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Metabolic and functional consequences of cytosolic 5'nucleotidase-1A overexpression in neonatal rat cardiomyocytes

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Abstract

Adenosine exerts a spectrum of energy preserving actions on the heart including negative chronotropic effects. The pathways leading to adenosine formation have remained controversial. In particular, although cytosolic 5' nucleotidases can catalyse adenosine formation in cardiomyocytes their contribution to the actions of adenosine has not been documented previously. We recently cloned two closely related AMP preferring cytosolic 5' nucleotidases (cN-IA and B); the A form predominates in heart. In this study we overexpressed pigeon cN-IA in neonatal rat cardiomyocytes using an adenovirus. cN-IA overexpression increased adenosine formation and release into the medium caused by simulated hypoxia and by isoproterenol in the absence and presence of inhibitors of adenosine metabolism. Adenosine release was not affected by an ecto-5'-nucleotidase inhibitor, α,β -methyleneADP but was by the nucleoside transporter, dipyridamole. The positive chronotropic effect of isoproterenol (130 ± 3 vs 100 ± 4 beats/ min) was inhibited (107 ± 3 vs 94 ± 3 beats/ min) in cells overexpressing cN-IA and this was reversed by addition of adenosine receptor antagonist, 8-(p-sulfophenyl)theophylline (120 ± 3 vs 90 ± 4 beats/ min). Our results demonstrate that overexpressed cN-IA can be sufficiently active in cardiomyocytes to generate physiologically effective concentrations of adenosine at its receptors.

Introduction

Adenosine exerts a spectrum of actions on the heart that include increasing coronary blood flow (2, 31), antagonizing the effects of catecholamines both pre- and post synaptically (18, 32) and prolonging atrioventricular conduction time (16). These actions are brought about by a family of cell surface adenosine receptors that have been extensively characterized (34). All the actions of adenosine on the heart tend to increase energy supply or reduce energy demand. Moreover, there appears to be a direct link between energy utilization and the rate of adenosine formation, which suggest that adenosine functions physiologically as a cardioprotective metabolite (23). The role of adenosine in ischaemic preconditioning (22) may also be an extension of this concept.

An ecto-5' nucleotidase (e-N) can catalyse the terminal dephosphorylation of AMP to adenosine. Biochemical experiments with inhibitors, and cloning, demonstrated conclusively that this enzyme is only capable of metabolizing extracellular nucleotides, in a cascade with other ecto-enzymes (39). Consistent with this, adenosine derived from AMP perfused through the heart requires e-N (25). However, a link between intracellular energy utilization, nucleotide release and e-N activity is at best controversial. In neonatal myocytes, adenosine formation induced by chemical hypoxia occurs exclusively intracellularly (21), although the extracellular pathway may also contribute in adult myocytes (4). The situation in the whole heart is more complicated due to the contribution made by the vasculature and nerve terminals. Experimental evidence has been presented in favour (13, 15) and against (3, 33) the involvement of the ecto-pathway under normoxic and hypoxic conditions. Deussen and colleagues (7) used trapping of adenosine through S-adenosylhomocysteine hydrolase and mathematical modelling to estimate the contribution of cytosolic and extracellular pathways to adenosine production in normoxic guinea-pig hearts. They concluded that 8 to 11% of adenosine production derived from extracellular metabolism. However, increased adenosine

formation in hearts stimulated by catecholamines (8, 9) apparently does not involve e-N (3, 19).

Alternatively, adenosine could be formed from cytosolic AMP, which rises during ATP breakdown owing to the myokinase equilibrium, by the action of a cytosolic 5' nucleotidase (cN). Adenosine might then be released from cells via equilibrative nucleoside transporters (17). An IMP-preferring enzyme, cN-II, was cloned and overexpressed and shown to selectively catalyse conversion of IMP to inosine rather than AMP to adenosine (28). An AMP-preferring enzyme was originally cloned from pigeon (30) and then from man and mouse (14, 29). When overexpressed, this enzyme selectively catalyzed conversion of AMP to adenosine. Furthermore, selective inhibitors of cN-I inhibit adenosine formation from rat adult myocytes under chemical hypoxia (11). Interestingly two cN-I genes were identified; cN-IA, the form originally purified and cloned from pigeon, is abundantly expressed in pigeon and human heart (14, 30). cN-IB is abundant in the testis, where it was also cloned as human autoimmune infertility related antigen (Genbank AF356185). Doubts remain regarding the role of cN-I in the myocardial actions of adenosine, in particular because its K_m for AMP measured in the test tube is around 5 mM, much higher than predicted cytosolic AMP concentrations. In addition most previous studies of cN-I activity have used inhibitors of adenosine kinase and adenosine deaminase to prevent further metabolism. While these studies provide absolute rates of adenosine formation, they leave unanswered the question whether sufficient adenosine can escape metabolism so as to carry out physiological effects at extracellular receptors. In the present studies, we overexpressed pigeon cN-IA in rat cardiomyocytes using adenovirus mediated gene transfer. We then studied adenosine formation under simulated hypoxia with and, importantly, without inhibitors of adenosine metabolism. Finally we investigated the influence of cN-IA

overexpression on a physiological response, namely beating rate in cardiomyocytes stimulated with isoproterenol.

Material and Methods

Materials. Tissue culture products were from Invitrogen. Molecular biology reagents, nucleotides and nucleosides and the cell cytotoxicity test (LDH measurement) were from Roche Biochemicals. Reagents for Western blotting were from Amersham and those for immunocytochemistry from DAKO. General laboratory reagents and chemicals were obtained from Sigma.

Construction of a recombinant adenoviral vector. The coding sequence of pigeon cN-IA was excised with BamHI and KpnI, and subcloned into the shuttle vector pDC 515 downstream the mouse CMV promoter (Microbix Biosystems Inc. Canada). Replication-deficient adenovirus was generated by site-specific FLP mediated recombination of the cotransfected shuttle and genomic plasmids in 293 cells. Viral stocks were plaque purified, amplified, CsCl banded and titrated according to the manufacture's instructions.

