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Isolation and characterization of pigeon breast muscle cytosolic 5′-nucleotidase-I (cN-I)

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5′-Nucleotidase specific towards dCMP and AMP was isolated from avian breast muscle and characterized. It was found to be similar to a type-I form (cN-I) identified earlier as the AMP-selective 5′-nucleotidase responsible for adenosine formation during ATP breakdown in transfected COS-7 cells. Expression pattern of the cN-I gene in pigeon tissues indicated breast muscle as a rich source of the transcript. We purified the enzyme from this source using two-step chromatography and obtained an active homogenous preparation, free of *ecto*-5′-nucleotidase activity. The tissue content of the activity was calculated at 0.09 U/g wet weight. The specific activity of the enzyme preparation was 4.33 U/mg protein and it preferred dCMP and AMP to dAMP and IMP as a substrate. Its kinetic properties were very similar to those of the enzyme purified earlier from heart tissue. It was strongly activated by ADP. Inhibition by inorganic phosphate was more pronounced than in heart-isolated cN-I. Despite this difference, a similar physiological function is suggested for cN-I in both types of muscle.

Keywords: 5′-nucleotidase, pigeon, skeletal muscle, tissue distribution, enzyme purification

In a living cell, ATP concentration is the primary sensor of the energetic state, and the balance between its production and consumption is critical to the fate of cells. Most ATP homeostasis regulatory mechanisms are based on feedback loops, involving ATP metabolites in the suppression of its catabolism. One such example is adenosine, produced from AMP by 5′-nucleotidases (5′-NT). Many isoforms of 5′-NT have been found in organisms ranging from bacteria through plants to vertebrates. Studies of the cellular distribution of mammalian 5′-(deoxy)nucleotidases and their sequences have led to a distinction between cytoplasmic, mitochondrial and membrane-bound (*ecto*-)forms (recently reviewed by Bianchi & Spychala, 2003). *Ecto*-5′-nucleotidases are components of signaling cascades that hydrolyze extracellular ATP to adenosine, which in turn exerts pleiotropic effects on the surrounding tissue (Zimmermann, 1992). Recent data stress the importance of the enzyme in both the neuronal system and muscle tissue (Zimmermann & Braun, 1999).

Soluble cytosolic forms of 5′-NT have not been fully characterized yet and are still a subject of much controversy. There are variants of this enzyme differing in substrate preferences, kinetic and regulatory properties and cellular localization. cN-II is an IMP-preferring form which regulates the cellular nucleotide pool, but its role in adenosine formation is less clear (Sala-Newby *et al.*, 2000). 5′-Nucleotidase-I (cN-I), earlier denominated as 'AMP-selective', first discovered and characterized in the pigeon heart (Skladanowski & Newby, 1990), is widely distributed in mammalian myocardial and endothelial tissue (Truong *et al.*, 1988; Yamazaki *et al.*, 1991; Darvish & Metting, 1993). It is believed that cN-I is responsible for the production of adenosine during the imbalance of energy supply and demand such as under ischemic or hypoxic conditions. The adenosine then produced exerts, *via* different receptors, a negative inotropic, chronotropic and dromotropic effect on the heart muscle and dilates coronary vessels (Shryock & Belardinelli, 1997). Generally speaking, it is known to act as a mediator in ischemic preconditioning, a mechanism that can prevent heart damage after a fairly long period of oxygen deficiency (for example Cross *et al.*, 2002).

Until now cN-I has been purified and characterized from the heart of many species, including rabbit (Yamazaki *et al.*, 1991), pigeon (Skladanowski

Abbreviations: AOPCP, adenosine α,β-methylenediphosphate; cN-I, cN-IA, cN-IB, cN-II, cytosolic forms of 5′-nucleotidase (types I, IA, IB, II); DMPC, dimethylpyrocarbonate; DTT, dithiothreitol; 5′-NT, 5′-nucleotidase (general); PMSF, phenylmethylsulphonyl fluoride; RT-PCR, reverse trancriptase polymerase chain reaction.

