidase supports the involvement of the membrane-bound enzyme pool in formation of adenosine. The involvement of ecto-5′-nucleotidase is also supported by recent studies which indicate that adenosine is formed at the extracellular surface [17]. Similarly, there is good evidence that adenosine release during hypoxia is due to the dephosphorylation of AMP released from cardiac myocytes, a process catalysed by ecto-nucleotidase located on endothelial cells [14]. Together, those studies and the present results indicate that the involvement of ecto-5′-nucleotidase in adenosine formation cannot be considered a moot point.

These results support the hypothesis of Rubio *et al.* [54], in which it was reasoned that a decrease in [CrP], together with increased release of Mg^{2+} from ATP, was responsible for allosteric activation of membrane-bound enzyme. The present results demonstrate that, owing to significant changes in $[Mg^{2+}]_{free}$, the concentrations of the active form of the inhibitors, unchelated [ATP] and [ADP], both fall during cardiac stimulation and hypoxia. The mechanism by which cytosolic effectors could alter the membrane-bound enzyme activity remains unclear at this stage. Since there may be an intracellular pool of membrane-bound enzyme constituting as much as 50% of the total membrane-bound activity [55], it may be that this recycling pool is available for regulation by cytosolic factors, although there is no evidence of this. Alternatively, since the 5′-nucleotidase is an integral protein spanning the plasma bilayer, it may be that cytosolic factors can interact with internal regulatory sites while the catalytic site faces the extracellular space, or adenosine may be formed at the cytoplasmic side of the ecto-5′-nucleotidase and subsequently transported to the extracellular space [14–17].

The calculated activities of the ATP-activated cytosolic 5′-nucleotidase are inconsistent with a role in adenosine formation under all conditions examined in this study. The enzyme does not appear to possess appropriate kinetic properties to allow it to participate significantly in the synthesis of purines observed in the coronary effluent of stimulated, hypoxic or ischaemic rat hearts (Fig. 3). This enzyme pool, which is known to be present in myocardial tissue, appears to remain largely inhibited during all conditions examined (Fig. 3). Calculated activity remains stable at all times. This is compatible with the observed decline in ATP (an activator), the increase in P_i (an inhibitor), and increase in IMP, the preferred substrate of this enzyme [24,28]. The changes in these regulators appear effectively to counter the increased supply of substrate, AMP (Table 5). It is more likely that this enzyme pool is responsible for the dephosphorylation of IMP to inosine [28]. Interestingly, an ATP-activated cytosolic enzyme which displays a preference for AMP as substrate has been recently isolated from rat heart [30]. This enzyme has not been fully characterized, and therefore cannot be examined in

the present study. However, it is possible that an enzyme with these characteristics might be important in the formation of adenosine.

Although the ATP-inhibited cytosolic enzyme examined in this study may contribute in part to extracellular adenosine formation in other tissues, as has been suggested [31,32], it is not known whether this enzyme is present in the myocardium. The calculated activity of this enzyme does appear to increase significantly under the conditions examined (Fig. 4), although the theoretical activity changes do not account for the total release of purines observed (Fig. 1). Since it is not known whether this enzyme is present in myocardial tissue, its role in adenosine formation cannot be ascertained. The calculated activity changes of the lysosomal enzyme are similar to those for the ATP-inhibited cytosolic enzyme, but of lesser magnitude (Fig. 5). The activity of the lysosomal enzyme did not increase sufficiently to account for the observed efflux of purines, and the relations between activity and [AMP] (Fig. 5) were not consistent with those observed between purine release and [AMP] (Fig. 1). The physiological significance of a lysosomal enzyme in the formation of extracellular adenosine is unclear. Similarly, it is not clear how cytosolic metabolites could affect the activity of this enzyme.

This study was undertaken in order to examine the relations between observed purine formation and calculated alterations in 5′-nucleotidase activity. Although the exact enzyme activity is not measured in this study, the theoretical changes in various enzymes have been calculated, based on the changes in the known allosteric regulators and substrates for these enzymes, and these are compared with measured adenosine formation. The results do not provide conclusive evidence; however, this study indicates that the metabolic changes and release of purines associated with isoprenaline infusion, graded hypoxia and underperfusion in heart are fully consistent with the involvement of an enzyme with the kinetic characteristics of the ecto-5′-nucleotidase found in cardiac tissue. Although it has been shown that adenosine formation occurs in the absence of ecto-enzyme activity [18–21], recent evidence does support a role for ecto-5′-nucleotidase in formation of adenosine [14,17]. The present results for the changes in cytosolic activators and inhibitors of 5′-nucleotidase support this possibility. The ATP-inhibited cytosolic enzyme which has been purified from other tissues also possesses appropriate characteristics for an enzyme involved in formation of adenosine under conditions of increased ATP hydrolysis; however, this enzyme may not be present in cardiac tissue. The characteristics of the ATP-activated cytosolic enzyme do not appear to be consistent with the purine formation measured in these hearts. Although the ATP-activated cytosolic enzyme has been studied extensively and has been proposed as the source of extracellular adenosine [18–21,24–26,29], the maximal activity under resting conditions and allosteric inhibition of this enzyme under conditions of increased ATP utilization are not consonant with an important role in formation of adenosine. It is likely that this enzyme is important in the dephosphorylation of IMP to inosine, as has been proposed [28]. The present results suggest that further work is required before the site of adenosine formation and the mechanism of regulation of the catalytic path are clarified.

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