Culture and infection of rat neonatal cardiomyocytes. Myocytes were isolated from 2-3 day-old rats by 4 cycles of digestion in 0.1% trypsin containing 0.02% EDTA in PBS. Digestion was stopped by addition of FCS to 20%. The dispersed cells were resuspended in MEM supplemented with 10% FCS, 100 µg/ml streptomycin, 100 U/ml penicillin and pre-plated for 1 hour to allow fibroblasts to adhere. The suspended myocytes were seeded on plates previously coated with 1 % gelatin in PBS for 1h. 10^6 myocytes were seeded in 35mm dishes for beating rate measurements or 5×10^5 on 12-well plates for metabolite assays. After 40 hours in culture more than 80% of the cells were beating spontaneously. The culture medium was then changed to 4:1 DMEM: M199 containing antibiotics and 1% FCS and adenoviral infection was performed. A previously described adenovirus expressing β -galactosidase (37) was used to control for the non-specific effects of viral infection.

Metabolic studies. Cells in 12 well dishes were washed in saline, transferred to KRH (containing in mM: NaCl, 120; KCl, 4.8; KH_2PO_4 , 1.2; MgSO_4 , 1.2; CaCl_2 , 1.3; HEPES, 25;

pH 7.3) and preincubated for 10 minutes at 37°C. Metabolic inhibition was triggered with 10 mM 2-deoxyglucose (DOG), 10 mM DOG plus 1 mM sodium dithionite (S₂O₄) or 10 mM DOG plus 0.5 mM sodium cyanide (CN). Control cells were incubated in control buffer (KRH containing 5.5 mM glucose and 1 mM sodium pyruvate). Adenosine formation caused by β -adrenergic stimulation with 20 μ M isoproterenol was measured in control buffer. In order to inhibit adenosine metabolism 10 μ M erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA), an inhibitor of adenosine deaminase (EC 3.5.4.4) (24) and 50 μ M 5'-amino-5'-deoxyadenosine (5'NH₂) to inhibit adenosine kinase (EC 2.7.1.20) (24) were included in some experiments. The nucleoside transport inhibitor dipyridamole was added at a final concentration of 10 μ M from a 10mM solution in DMSO. Cell incubations were terminated by the addition of 0.75 volumes of 1.6M HClO₄ and total purines and protein concentrations determined by HPLC as previously described (30). Separate experiments were conducted to measure adenosine in the medium by HPLC. To stop incubations, 300 μ l of the supernatant buffer were removed and frozen immediately. After carefully removing the rest of the buffer, 400 μ l of 1.2M HClO₄ were added to assay purines and protein in the cell pellet as described above. Cell viability was assessed by measurement of LDH in cell supernatants at the end of the incubations and expressed as a percentage of the total LDH present in parallel wells extracted in KRH containing 0.1% TritonX-100.

Activities of purine metabolizing enzymes. Cell extracts were prepared in buffer containing in mM: Na dimethylglutarate, 20; Na β -glycerophosphate, 20; KCl, 100; DTT, 0.1; 0.1% TritonX-100 and a Sigma protease inhibitory cocktail (P8340), pH 6.9. Assays for cytosolic 5'nucleotidase were performed in extraction buffer with near-saturating substrate concentrations (10mM AMP) in the presence of the activator ADP (1 mM), the selective e-N inhibitor, α,β -methyleneADP (50 μ M), an inhibitor of adenosine deaminase, pentostatin (2 μ M) and 5'-amino-5'-deoxyadenosine (10 μ M), (30). e-N was assayed in buffer containing

in mM: Tris-HCl, 50; Na β -glycerophosphate, 10; MgCl₂, 5 and 0.1% tritonX-100, pH 8.0 containing 0.2mM AMP as substrate in the presence of an inhibitor of adenosine deaminase, pentostatin (2 μ M) and the adenosine kinase inhibitor 5'-amino-5'-deoxyadenosine (10 μ M). AMP deaminase was assayed in buffer containing in mM: Na dimethylglutarate, 20, EDTA, 1; DTT, 0.1 and KCl, 150, pH 7.0 containing 15mM AMP as substrate and 1.5mM ATP as activator. Adenosine deaminase was measured in 50mM potassium phosphate buffer pH 7.4 containing 150 μ M adenosine. Aliquots of extracts containing 10-20 μ g of protein were incubated for between 0 and 10 min at 37 °C, the reactions were stopped by addition of HClO₄ followed by analysis of the products by HPLC as already described (30). Adenosine kinase was measured at 37 °C in aliquots containing 1.5-3 μ g of protein using ¹⁴C adenosine as substrate (5).

Effects of cN-IA overexpression on beating rates. Cells 35mm dishes were transferred to HEPES buffered RPMI1640 (0.8ml). Beating rates were determined by counting the beats of four groups of cells for 1min using a heated microscope stage maintained at 37°C. Determinations were made after equilibrating for 10 min under all conditions used.

Western blots and immunocytochemistry. The generation of the rabbit polyclonal antibody against peptides derived from the sequence of pigeon cN-IA and the methods used for western blotting have been described previously (30).

Statistical analysis. Results are expressed as mean \pm SEM. Statistical significance was assessed with one-way analysis of variance (ANOVA) followed by paired Student's t-test if appropriate or unpaired Student's t-test with Bonferroni correction for repeated measures. A value of p<0.05 was considered significant.

Results

Adenovirus-mediated overexpression of cN-IA in rat cardiomyocytes

Infection of neonatal rat cardiomyocytes with a multiplicity of infection of 10 plaque forming units (pfu) per cell of an adenovirus that drives β -galactosidase expression transduced more than 90% of cells (Fig. 1A). Infection with a similar virus engineered to express pigeon cN-IA led to overexpression of a protein of the correct size by western blotting using a previously described antibody against a peptide epitope in pigeon cN-IA (Fig. 1B). This was accompanied by an 18-fold increase in 5'-nucleotidase activity from 45 ± 6 to 824 ± 36 nmol.min⁻¹.mg protein⁻¹ (n=7). By immunocytochemistry using the same antibody as for western blotting more than 90% of cardiomyocytes in cN-IA-adenovirus-infected cultures stained for cN-IA (Fig 1D). The activity (nmol.min⁻¹.mg protein⁻¹) of other purine metabolizing enzymes was similar in β -galactosidase and cN-IA transduced cells as follows: eN 134 ± 16 versus 142 ± 4 ; adenosine kinase 3.9 ± 0.9 versus 3.8 ± 1.0 ; adenosine deaminase 12.5 ± 1.7 versus 12.1 ± 1.4 and AMP deaminase 125 ± 29 versus 119 ± 26 .