& Newby, 1996), rat (Truong *et al*., 1988), dog (Darvish & Metting, 1993) and man (Skladanowski *et al*., 1996). Using the pigeon heart, the richest known source of vertebrate cN-I, the enzyme has been cloned and sequenced (Sala-Newby *et al*., 1999). Expression profiling of pigeon tissues has shown a significant mRNA signal not only in the heart, but also in breast muscle, brain and testes (Sala-Newby *et al*., 1999). Two loci have been found in the human and mouse genome coding for cN-IA and cN-IB (Hunsucker *et al*., 2001; Sala-Newby & Newby, 2001).

Studies from numerous laboratories have presented various features of AMP metabolism and adenosine production during muscle contraction (for example see Hellsten & Frandsen, 1997). It is known that the level of adenosine production in muscles is fiber-type dependent and is higher in slow- than in fast-twitch muscles (Bockman & McKenzie, 1983; Whitlock & Terjung, 1987) perhaps due to active 5′-nucleotidase-I, the presence of which in skeletal muscular tissue, however, has never actually been confirmed. Our intention, therefore, was to purify putative cN-I 5′-nucleotidase from pigeon breast muscle and characterize its kinetic and regulatory properties. A comparison of the properties of the heart and skeletal muscle forms of the enzyme could lead to a better understanding of AMP metabolism and the role played by adenosine in these two tissues. Our hypothesis was that, because of the different functions of adenosine in heart and skeletal muscles, some of the cN-I regulatory modes must be tissue-specific. Our final aim was to compare the kinetic properties of both enzymes and to elucidate the differences in the regulation of their activity.

MATERIALS AND METHODS

Chemicals. Proteinase inhibitors, non-radioactive nucleotides except for IMP, BSA, CDP-Star were obtained from Roche Molecular Biochemicals (Switzerland). Chromatography beads and low molecular markers were from Pharmacia (Sweden). Brilliant blue, IMP, ammonium persulphate, Ficoll 400, bromophenol blue, sodium citrate were from Serva (Germany). The Reverse Transcription System and enzymes for PCR were from Fermentas (Lithuania). [2-^{3}H]AMP (18.6 μCi/mmol) was from Amersham Life Sciences (UK) and [α-^{32}P]dATP (250 Ci/mmol) from DuPont NEN (USA). Buffer components, potassium chloride, sodium chloride, magnesium chloride, sodium acetate, guanidinium thiocyanate, SDS, EDTA, PMSF, β-mercaptoethanol, ethidium bromide, glycerol, adenosine α,β-methylene-diphosphate, DTT, agarose, formaldehyde, citric acid, maleic acid, N,N-dimethylformamide, DMPC, phenol, isopropanol, chloroform, PPV, TEMED, salmon sperm and molecular weight markers for SDS/PAGE were purchased from Sigma (USA). All reagents were of the highest analytical grade available.

