Distinct Roles for Recombinant Cytosolic 5′-Nucleotidase-I and -II in AMP and IMP Catabolism in COS-7 and H9c2 Rat Myoblast Cell Lines

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Catabolism of AMP during ATP breakdown produces adenosine, which restores energy balance. Catabolism of IMP may be a key step regulating purine nucleotide pools. Two, cloned cytosolic 5′-nucleotidases (cN-I and cN-II) have been implicated in AMP and IMP breakdown. To evaluate their roles directly, we expressed recombinant pigeon cN-I or human cN-II at similar activities in COS-7 or H9c2 cells. During rapid (more than 90% in 10 min) or slower (30–40% in 10 min) ATP catabolism, cN-I-transfected COS-7 and H9c2 cells produced significantly more adenosine than cN-II-transfected cells, which were similar to control-transfected cells. Inosine and hypoxanthine concentrations increased only during slower ATP catabolism. In COS-7 cells, 5′-nucleotidase activity was not rate-limiting for inosine and hypoxanthine production, which was therefore unaffected by cN-II- and actually reduced by cN-I- overexpression. In H9c2 cells, in which 5′-nucleotidase activity was rate-limiting, only cN-II overexpression accelerated inosine and hypoxanthine formation. Guanosine formation from GMP was also increased by cN-II. Our results imply distinct roles for cN-I and cN-II. Under the conditions tested in these cells, only cN-I plays a significant role in AMP breakdown to adenosine, whereas only cN-II breaks down IMP to inosine and GMP to guanosine.

ATP levels are a key determinant of cellular function. Formation of adenosine from AMP represents an important regulatory mechanism in cellular ATP homeostasis. In the heart, for example, adenosine causes coronary vasodilatation (1), inhibits sinoatrial and atrioventricular conduction (2), antagonises production (3) and action of catecholamines (4), causes pain (5), and contributes to ischemic preconditioning (6). We proposed that adenosine acts as a "retaliatory metabolite," being formed when there is an excess of ATP breakdown over its formation and mediating the spectrum of actions designed to restore energy balance (7, 8).

The pathways of ATP catabolism and adenosine formation are described in Fig. 1. The nature of the enzyme responsible for cytosolic AMP catabolism to adenosine has been the subject of much controversy. Two forms of cytosolic 5′-nucleotidase (cN-I and cN-II)† have been implicated. The cN-I was very recently cloned and shown to catalyze adenosine formation during ATP breakdown (9). The role of cN-II is less clear. Levels of expression of cN-II are usually higher in cultured cells compared with the corresponding tissue. It has therefore been suggested that cN-II is particularly important in cells with high DNA synthesis or turnover (10). The cN-II was cloned from chicken, human, and ox (11, 12) and was later shown to have activity in vitro (12, 13). Stable cell lines overexpressing cN-II were recently shown to have slightly reduced total nucleoside triphosphate pools (14). However, the function of cN-II in cells undergoing ATP breakdown has remained untested.

From enzyme kinetic data, the cN-I displays a preference for AMP over IMP and is stimulated by ADP but not ATP (15, 16), implying that it is active against AMP during ATP catabolism. Furthermore, its pH regulation (17) and studies with selective inhibitors (18) have implicated cN-I in adenosine formation in the heart, adding weight to the cloning and expression data (9). The cN-II displays selectivity for IMP and GMP over AMP, is stimulated by both ATP and ADP, and is inhibited by inorganic phosphate (19). These data suggest a selective role for cN-II in catabolism of IMP and cast doubt on whether it is active during ATP catabolism. On the other hand, the $K_m$ for AMP of cN-II measured in the presence of optimal activator, ATP, is similar to that of cN-I (approximately 5 mM) (19), and studies with selective inhibitors have also implicated cN-II in adenosine formation (20) during ATP breakdown.