Effect of cN-IA overexpression on nucleotide metabolism under simulated hypoxia

Purine concentrations were measured in cardiomyocytes plus medium after incubation under increasingly severe conditions of metabolic poisoning to simulate the effects of hypoxia either in the presence or absence of inhibitors of adenosine kinase (5'NH₂) and adenosine deaminase (EHNA) (24). As shown in Table 1, 2-deoxyglucose (DOG), a glycolytic inhibitor, alone decreased ATP concentrations by approximately 40%. This was increased to approximately 55% by adding dithionite (S₂O₄) that consumes molecular oxygen, and to approximately 70% by adding cyanide, an inhibitor of cytochrome oxidase (Table 1). Transduction with β -galactosidase had no effect on baseline ATP concentration or ATP catabolism compared to untransduced cells (results not shown). The baseline ATP concentration was decreased significantly by 8% in cN-IA transduced cardiomyocytes (43 ± 3

nmol/mg protein, n=7) compared to β -galactosidase transduced cells (47 ± 2 nmol/mg protein, n=7, $p < 0.02$). Despite this, there was no difference in the percentage of ATP broken down between β -galactosidase and cN-IA transduced cells under any condition of chemical hypoxia (Table 1). Including inhibitors of adenosine metabolism did not affect either the baseline ATP concentration in β -galactosidase or cN-IA transduced cells (48 ± 3 and 44 ± 3 nmol ATP/mg protein, respectively, n=3, p=NS) or the percentage ATP breakdown during chemical hypoxia (Table 1). Cardiomyocyte viability measured by lactate dehydrogenase (LDH) release was $>98\%$ in both β -galactosidase and cN-IA transduced cells. Only in cN-IA transduced cells treated with DOG and cyanide was LDH marginally elevated (Table 1).

Transduction with cN-IA profoundly altered the pattern of ATP metabolites produced either in the presence or absence of inhibitors of adenosine metabolism (Fig 2A, B). These figures summarize the pattern of nucleotide metabolism in representative experiments. Overexpression of cN-IA increased adenosine production (Fig. 2A, B), even in the absence of inhibitors of adenosine metabolism (Fig. 2A). cN-IA overexpression increased adenosine production at the expense of IMP, inosine and hypoxanthine, as most clearly seen in the presence of inhibitors of adenosine metabolism (Fig. 2B). This demonstrates competition between cN-IA and AMP deaminase for the available cytosolic AMP. Only with DOG and cyanide, when ATP is most depleted, did AMP accumulate in β -galactosidase transduced cells (Fig 2A, B). This is probably due to the inhibition of AMP deaminase known to occur under such conditions (20). With DOG and cyanide, cN-IA overexpression reduced the final AMP concentration. Averaged data from several experiments (Fig. 3A, B) demonstrates that even under base line conditions, cN-IA overexpression significantly increased the concentration of adenosine in the presence or absence of inhibitors of adenosine metabolism (Fig 3A, B). Moreover, cN-IA overexpression dramatically potentiated the stimulatory effects of simulated hypoxia on adenosine concentration (Fig. 3A, B). The patterns of changes after

simulated hypoxia were similar in the absence of inhibitors of adenosine metabolism, although the absolute levels of adenosine achieved were approximately halved (Fig. 3A, B). As expected, there was correspondingly more inosine and hypoxanthine in the absence of inhibitors of adenosine metabolism than in their presence (Fig. 2A, B). In agreement with our previous work in rat neonatal cardiomyocytes (21), inclusion of the selective e-N inhibitor, AOPCP (50 μ M) did not affect adenosine formation under any condition of simulated hypoxia (data not shown and see below).

We conducted a separate set of experiments in which medium and cells were analysed separately. Values for adenosine concentration in the medium (Fig. 4) were similar to those measured in cells plus medium in Fig. 3B, which shows that most of the adenosine was present in the medium, as we previously reported (21). Medium adenosine concentrations were not affected by inclusion of AOPCP either under baseline or after treatment with DOG and cyanide. However the nucleoside transport inhibitor, dipyridamole, dramatically reduced medium adenosine concentrations. Adenosine concentrations in the cells were below the limits of accurate measurement, except in the case of DOG plus cyanide plus dipyridamole, when they were 4-fold the extracellular concentration. Conversely, ATP, ADP or AMP concentrations were not measurable by HPLC in the medium under any condition (data not shown). These data confirm our previous study (21), which showed that the overwhelming majority of adenosine is formed intracellularly in neonatal rat cardiomyocytes, independently of e-N, and then transported out. Not surprisingly, overexpression of cN-IA increased intracellular adenosine formation.

Effect of cN-IA overexpression on nucleotide metabolism and beating frequency after stimulation with isoproterenol

The increase in total (cells plus medium) adenosine concentration resulting from overexpression of cN-IA persisted in the presence of 20 μ M isoproterenol whether or not

inhibitors of adenosine metabolism were also present (Fig. 5A). In a separate series of experiments we measured adenosine concentration in the medium alone. Medium adenosine concentrations were also significantly greater in cN-IA than β -galactosidase transduced cells and this difference again persisted in the presence of isoproterenol (Fig. 5B). The difference between cN-IA and β -galactosidase transduced cells was preserved in the presence of AOPCP but lost in the presence of dipyridamole (Fig. 5B). These data confirm that cN-IA overexpression led to an increase in adenosine formation and release, which was present both before and after isoproterenol treatment.