Purification of 5′-nucleotidase. Pigeon breast muscle and brain cN-I were purified using a modification of the previously described methods for the pigeon heart (Skladanowski & Newby, 1990) and human heart (Skladanowski *et al*., 1996) enzymes. Pigeons (*Columba livia*) of either sex from a local breeding farm were killed by decapitation. All isolation and purification steps were performed at 4°C. Breast muscles (60 g) or brains (10 g) were trimmed of fat and large vessels, cut into small pieces and homogenized in an MSE homogenizer (2 × 1 min at full speed with a 30 s break) in 7.5 volumes of buffer A containing 20 mM DMG, 0.14 M KCl, 0.1 mM DTT, 0.2 mM PMSF and proteinase inhibitors, pH 7.0. The homogenate was centrifuged at 27500 r.p.m. for 1 h and protein precipitated from the resulting supernatant with ammonium sulphate (70% saturation) during 2–3 h with continuous stirring. The protein suspension was left on ice for 2–3 h and afterwards collected in pellet form at 12000 r.p.m. (15 min). The protein precipitate was then dissolved in low-salt buffer B (1 mM EDTA, 0.1 mM DTT, 0.05 M Hepes, 0.2 mM PMSF, 25% glycerol, pH 7.0) containing a proteinase inhibitor mix. The remaining ammonium sulphate was removed on a Sephadex G-25 column equilibrated with buffer B. The salt-free protein solution was applied to a phosphocellulose P-11 column (2 × 10 cm) previously equilibrated with buffer B. The protein was eluted in 1 ml fractions with a linear gradient of 0–0.8 M NaCl. The fractions containing cN-I activity were pooled, desalted on a Sephadex G-25 column, and reapplied on a phosphocellulose P-11 column. The final product was used for the kinetic experiments. Alternatively, affinity chromatography on AMP-Sepharose 4B was performed as the last step. The column (1 × 3 cm) was then equilibrated with buffer B and the concentrated active protein eluted with a linear gradients of 0–0.4 mM ADP and 0–0.3 M NaCl. The enzyme thus obtained (4.33 units/mg protein) was used in the Western blot experiments.

Enzyme assays. To avoid enzyme inactivation, all kinetic measurements were performed in one day on freshly purified enzyme. cN-I was assayed either with AMP labeled with [2-^{3}H]AMP or with non-radioactive AMP, dAMP, IMP and dCMP as substrates. In the radiometric assay, the mixture contained 20 μl of 5× incubation buffer (final concentration: 100 mM TES, 10 mM $MgCl_2$, 30 mM NaCl, 10 mM AMP and 1 MBq of [2-^{3}H]AMP) and 60 μl of water. ADP (final concentration 1 mM) was added when needed. The reaction was initiated by adding 20 μl (13 μg) of enzyme preparation and carried out at 37°C for 10–20 min. Assays were terminated by boiling for 4 min. The product accumulation was confirmed linear with the protein concentrations and the reaction time. Af-

ter the reaction had been stopped, each sample was cooled on ice, diluted with 500 μl of deionized water and centrifuged at 12000 r.p.m. for 5 min. Aliquot of 500 μl of the supernatant was applied to anionite Poly-Prep Prefilled Chromatography Columns (Bio-Rad) and the eluate was collected. Adenosine was eluted with an additional 500 μl of water and the radioactivity determined by scintillation counting. One unit of enzyme activity was taken as the amount needed to convert 1 μmole of substrate per minute at 37°C. When unlabeled substrates were used, the reaction rate was measured either by estimation of phosphate liberated from AMP or IMP according to Itaya and Ui (1966) or by HPLC estimation of nucleosides and nucleotides. Both methods were found to give the same results. The reaction was initiated by adding 30 μl of the enzyme solution to 50 μl of the assay mixture (+/–1 mM ADP, 30 mM NaCl, 50 mM DMG, 10 mM $MgCl_2$, substrates at 10^{-6}–5 × 10^{-3} M) and allowed to proceed for 5–30 min at 37°C. The reaction was stopped either by boiling the mixture for 4 min (for inorganic phosphate estimation) or by adding 50% $HClO_4$ to the test tube (for nucleoside estimation by HPLC). Ice-cooled samples were then centrifuged at 3000 r.p.m. and the supernatant diluted 1:1 with water, then 50 μl of the resulting mixture was added to 1 ml of the Itaya–Ui reagent, and the absorbance at 660 nm measured. Alternatively, $HClO_4$-deproteinized sample was neutralized with 1.2 M K_2CO_3/30 mM EDTA and centrifuged over 15 min at 14000 r.p.m. The supernatant was filtered twice and applied to reverse phase HPLC according to Smolenski *et al*. (1990), but performed at pH 6.7 and using modified gradients of B (15% acetonitrile in buffer A containing 150 mM KCl and 150 mM KH_2PO_4) linearly changed in time: 0 min, 0% B; 0.1 min, 3% B; 3 min, 9% B; 7.5 min, 100% B; 8.5 min, 0% solvent B for 3.5 min. All assays were done in triplicate.