The availability of cDNA clones for cN-I and cN-II has allowed us for the first time to compare their ability to catalyze AMP and IMP degradation directly in the same cell types. The overexpression strategy employed here allowed us to increase the activity of cN-I or cN-II selectively, without altering the levels of the other enzymes in the pathway. We chose to study two different cell types, COS-7 and H9c2 cells. COS-7 cells were chosen because of their ease of transfection and because flux through AMP deaminase was low compared with cytosolic 5′-nucleotidase (see Fig. 1). Hence, IMP did not accumulate, and this was an ideal cell line in which to compare the activity of cN-I and cN-II in adenosine formation. In order to compare the effect of cN-I and cN-II overexpression on IMP degradation, it was necessary to use a cell line in which AMP deaminase was greater than cytosolic 5′-nucleotidase activity and hence IMP accumulated. Skeletal muscle contains the highest concentration of AMP deaminase (21); here, AMP deaminase plays a role

† The abbreviations used are: cN, cytosolic 5′-nucleotidase; β-gal, β-galactosidase; DOG, 2-deoxy-D-glucose; FCCP, carbonyl cyanide p-(trifluoromethoxy) phenyl-hydrazone; GFP, green fluorescent protein; TES, 2-N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid.

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Divergent Roles of Cytosolic 5′-Nucleotidases

Table I

| Cell line | Transfection | cN-I activity | cN-II activity |
|-----------|--------------|---------------|---------------|
| COS-7(n=5) | β-gal | 0.026 ± 0.015 | 0.021 ± 0.006 |
| cN-I | 0.18 ± 0.06 | ND |
| cN-II | ND | 0.20 ± 0.07 |
| H9c2(n=3) | GFP | 0.012 ± 0.004 | 0.016 ± 0.001 |
| cN-I | ND | 0.042 ± 0.006 |
| cN-II | ND | 0.033 ± 0.007 |

allowed the calculation of $V_{max}$. To measure cN-II activity, cells were extracted with a buffer containing 10 mM TES, 120 mM KCl, 1 mM EDTA, 0.1 mM dithiothreitol, 0.1% Triton X-100, pH 6.5. Aliquots of cell extract were incubated for 0 and 5 min at 37 °C with an equal volume of the substrate mix to yield final concentrations of 70 mM Naβ-glycerophosphate (pH 6.5), 35 mM MgCl₂, 5 mM ATP, and 2 mM IMP to measure $V_{max}$ or with a range of IMP concentrations for $K_m$ determinations. The effect of phosphate (at 1 mM ATP), ATP, and FCCP were investigated at 0.25 mM IMP, and the substrate preference was investigated at 2 mM AMP or GMP. Adenosine and inosine concentrations were measured by high pressure liquid chromatography and protein concentration by BCA assay, as described above.

Statistical Methods—Each experiment consisted of at least duplicate observations. Averaged observations for at least three independent experiments are expressed as mean ± S.E. When only two experiments were carried out, the range is given. Comparisons are made by Student’s t test using paired data if available.

RESULTS

Characterization of the cN-I and cN-II cDNA Clones—As described previously (9), the pigeon heart cN-I expressed in COS-7 cells had characteristics similar to the enzyme purified from pigeon, rat, rabbit, and human hearts. It had a preference for AMP over IMP, a $K_m$ for AMP of approximately 3 mM. Human recombinant cN-II expressed in COS-7 cells had a $K_m$ value measured in the presence of a maximum activating concentration of 5 mM ATP of 0.2 and 0.06 mM ATP. It preferred GMP to IMP to AMP (1.33:10.33 or 1.33:10.27 in two separate experiments). Removal of ATP from the assay reduced activity by 68 and 64%, whereas addition of 5 mM phosphate inhibited activity in the presence of 1 mM ATP by 98 and 99%. All of these properties are consistent with those of the purified enzymes from humans and other species (19, 29).

ATP Catabolism and Adenosine Formation in COS-7 Cells Overexpressing cN-I or cN-II—We diluted the cN-I and cN-II plasmids with control pSVβ-gal plasmid in order to achieve similar levels of 5′-nucleotidase activity in transfected cells (Table I). The specific activity of the enzymes was compared with that in control cells that had been transfected to express β-gal alone. Homogenate cN-I activity was increased 7-fold, and cN-II activity was increased 10-fold, respectively (Table I). Between 24 and 30% of the cells counted, respectively. Expression of β-gal was measured histochemically as described previously (26), as a measure of transfection efficiency.