Isoproterenol significantly and reversibly increased the beating frequency of cardiomyocytes transduced with β -galactosidase (Fig. 6A). The effect of isoproterenol was not reversed by including inhibitors of adenosine metabolism but was by adding 50 μ M adenosine, which had no effect on beating frequency in the absence of isoproterenol (Fig. 4A and data not shown). By contrast, isoproterenol did not increase beating frequency in cardiomyocytes overexpressing cN-IA (Fig. 6A). To confirm that these effects were mediated through adenosine receptors, the experiments were repeated in the presence of a nonselective adenosine receptor antagonist, 8-sulphophenyltheophylline (8-SPT). 8-SPT had no effect on the ability of isoproterenol to stimulate beating frequency but partially antagonized the inhibition of this by added adenosine (Fig. 6B). More importantly, however, 8-SPT completely abolished the inhibitory effect of cN-IA overexpression on isoproterenol induced beating frequency (Fig. 6B).

Discussion

Cytosolic 5' nucleotidases were first characterized and named based on kinetic and physical properties during purification from avian, rodent and human hearts. The so-called cN-II, was the first to be cloned from chicken and man (26). It is a tetramer of 60 kDa subunits and prefers IMP ($K_m \sim 0.2\text{mM}$) over AMP ($K_m \sim 5\text{ mM}$). It is activated *in vitro* by ATP or ADP and inhibited by inorganic phosphate, which implies a biphasic response during ATP breakdown that has been confirmed in whole cell experiments (38). The so-called cN-I was later cloned from pigeon, man and mouse (14, 29, 30). Although cN-I and II may share similar catalytic residues (1), they are genetically unrelated. The purified enzyme is a tetramer of 40 kDa subunits that has a preference for AMP ($K_m \sim 5\text{ mM}$) compared to IMP ($K_m \sim 20\text{ mM}$). It is insensitive to inorganic phosphate and requires a nucleoside diphosphate (e.g. ADP) for maximum activity (nucleoside triphosphates cannot act as substitutes). These kinetic characteristics suggest that the enzyme is active during ATP catabolism when AMP, ADP and inorganic phosphate concentrations rise. Inhibitors selective for cN-I over cN-II are dideoxynucleosides (particularly ddC and ddU) and these have been shown to decrease adenosine production from rat myocytes (10). This was the first direct evidence that cN-I could contribute to adenosine formation in cells. The role of cN-I was confirmed by plasmid based overexpression studies, which demonstrated the ability of cloned cN-IA to produce adenosine in established cell lines in the presence of inhibitors of adenosine metabolism (28, 30).

Sequencing studies recently distinguished two isoforms of cN-I encoded by genes on human chromosomes 1 and 2, respectively. The form purified and kinetically characterized from pigeon heart and later used for protein sequencing and cloning was designated cN-IA (29). The kinetic properties of cN-IB have yet to be fully elucidated, although its basic characteristics in terms of K_m , activation by AMP, insensitivity to inorganic phosphate and

inhibition by dideoxynucleosides appear similar to cN-IA. Moreover both isoforms can generate adenosine during ATP catabolism when overexpressed in COS-7 cells (29). Since cN-IA is abundantly expressed in hearts while cN-IB is poorly expressed in heart and predominant in testis we chose to focus on cN-IA in these experiments. Furthermore since the kinetic properties of purified and cloned cN-IA are highly conserved across species from pigeon to man (35, 36) we chose to overexpress the pigeon enzyme, for which we had suitable full-length clones.

The high K_m for AMP of cN-I ($\sim 5\text{mM}$) has cast doubts on its physiological function in myocardial adenosine formation because the predicted concentrations of free cytosolic AMP in cardiomyocytes are in the micromolar range. On the other hand, AMP deaminase also has a relative high K_m for AMP $\sim 0.6\text{ mM}$ that increases to $\sim 2.4\text{mM}$ in the absence of ATP (20). Some cellular fractionation and histological evidence has implied that both cN-I and AMP deaminase may be associated with contractile fibres and therefore in a microenvironment exposed to higher AMP concentrations (27, 30). A further concern has been that any adenosine produced in the cytoplasm would have to escape metabolism by adenosine kinase and adenosine deaminase and be transported out through the equilibrative nucleoside transporter before it could activate extracellular adenosine receptors. There remained doubts therefore whether cN-I could sufficiently elevate adenosine concentrations in primary cardiomyocytes in the face of adenosine metabolism to act at adenosine receptors. To investigate this question we first had to develop an adenovirus, which we showed capable of achieving high-level expression of cN-IA in primary cardiomyocytes. Our second goal was to measure adenosine formation and release during simulated hypoxia in the presence of inhibitors of adenosine metabolism. Although these experiments were not designed to measure initial rates of adenosine formation, we can calculate that 20 nmoles of adenosine were produced over 10 minutes in cN-IA transduced cells, which compares with the V_{\max} of

nearly 900 nmol.min⁻¹.mg protein⁻¹. Clearly the enzyme operates under far from saturating conditions, consistent with the low free cytosolic AMP concentrations. Furthermore, by suppressing IMP, inosine and hypoxanthine concentrations, we showed that cN-IA effectively competed for substrate with AMP deaminase. Most importantly, we showed for the first time, that the enzyme was sufficiently active to elevate adenosine concentrations even in the absence of inhibitors of adenosine metabolism.

All previous studies demonstrating adenosine formation from recombinant cN-I overexpression have used extreme conditions of ATP depletion. We showed here, however, that overexpression of cN-I provoked adenosine formation and elevated extracellular adenosine concentrations even under baseline conditions, when ATP levels were only 8% less than in β -galactosidase transduced cells.