Data were graphed using the SigmaPlot program (version 8.0), and the kinetic parameters were derived using linear regression (Hill equation, three parameters, according to the method described by Marszalek *et al*., 1989). Protein concentration was determined according to Bradford (1976) with bovine serum albumin as standard.

SDS/PAGE and Western blot analysis. Electrophoresis was performed in 10% gel as described by Laemmli (1970). To determine the size of the proteins obtained, wide-range molecular markers (6.5–205 kDa, Sigma) or low molecular markers in the 14–94 kDa range (Pharmacia Biotech) were used. The gel was either stained with Coomassie R-250 reagent or transferred to an Immobilon P membrane. For Western blots either 1–10 μg pure enzyme or 100–150 μg of proteins from the crude extract were separated. Pigeon tissues were homogenized in a buffer containing 100 mM Hepes, pH 7.0, 10 mM $MgCl_2$, 30 mM NaCl, 0.2 mM PMSF and the proteinase inhibitor mix and centrifuged at 14000 r.p.m. for 15 min. After transfer, the membranes were incubated with anti cN-I rabbit polyclonal antibody (kindly provided by Graciela Sala-Newby from the Bristol Heart Institute, UK) at a 1:100 dilution overnight, followed by alkaline phosphatase-conjugated anti-rabbit antibody (Sigma) at 1:5000. Bands were visualized by chemiluminescence using the ECL system with CDP-Star as a substrate (Roche Molecular Biochemicals).

Northern blot analysis. Total cellular RNA was isolated from specimens of male pigeon tissues by the method of Chomczynski and Sacchi (1987). RNA was fractionated on 1.2% agarose gel containing 37% formaldehyde, transferred to a nylon membrane (Roche Molecular Biochemicals, Germany) by capillary transfer overnight at 4°C and then UV cross-linked. cDNA probes (each 200 ng) corresponding to open reading frame of either cN-I or 18S RNA (obtained from G.B. Sala-Newby, Bristol Heart Institute, UK) were labeled with 50 μCi [α-^{32}P]dATP in a random hexamer priming procedure. The reaction with 5 U of *Klenow* polymerase was carried out overnight at room temperature and was terminated by the addition of 1× SET (10 mM Tris/Cl, 1 mM EDTA, 1% SDS). The resulting mixture was then separated on a Sephadex G-50 column equilibrated with TE/NaCl buffer and eluted with 1× TES (50 mM Tris/HCl, 1 mM EDTA, 150 mM NaCl, 1% SDS). Radioactivity in the fractions was determined by scintillation counting. Membranes were prehybridized at 60°C for 2–4 h in a solution containing 5× Dernhardt's reagent, 0.5% SDS, 20× SSC (saline-sodium citrate buffer) and 10 mg/ml denatured salmon testes DNA. Hybridization was performed for 16–18 h at 60°C in a solution containing 0.5% SDS, 20× SSC and 10 mg/ml denatured salmon testes DNA with 2 × 10^6 c.p.m./ml of labeled probe. Further treatment was carried out according to the procedure described by Sala-Newby *et al*. (1999). Autoradiography on XAR-5 film was performed with an intensifying screen for 24–72 h at –70°C.

RT-PCR. The reverse transcriptase reaction was carried out using 1–2 μg of total RNA as a template with the Reverse Transcription System (Fermentas, Lithuania) and oligo(dT) as a primer, according to the producer's guide. Subsequent PCR amplification with a 1/50 part of the sample was carried out with 1 U of Taq DNA polymerase and 10 pmols of 3′- and 5′-primers (5′-GCCAAGGCTTTCTACGACAAC-3′ and 5′-GCTTCTCCTGTGCCGGCTTC-3′). The PCR scheme for cN-I was as follows: 94°C for 1 min, 60°C for 2 min and 72°C for 1 min; number of cycles: 38. The resulting products were visualized by electrophoresis on 1% agarose gel containing 0.5 μg/ml ethidium bromide. Positive control was visualized using GAPDH primer pairs,

the sequences of which have already been published (Sala-Newby *et al*., 1999).