Measurement of Nucleoside, Nucleotide, and Protein Concentrations—Addition of 0.75 volumes of 1.6 M HClO₄ was used to terminate reactions followed by neutralization of supernatants with 0.125 volumes of 3 M K₂HPO₄. Nucleosides and nucleotides were measured by reverse phase high pressure liquid chromatography as described previously (27), but performed at pH 6.7 and using a modified gradient of B (15% acetonitrile in Buffer A containing 150 mM KCl and 150 mM KH₂PO₄) changed linearly as follows: 0 min, 0%; B: 1 min, 3%; B: 3 min, 9%; B: 7 min, 100%; B: 8.5 min, 0% solution B for 3.5 min. Protein concentrations were measured in the HClO₄ pellets resuspended in 1 M NaOH using a micro-BCA assay (Pierce).

Characterization of Recombinant cN-I and -II in Cell Extracts—To measure cN-I activity, control- and cN-I-transfected cells were extracted with a buffer containing 40 mM N-dimethylglycine, 200 mM KH₂PO₄, pH 6.7, and protein concentrations were determined by using spectrophotometric absorbance measurements.

The initial concentration of ATP was 146 ± 19, 139 ± 21, and 144 ± 24 nmol mg of protein⁻¹ for β-gal-, cN-I-, and cN-II-transfected cells, respectively, and remained unchanged during incubations without metabolic poisons for up to 10 min (results not shown).

We measured the breakdown of ATP to ADP, AMP, and its further metabolites adenosine, inosine, and hypoxanthine (Fig. 1). Incubation of the cells with inhibitors of adenosine deaminase and adenosine kinase allowed the measurement of the rates of adenosine formation without further metabolism back to AMP or to inosine and hypoxanthine. To control for the effectiveness of inhibition, we measured metabolism of 2 μM exogenous adenosine. With inhibitors present, 105 ± 6% was
recovered after a 10 min incubation at 37 °C (n = 3). In the experiments with metabolic inhibitors (see below), inosine and hypoxanthine did not arise by deamination of adenosine but from metabolism of AMP to IMP by AMP deaminase followed by catabolism of IMP (Fig. 1). COS-7 cells did not contain detectable xanthine oxidase (results not shown), and hence, allopurinol was not used to inhibit further metabolism of hypoxanthine to uric acid.

We investigated ATP breakdown and adenosine formation under two conditions of metabolic inhibition. The amount of lactate dehydrogenase present in the supernatants remained unaffected and below 5% under all conditions tested. Treatment of control and cN-II-expressing COS-7 cells with 10 mM DOG + 1 μM FCCP for 10 min caused 91 ± 1 and 91 ± 1% falls in the ATP concentration after 10 min, respectively (Fig. 2a). After 10 min, 74 ± 1 and 74 ± 1%, respectively, of the fall in ATP concentration was accounted for by increased AMP and 8.6 ± 1.7 and 9.3 ± 2.1% by adenosine. Although cN-I-transfected cells showed a similar 91 ± 2% fall in ATP concentration (Fig. 2a), only 48 ± 2% was accounted for by increased AMP (p < 0.01 versus β-gal or cN-II), whereas 31 ± 3% was accounted for by increased adenosine (p < 0.02 versus β-gal or cN-II). Measurements of absolute adenosine concentration (Fig. 3a) show that adenosine accumulation in cN-I-transfected cells was nonlinear but significantly greater than in β-gal- or cN-II-transfected cells at 5 and 10 min. There was no difference in adenosine accumulation between β-gal- and cN-II-transfected cells (Fig. 3a). The concentration of IMP (the preferred substrate of cN-II) remained below 0.25 nmol/mg of protein−1 under all conditions of incubation and did not accumulate during ATP breakdown (Fig. 2a). This demonstrates that the activity of adenosine deaminase was low under these conditions. Indeed inosine and hypoxanthine, the ultimate products of AMP deamination (Fig. 1), did not accumulate either under these conditions (Fig. 3b). These data indicate that even when the competing substrate, IMP, remained at very low concen-
trations, only cN-I and not cN-II overexpression increased adenosine production.