Finally we sought to investigate whether cN-IA over expression could influence a response to adenosine mediated by extracellular adenosine receptors. Since neonatal myocytes are anchored to the substratum, cell shortening could not be quantified. Hence the most convenient parameter to measure was beating frequency. As previously shown (12), isoproterenol exerts a positive chronotropic effect on neonatal cardiomyocytes. This does not appear to be under tonic control by adenosine, because 8-SPT, a nonselective competitive inhibitor of adenosine receptors, had no stimulatory effect on the beating rate. However, added adenosine reversed the effect of isoproterenol, and this appeared to be receptor-mediated. The partial effect of 8-SPT can be explained by the relatively high concentration of added adenosine used. Overexpression of cN-I produced a similar effect to added adenosine, which was in this case fully reversed by 8-SPT. The increase in adenosine formation and release by cN-IA overexpression persisted in the presence of isoproterenol and, from inhibitor experiments, was again independent of e-N but depended on nucleoside transport. The concentration of adenosine found in β -galactosidase transduced and cN-IA transduced

cardiomyocytes in the presence of isoproterenol ranged from 0.1-0.6 μ M, which is in the range known to be effective at adenosine receptors (34). These results show, again for the first time, that cN-IA can generate sufficient concentrations of adenosine to exert physiological effects on cell surface receptors even in the face of adenosine metabolism.

Limitations. While our overexpression studies demonstrate the potential of cN-IA to produce adenosine in cardiomyocytes, they clearly do not directly address the role of cN-IA in regulating myocardial adenosine concentrations and physiological actions in the heart. This will ultimately require transgenic and knockout experiments in mice for which our present experiments provide necessary justification. The extracellular compartment in the intact myocardium is much smaller in proportion to that in isolated cardiomyocyte cultures and this would tend to potentiate the extracellular concentration of any adenosine formed in the cytoplasm and released from the nucleoside transporter. In this sense our experiments in isolated cardiomyocytes represent rather stringent conditions to test the role of cN IA, which we compensated for by overexpressing the enzyme. A further limitation of our study is that we focused on the cardiomyocytes. Since endothelial cells also contribute to adenosine formation in the heart (6), future studies addressing the role of cN-I in those cells would also be valuable.

In conclusion, our studies demonstrate for the first time the activity of cloned cN-IA in primary rat cardiomyocytes. They demonstrate that cN-IA is sufficiently active to significantly increase adenosine concentration in the absence of inhibitors of adenosine metabolism and mediate physiological responses to adenosine at extracellular receptors.

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References

1. **Allegrini, S, Scaloni, a, Ferrara, L, Pesi, R, Pinna, P, Sgarrella, F, Camici, M, Eriksson, S, and Tozzi, M G.** Bovine Cytosolic 5'-Nucleotidase Acts through the Formation of an Aspartate 52-Phosphoenzyme Intermediate. *J Biol Chem* 276: 33526-33532, 2001.
2. **Berne, R M.** Regulation of coronary blood flow. *Physiol Rev* 44: 1-29, 1964.
3. **Borst, M M, and Schrader, J.** Adenine Nucleotide Release From Isolated Perfused Guinea Pig Hearts and Extracellular Formation of Adenosine. *Circ Res* 68: 797-806, 1991.
4. **Bukoski, R D, and Sparks, H V.** Adenosine production and release by adult rat cardiocytes. *J Mol Cell Cardiol* 18: 595-605, 1986.
5. **de Jong, J W, and Kalkman, C.** Myocardial adenosine kinase: Activity and localization determined with rapid radiometric assay. *Biochim Biophys Acta* 320: 388-396, 1973.
6. **Deussen, A, Möser, G, and Schrader, J.** Contribution of coronary endothelial cells to cardiac adenosine production. *Pflügers Arch.* 406: 608-614, 1986.
7. **Deussen, A, Stappert, M, Schafer, S, and Kelm, M.** Quantification of extracellular and intracellular adenosine production : understanding the transmembranous concentration gradient. *Circulation* 99: 2041-2047, 1999.
8. **DeWitt, D F, Wangler, R D, Thompson, C I, and Sparks, H V.** Phasic Release of Adenosine during Steady State Metabolic Stimulation in the Isolated Guinea Pig Heart. *Circ Res* 53: 636-643, 1983.
9. **Dobson, J G, and Schrader, J.** Role of extracellular and intracellular adenosine in the attenuation of catecholamine evoked responses in guinea pig heart. *J Mol Cell Cardiol* 16: 813-22, 1984.

10. **Garvey, E P, Lowen, G T, and Almond, M R.** Nucleotide and nucleoside analogues as inhibitors of cytosolic 5'- nucleotidase I from heart. *Biochemistry* 37: 9043-9051, 1998.
11. **Garvey, E P, and Prus, K L.** A specific inhibitor of heart cytosolic 5'nucleotidase I attenuates hydrolysis of adenosine 5'-monophosphate in primary rat myocytes. *Arch Biochem Biophys* 364: 235-240, 1999.
12. **Gulick, T, Pieper, S, Murphy, M, Lange, L, and Schreiner, G.** A new method for assessment of cultured cardiac myocyte contractility detects immune factor-mediated inhibition of beta-adrenergic responses. *Circulation* 84: 313-321, 1991.
13. **Headrick, J P, Matherne, G P, and Berne, R M.** Myocardial Adenosine Formation During Hypoxia: Effects of Ecto-5' Nucleotidase Inhibition. *J Mol Cell Cardiol* 24: 295-303, 1992.
14. **Hunsucker, S A, Spychala, J, and Mitchell, B S.** Human cytosolic nucleotidase-I: Characterization and role in nucleoside analog resistance. *J Biol Chem* 276: 10498-10504, 2001.
15. **Imai, S, Chin, W P, Jin, H, and Nakazawa, M.** Production of AMP and adenosine in the interstitial fluid compartment of the isolated perfused normoxic guinea pig heart. *Pflügers Arch.* 414: 443-449, 1989.
16. **Jenkins, J R, and Belardinelli, L.** Atrioventricular nodal accommodation in isolated guinea pig hearts: physiological significance and role of adenosine. *Circ Res* 63: 97-116, 1988.
17. **Jennings, L L, Cass, C E, TRitzel, M W L, Yao, S Y M, Young, J D, Griffiths, M, and Baldwin, S A.** Adenosine Transport: Recent Advances in the Molecular Biology of Nucleoside Transporter Proteins. *Drug Dev Res* 45: 277-287, 1998.