RESULTS AND DISCUSSION

Presence of cN-I in pigeon tissues

Previous experiments by Sala-Newby and coworkers (1999) have shown diverse cN-I expression in pigeon tissues such as heart, breast muscle and brain. We isolated RNA from a broader panel of tissues: *musculus gastrocnemius, musculus biceps brachii*, breast muscle, heart, brain, testis, liver and kidney.

The data presented in Fig. 1 show cN-I gene expression in these pigeon tissues. It can be seen that heart expressed a very high level of the approx. 2.3 kb cN-I transcript, while a slightly lower level of expression was found in breast muscle and brain (Fig. 1A). Upon overexposure, expression could also be seen in testis and in *m. gastrocnemius*. Specific mRNA was not observed for *m. biceps brachii*, liver or kidney. Northern blot results were confirmed by RT-PCR analysis. RT-PCR products of the correct size (1000 bp, corresponding to the open reading frame of cN-I cDNA) were obtained from all the pigeon tissues tested except for *m. biceps brachii*, liver and kidney (Fig. 1B). The reaction leading to overproduction of *GAPDH* fragments was taken to be the control. The cN-I gene fragment of 1000 bp (provided by G.B. Sala-Newby from the Bristol Heart Institute, UK) served as a molecular mass standard. The presence of specific protein was found to correspond with mRNA levels. Figure 1C shows that the antibodies used for Western blotting detect the cN-I 43 kDa product in tissue extracts from heart, breast muscle, *m. gastrocnemius*, brain and testis.

Our interest focused on how the relatively high expression of cN-I in breast and *gastrocnemius* muscle contributes to adenosine production in these tissues. Both are classic examples of slow-twitch oxidative muscles in pigeon, where oxidative metabolism dominates over the glycolytic pathway, whereas *m. biceps brachii* is mostly composed of fast-twitch glycolytic muscle fibers (Hellsten & Frandsen, 1997). The level of mRNA expression was much lower in *m. biceps brachii* than in *m. gastrocnemius* and breast muscle. There is good evidence in the literature that slow-twitch oxidative muscle fibers tend to produce more adenosine as a result of AMP dephosphorylation (Bockman & McKenzie, 1983). It is believed that most of the metabolite is produced as a result of the extracellular ATP degradation cascade in which *ecto*-5′-nucleotidase takes part (Whitlock & Terjung, 1987). Although the role of 5′-nucleotidase-I in AMP metabolism in muscles has not been studied in detail, it is reasonable to assume that at least part of the adenosine is produced as a result of the action of this enzyme.