Because cN-II is activated by ATP, we also conducted experiments under conditions of slower ATP decline. Incubation of β-gal-and cN-II-transfected COS-7 cells for 10 min in the presence of 5 mM DOG alone caused a 35 ± 4 and 34 ± 2% fall in the ATP concentration, respectively (Fig. 2b). Of this, 8.4 ± 3.3 and 11 ± 6% accumulated as AMP, and 5.3 ± 2.0 and 5.9 ± 1.7% accumulated as adenosine, respectively (Fig. 2b). Cells overexpressing cN-I also lost 35 ± 7% of their ATP and 8.3 ± 5.5% was accounted for by AMP (Fig. 2b). Adenosine accumulation accounted for 24 ± 6.3% of ATP breakdown, significantly more (p < 0.04) than in β-gal- or cN-II-transfected cells (Figs. 2b and 3c). Hence, even under these conditions, only cN-I and not cN-II increased adenosine formation (Fig. 3c).

AMP deaminase was active under these conditions, and AMP deamination proceeded mainly to inosine and hypoxanthine (Figs. 2b and 3d). Inosine and hypoxanthine accumulated to a similar extent in β-gal- and cN-II-transfected cells (Fig. 3d). As expected, cN-I overexpression caused a converse decrease in the accumulation of inosine and hypoxanthine because catalysis was diverted to adenosine (Figs. 2b and 3d). The concentration of IMP remained below 0.4 nmol/mg protein in all transfected cells under these conditions. Hence, even under these conditions, the activity of AMP deaminase was rate-limiting, and the endogenous activity of 5′-nucleotidase in all cells was therefore sufficient for production of inosine, and hypoxanthine. It was therefore impossible to measure the effect of cN-I or cN-II on catalysis of IMP in COS-7 cells.

ATP Catabolism and Adenosine Formation in H9c2 Cells Overexpressing cN-I or cN-II—In order to investigate the effect of cN-I or cN-II overexpression on IMP breakdown, it was necessary to find a cell line in which AMP deaminase was not rate-limiting. Skeletal muscle contains the highest concentration of AMP deaminase (21). We therefore chose to reexamine the effect of cN-I and cN-II overexpression in H9c2 cells, which have a mixed heart/skeletal muscle phenotype.

H9c2 cells were cotransfected with a plasmid coding for enhanced GFP, together with one for cN-I or cN-II, so as to express similar activities of 5′-nucleotidase (Table I) or with GFP alone, as control. The proportion of cells transfected, as determined by GFP fluorescence, was 20–25%. Activities of cN-I and cN-II were some 5–6-fold lower than in COS-7 cells (Table I). ATP concentrations were similar in GFP-, cN-I-, and cN-II-transfected cells (103 ± 4, 94 ± 4, and 101 ± 9 nmol/mg of protein, respectively) in the absence of metabolic inhibitors. ATP breakdown was induced either by DOG + FCCP or by DOG alone, and the same metabolites were measured as for COS-7 cells. The concentration of guanosine was also measured, as it is a potential product of 5′-nucleotidase activity. Cell viability by release of lactate dehydrogenase and propidium iodide staining was above 95% under all conditions used.

The purine metabolite concentrations are summarized in Fig. 4 and Table II. The major difference between H9c2 and COS-7 cells was that IMP clearly accumulated during ATP breakdown, especially with DOG plus FCCP, indicating that AMP deaminase activity was not rate-limiting (Fig. 4). ATP breakdown was similar in GFP-, cN-I-, and cN-II-transfected cells. Adenosine accumulation occurred in cN-I-overexpressing cells even in the absence of metabolic poisons (Table II), as previously shown in COS-7 cells (9). Under both conditions of ATP breakdown, cN-I overexpression significantly accelerated adenosine formation, as demonstrated above for COS-7 cells. However, cN-II overexpression did not increase adenosine formation under either condition of ATP breakdown (Table II). Overexpression of cN-II increased inosine and hypoxanthine accumulation in the presence of 5 mM DOG (Table II). The concentration of guanosine was also significantly increased (Table II). Both measures clearly indicate that cN-II is active under these conditions and is capable of IMP and GMP catabolism. By contrast, overexpression of cN-I did not increase inosine, hypoxanthine, or guanosine concentrations relative to GFP-transfected cells, demonstrating that it does not contribute to the metabolism of IMP under these conditions.