18. **Khan, M T, and Malik, K U.** Inhibitory effect of adenosine and adenine nucleotides on potassium-evoked efflux of [3H]-noradrenaline from the rat isolated heart: lack of relationship to prostaglandins. *J Pharmac* 68: 551-561, 1980.
19. **Lorbar, M, Fenton, R A, and Dobson, J G.** ATP as a source of interstitial adenosine in the rat heart. *Can J Physiol Pharmacol* 77: 579-588, 1999.
20. **Mahnke-Zizelman, D K, D'Cunha, J, Wojnar, J M, Brogley, M A, and Sabina, R L.** Regulation of rat AMP deaminase 3 (isoform C) by development and skeletal muscle fibre type. *Biochem. J.* 326: 521-29, 1997.
21. **Meghji, P, Holmquist, C A, and Newby, A C.** Adenosine formation and release from neonatal-rat heart cells in culture. *Biochem. J.* 229: 799-805, 1985.
22. **Nakano, A, Cohen, M V, and Downey, J M.** Ischemic preconditioning: from basic mechanisms to clinical applications. *Pharmacol Ther* 86: 263-75, 2000.
23. **Newby, A C.** Adenosine and the concept of 'retaliatory metabolites'. *Trends Biochem Sci* 9: 42-44, 1984.
24. **Newby, A C.** The interaction of inhibitors with adenosine metabolising enzymes in intact isolated cells. *Biochem Pharmacol* 30: 2611-2615, 1981.
25. **Obata, T, and Yamanaka, Y.** Tyramine produces interstitial adenosine-mediated activation of ecto-5'-nucleotidase in rat heart in vivo. *Eur J Pharmacol* 374: 25-31, 1999.
26. **Oka, J, Matsumoto, A, Husokawa, Y, and Inoue, S.** Molecular cloning of human cytosolic purine 5'-nucleotidase. *Biochem Biophys Res Commun* 205: 917-922, 1994.
27. **Rundell, K W, Tullson, P C, and Terjung, R L.** Altered kinetics of AMP deaminase by myosin binding. *Am J Physiol Cell Physiol* 263: C294-C299, 1992.

28. **Sala-Newby, G B, Freeman, N V E, Skladanowski, A C, and Newby, A C.** Distinct roles for recombinant cytosolic 5'-nucleotidase-I and -II in AMP and IMP catabolism in COS-7 and H9c2 rat myoblast cell lines. *J Biol Chem* 275: 11666-11671, 2000.
29. **Sala-Newby, G B, and Newby, A C.** Cloning of a mouse cytosolic 5'-nucleotidase-I identifies a new gene related to human autoimmune infertility-related protein. *Biochim Biophys Acta* 1521, 2001.
30. **Sala-Newby, G B, Skladanowski, A C, and Newby, A C.** The mechanism of adenosine formation in cells: cloning of cytosolic 5'nucleotidase I. *J Biol Chem* 274: 17789-17793, 1999.
31. **Schrader, J.** Adenosine. A homeostatic metabolite in cardiac energy metabolism. *Circulation* 81: 389-391, 1990.
32. **Schrader, J, Baumann, G, and Gerlach, E.** Adenosine as inhibitor of myocardial effects of catecholamines. *Pflügers Arch* 372: 29-35, 1977.
33. **Schutz, W, Schrader, J, and Gerlach, E.** Dfferent sites of adenosine formation in the heart. *Am J Physiol Heart Circ Physiol* 240: H963-H970, 1981.
34. **Shryock, J C, and Belardinelli, L.** Adenosine and Adenosine Receptors in the Cardiovascular System: Biochemistry, Physiology, and Pharmacology. *Am J Cardiol* 79: 2-10, 1997.
35. **Skladanowski, A C, and Newby, A C.** Partial purification and properties of an AMP-specific soluble 5'-nucleotidase from pigeon heart. *Biochem. J.* 268: 117-122, 1990.
36. **Skladanowski, A C, Smolenski, R T, Tavernier, M, de Jong, J W, Yacoub, M H, and Seymour, A M L.** Soluble forms of 5'-nucleotidase in rat and human heart. *Am J Physiol Heart Circ Physiol* 270: H1493-H1500, 1996.

37. **Wilkinson, G W G, and Akrigg, A.** Constitutive expression from the CMV major IE promotor in a defective adenovirus vector. *Nucleic Acid Res* 20: 2233-2239, 1992.
38. **Worku, Y, and Newby, A C.** The mechanism of adenosine production in rat polymorphonuclear leucocytes. *Biochem. J.* 214: 325-330, 1983.
39. **Zimmermann, H.** Extracellular metabolism of ATP and other nucleotides. *Naunyn-Schmiedeberg's Arch Pharmacol* 362: 299-309, 2000.

Table 1 Effect of cN-I overexpression and metabolic inhibitors on decreases in ATP concentration and cell viability

Treatment	ATP decrease (% KRH control)		LDH release (% total)	
	β -galactosidase	cN-I	β -galactosidase	cN-I
KRH			1.0 \pm 0.1	1.2 \pm 0.1
DOG	43 \pm 8	37 \pm 8	1.2 \pm 0.2	1.3 \pm 0.1
DOG+dithionite	56 \pm 15 ^{\$}	54 \pm 12 ^{\$}	1.2 \pm 0.1	1.1 \pm 0.1
DOG+cyanide	65 \pm 6 [†]	71 \pm 6 [†]	1.0 \pm 0.1	1.1 \pm 0.2
KRH+EHNA+5'NH ₂			1.1 \pm 0.2	1.1 \pm 0.3
DOG+EHNA+5'NH ₂	49 \pm 10	39 \pm 7	1.1 \pm 0.1	1.3 \pm 0.3
DOG+dithionite	62 \pm 16 ^{\$}	56 \pm 14 ^{\$}	1.0 \pm 0.1	1.1 \pm 0.1
+EHNA+5'NH ₂				
DOG+cyanide	71 \pm 8	75 \pm 7 [†]	1.2 \pm 0.1	1.7 \pm 0.2 *
+EHNA+5'NH ₂				

ATP concentrations measured by hplc are expressed as a percentage of the corresponding values obtained from β -galactosidase or cN-I transduced cells incubated in Kreb's Ringer Hepes buffer (KRH) (47 \pm 2 versus 43 \pm 3 nmol ATP/mg protein, n=7, p<0.05) or KRH+EHNA+ 5'-amino-5'-deoxyadenosine (5'NH₂) (48 \pm 3 vs 44 \pm 3 nmol ATP/mg protein, n=3, p=NS). LDH released into the medium is expressed as a percentage of the value in homogenates. Values are mean \pm SEM of 3 separate experiments or otherwise stated. * p<0.05 versus β -galactosidase transduction. ^{\$} p<0.003 when all experiments with dithionite are compared to DOG alone. [†] p<0.01 for cyanide versus DOG alone, p<0.0001 when all experiments with cyanide are compared to DOG alone.