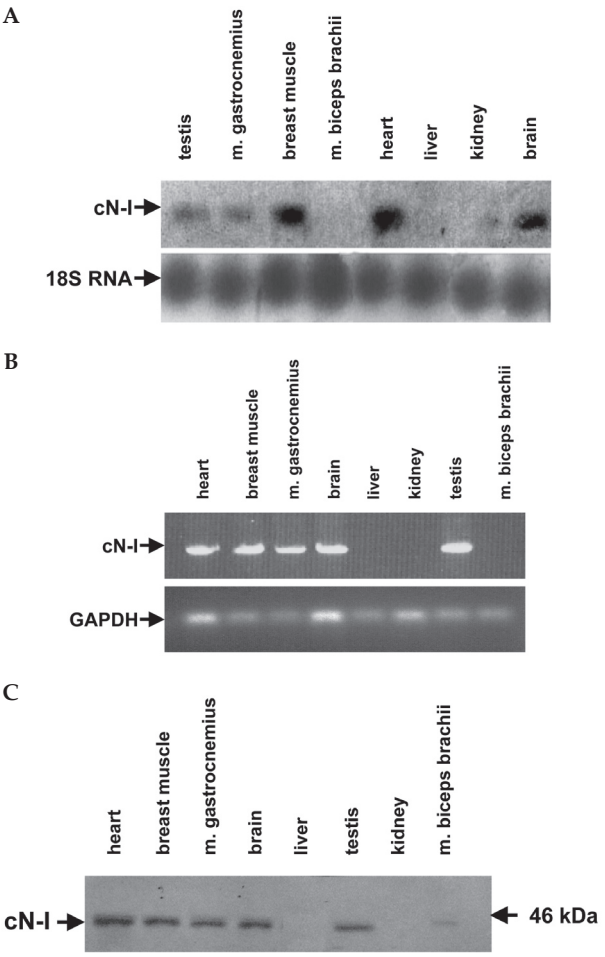

Figure 1. Expression of cN-I in various pigeon tissues. **A.** Northern blot analyses were performed with 20 μg of total RNA isolated from pigeon tissue. Hybridization was with 200 ng of either cN-I (upper panel) or 18S RNA cDNA probe (lower panel) labeled with 50 μCi [α-^{32}P]dATP. Membranes were exposed against XAR-5 film as in Materials and Methods. Lanes: 1, testis; 2, *musculus gastrocnemius*; 3, breast muscle; 4, *biceps brachii*; 5, heart; 6, liver; 7, kidney; 8, brain. **B.** RT-PCR analysis. 1% Agarose gel showing bands for cN-I (upper panel) and for *GAPDH* in corresponding tissues (lower panel). Lanes: 1, heart; 2, breast muscle; 3, *musculus gastrocnemius*; 4, brain; 5, liver; 6, kidney; 7, testis; 8, *biceps brachii*. **C.** Western blot analysis of cN-I protein distribution. Tissue extracts were fractionated on 10% polyacrylamide gel at 150 μg protein per lane, transferred onto Immobilon P membrane and detected with anti-cN-I antibody. Lanes: 1, heart; 2, breast muscle; 3, *musculus gastrocnemius*; 4, brain; 5, liver; 6, testis; 7, kidney; 8, *musculus biceps brachii*.

Interestingly, the enzyme from skeletal muscle has never been purified or studied before. In order to verify the possible role of 5′-nucleotidase-I in the production of adenosine in this tissue, we chose breast muscle as an abundant source of cN-I for studying the basic kinetic and regulatory properties of the purified enzyme.

Purification of pigeon breast cN-I

Pigeon breast muscle 5′-nucleotidase was purified from fresh tissue homogenate by ammonium sulphate precipitation followed by either doubled phosphocellulose or by consecutive phosphocellulose and AMP-Sepharose 4B chromatographies. The purification was performed according to the method published for pigeon (Skladanowski & Newby, 1990) and human (Skladanowski *et al*., 1996) heart with few modifications. They included: (i) desalting on a Sephadex G-25 column instead of dialysis that led to a dramatic loss of activity, and (ii) elution from AMP-Sepharose 4B with a two-component-gradient in which NaCl and ADP concentrations increased in parallel. A typical purification procedure is presented in Table 1.

The first step yielded a protein that eluted as a single peak at 0.4–0.5 M NaCl and had a specific activity of 0.6 units/mg protein (Fig. 2A). Rechromatography yielded an unchanged elution profile (not shown) and the specific activity reached 1.42 units/mg protein. An accompanying faint band in SDS/PAGE is still visible in addition to the main one. Replacement of the second phosphocellulose column by affinity chromatography on AMP-Sepharose 4B caused a greater increase in specific activity and raised the purification rate with an acceptable yield (Fig. 2B). SDS/PAGE analysis of samples from each step is depicted on Fig. 2C and shows an apparently homogenous enzyme preparation of 43 kDa from the last step. The molecular size was found to be the same as for the other species of purified cN-I from heart tissue (Truong *et al*., 1988; Skladanowski & Newby, 1990; Yamazaki *et al*., 1991; Darvish & Metting, 1993). Purification of the breast muscle enzyme provided a pure, active, albeit low-stability protein. The preparation appeared to be free of AMP-deaminase activity, which was removed on the phosphocellulose P-11 column (not shown). This step produced a different elution profile than that for human myocardial 5′-nucleotidase, where two activities, the second one at a higher ionic strength being the cN-II form, were observed (Skladanowski *et al*., 1996).