Overexpression of cN-II did not increase inosine, hypoxanthine, or guanosine concentrations during severe ATP depletion with DOG plus FCCP, which, together with the greater accumulation of IMP (Fig. 4), indicates that cN-II is inactive
under these conditions. Overexpression of cN-I was also without effect on IMP and GMP metabolites under these conditions.

**DISCUSSION**

Kinetic, molecular, and inhibitor studies have previously implicated cN-I in adenosine formation (9, 15, 16, 18). However, the role of cN-II in adenosine formation has remained unclear. Ours is the first study to compare directly the roles of cN-I and cN-II in AMP catabolism to adenosine in the same cell type during ATP breakdown. The major conclusion is that only cN-I and not cN-II catalyzes significant formation of adenosine under rapid or more progressive conditions of ATP depletion. This conclusion applied equally to two cell types of widely different origin, namely COS-7 or H9c2 cells. However, the lack of AMP catalysis through cN-II is all the more remarkable in COS-7 cells, given that IMP concentrations remained very low, owing to the relatively low activity of AMP deaminase, even when ATP levels were less severely depleted. Hence competition between IMP and AMP for metabolism by cN-II could not explain its inability to produce adenosine.

Our studies could not clearly establish whether cN-I or cN-II is capable of AMP catabolism in COS-7 cells, because AMP deaminase was rate-limiting under all the conditions investigated. We therefore addressed this issue in H9c2 cells, in which IMP was shown to accumulate during ATP breakdown and hence 5'-nucleotidase activity rather than AMP deaminase was rate-limiting for the pathway. In H9c2 cells, it was clear that cN-II could contribute to IMP catabolism under conditions of progressive ATP breakdown but not when ATP pools were rapidly depleted. These observations are consistent with previous in vitro and in vivo data (30, 31) showing that cN-II activity is biphasically related to energy charge. Activity of cN-II is increased during moderate falls in energy charge but strongly inhibited by profound falls. The failure of cN-I to catalyze IMP breakdown is consistent with its preference for AMP in vitro. The kinetic properties of the enzymes from pigeon, rabbit, rat, and human are remarkably similar (15, 16, 32). It is therefore unlikely that our results arise from the use of a pigeon enzyme in mammalian cells. Putative human (GenBank accession number AA446194) and mouse (GenBank accession number AA1429147) homologues exist for pigeon cN-I and show considerable protein sequence homology. Our present results further support a physiological role for cN-I in adenosine formation, which opens up the possibility of varying adenosine formation rates by manipulating the enzyme activity. Increasing cN-I activity would be expected to promote a variety of cytoprotective actions.

In contrast to cN-I, the role of cN-II appears to be restricted to IMP rather than AMP catabolism. IMP can arise by action of AMP deaminase on AMP, during ATP breakdown, but is also the product of de novo purine synthesis, under conditions of abundant ATP. Activity of cN-II may therefore be a key step controlling the balance of purine accumulation within cells and hence the upper limit of the purine nucleotide pool (33). Interestingly, work from Bianchi and co-workers (14) shows that stable cell lines overexpressing cN-II have somewhat lower total ATP and other purine trinucleotide levels. It would be interesting, in future studies, to examine the responses of these cell lines to mild and severe metabolic stresses. In the present experiments, we did not observe any effects of cN-I or cN-II overexpression on baseline ATP levels in COS-7 or H9c2. However, transient transfection occurred in only 20–30% of cells, which may confound these measurements. Overexpression of cN-I at 10-fold higher levels than used here did produce a decrease in baseline ATP concentration in COS-7 cells (9).
In conclusion, our present results clearly demonstrate that cN-I and cN-II play distinct roles in AMP and IMP catabolism, respectively.

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