Fig. 1 Overexpression of β -galactosidase and cN-I in cardiomyocytes

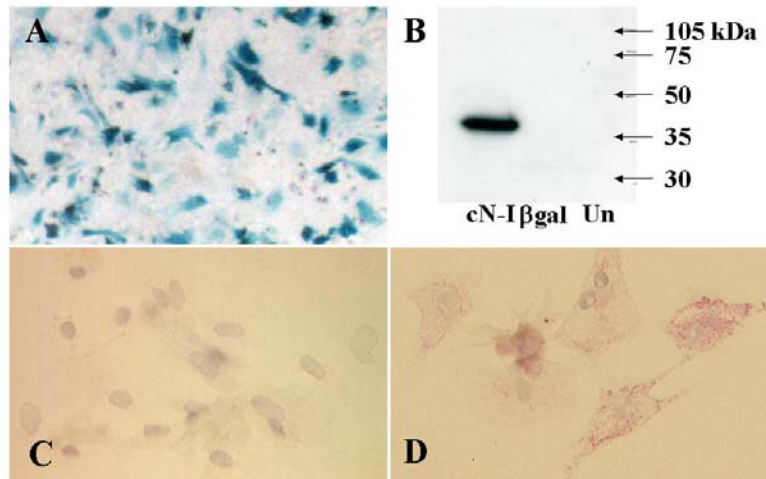


Fig. 2 Relative purine concentrations in β -galactosidase and cN-I transduced cardiomyocytes plus medium

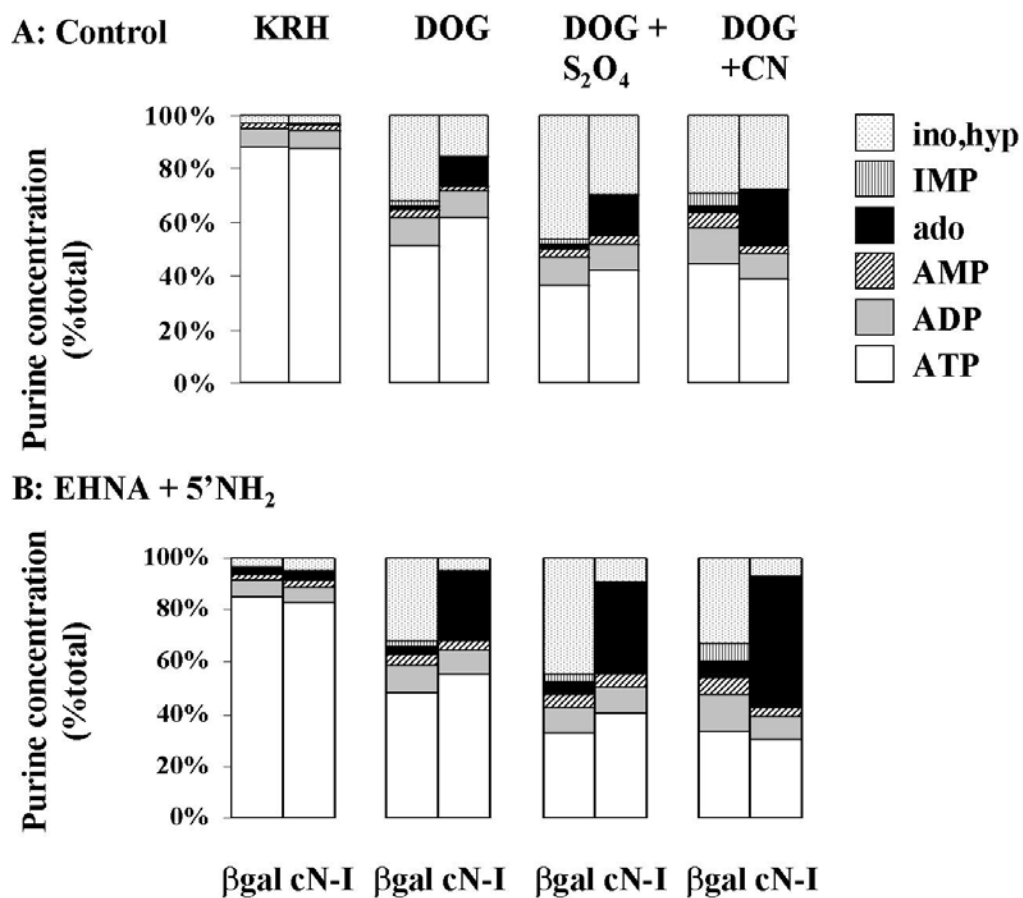


Fig. 3 Adenosine concentrations in β -galactosidase and cN-I transduced cardiomyocytes plus medium