Substrate preferences and kinetic parameters

To exclude the probable cross-reactivity with *ecto*-5′-nucleotidase, very abundant in skeletal muscle, we checked the impact of its specific inhibitor, AOPCP (adenosine α,β-methylenediphosphate). The effect measured in the presence of 10 mM AMP as a substrate, without ADP but with 100 μM AOPCP, a concentration which completely inhibits *ecto*-5′-nucleotidase, was a depletion of the cN-I reaction rate only by 30% (not shown). This might be caused by contamination with *ecto*-form but this is low proba-

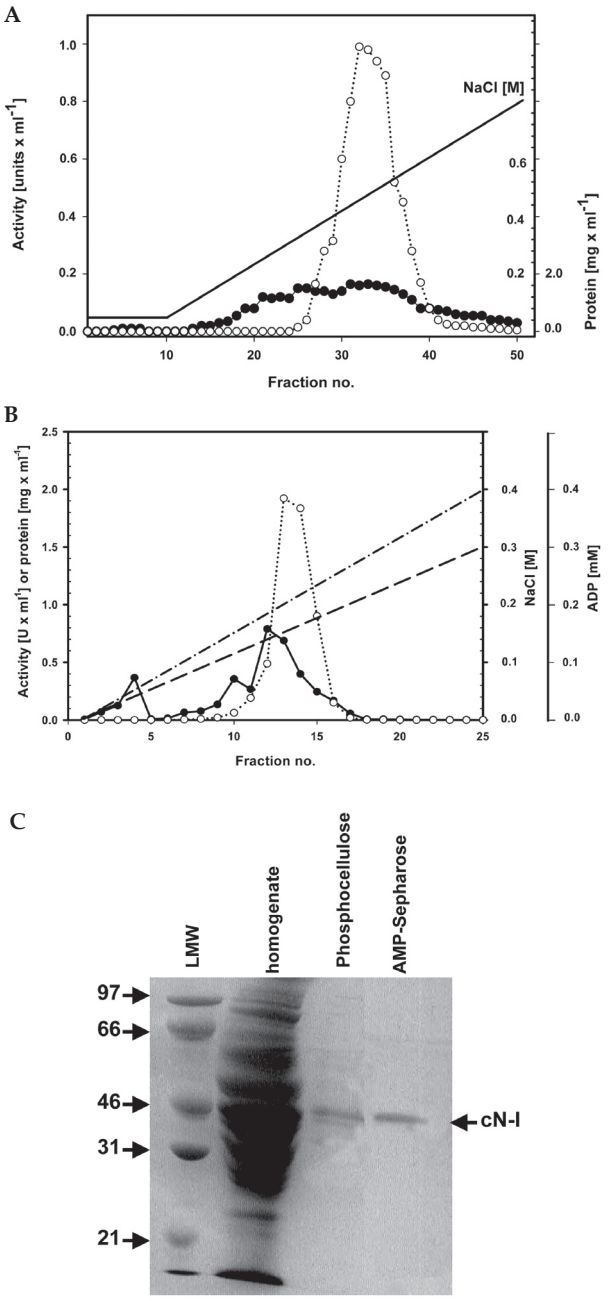

Figure 2. Purification of pigeon breast muscle 5′-nucleotidase-I (cN-I).
A. Phosphocellulose P-11 chromatography. Desalted $(NH_4)_2SO_4$-precipitate was loaded onto a phosphocellulose P-11 column previously equilibrated with buffer B. Protein (closed circles) was eluted with a linear gradient of 0–0.8 M NaCl (diagonal solid line) in buffer B. Fractions of 1 ml were collected. cN-I activity (open circles) was detected as a single peak and pooled. **B.** AMP-Sepharose 4B chromatography of pooled fractions from phosphocellulose P-11. The column was equilibrated with buffer B and the protein (solid line) was eluted with a linear gradient of 0–0.4 mM ADP (dash-dotted line) and 0–0.3 M NaCl (long-dashed line). cN-I activity (dotted line) was found as a single peak and pooled. **C.** SDS/PAGE patterns of pigeon breast muscle cN-I at various stages of purification. Lanes: 1, low molecular markers; 2, crude homogenate; 3, pooled active fractions after phosphocellulose P-11 chromatography; 4, enzyme purified on AMP-Sepharose 4B.

Table 1. Purification of 5′-nucleotidase-I (cN-I) from pigeon breast muscle

Stage	Total protein [mg]	Activity [U/ml]	Specific activity [U/mg]	Purification fold	Yield [%]
Homogenate	127	0.012	0.033	1	100
Ammonium phosphate precipitate	51	1.40	0.082	2.5	99.8
Phosphocellulose pool	6.3	0.38	0.60	18	90.2
AMP-Sepharose 4B pool	0.2	0.52	4.33	131	20.6

The results are representative of five similar experiments. Sixty grams of breast muscle was taken for each purification.

ble in view of apparent homogeneity in the SDS gel. We rather suggest that AOPCP can compete for the ADP-binding site and remove traces of ADP derived from the elution gradient.