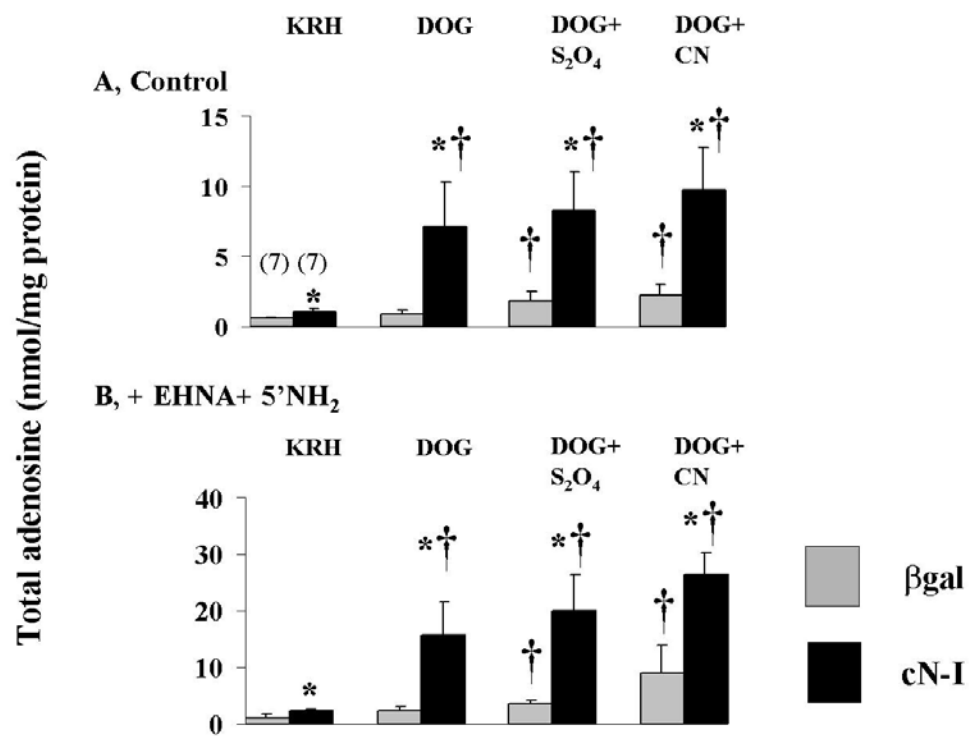


Fig. 4 Effects of AOPCP and dipyridamole on extracellular adenosine concentrations in β -galactosidase and cN-I transduced cardiomyocytes

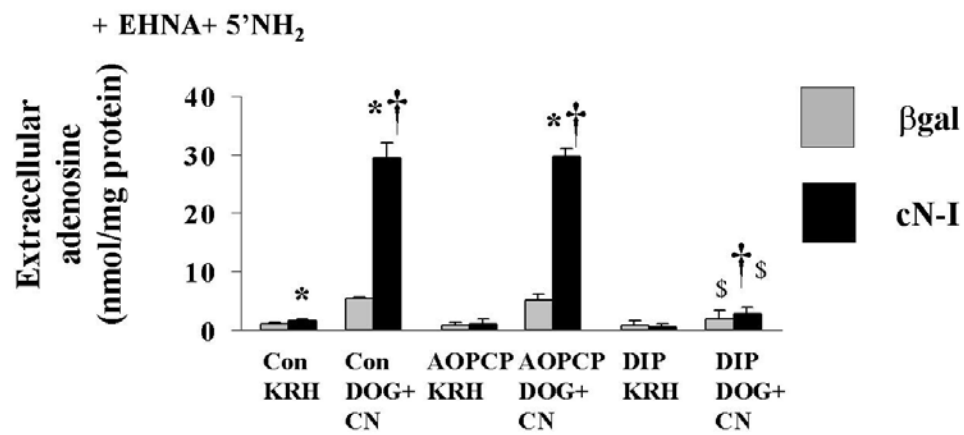


Fig. 5 Adenosine concentrations in isoproterenol-treated β -galactosidase and cN-IA transduced cardiomyocytes

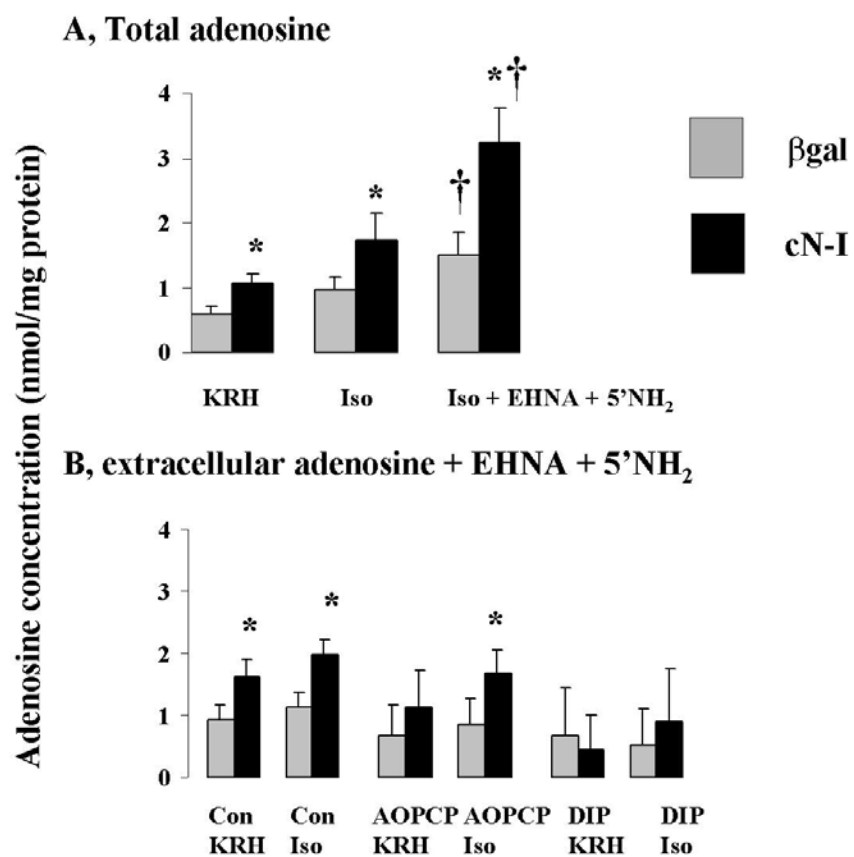


Fig. 6 Effect of adenosine on beating frequency with and without isoprenaline