Figure 3 shows the AMP-saturation curve for pigeon breast muscle 5′-nucleotidase in the presence and absence of 1 mM ADP. Such an ADP concentration has been widely reported as being optimal for the activation of cN-I (Skladanowski & Newby, 1990; Yamazaki *et al*., 1991; Darvish & Metting, 1993). The half-saturation value and the maximum velocity were calculated using nonlinear regression method (Marszalek *et al*., 1989) at 0.29 ± 0.03 mM AMP and 331 ± 15 mU/mg enzyme. The lack of ADP brought about a significant decrease in the reaction rate and a different curve shape. This change of shape from hyperbolic to sigmoidal in the absence of ADP indicates that this compound functions as an allosteric activator of cN-I.

A list of kinetic parameters of the pigeon breast muscle enzyme is presented in Table 2. Similarly as with recombined human cN-I (see Hunsucker *et al*., 2001) but not as distinctly, it shows high affinity towards dCMP which exceeds even that for AMP. Assessing by the ratio $V_{max}/K_{0.5}$, the preference for AMP dephosphorylation over IMP dephosphorylation is clear. A high AMP/IMP ratio, calculated here for 15, seems to be a common feature for 5′-nucleotidases-I purified from various sources. The high AMP/dAMP ratio matches the previous. The high affinity towards dCMP (apparent $K_{0.5}$ equal to 0.04 mM) suggests an important role in the regulation of physiological cytidine nucleotide/nucleoside pool. As this feature has been found for the breast muscle enzyme but not for the heart isoform, it might be then muscle fiber type-specific.

Inorganic phosphate can inhibit the enzymatic activity of 5′-nucleotidases isolated from various sources (Truong *et al*., 1988; Skladanowski & Newby, 1990; Yamazaki *et al*., 1991). Figure 4 shows the inhibitory effect of ortophosphate on the reaction rate with 1 mM AMP. Half-millimolar P_i decreased the reaction rate almost threefold, with no further decrease at higher concentrations. It seems that breast muscle cN-I is much more susceptible to inhibition by phosphate than its heart analogue (apparent K_i equal to about 20 mM; Skladanowski & Newby, 1990), and P_i is not a competitive but rather an allosteric type of inhibitor.

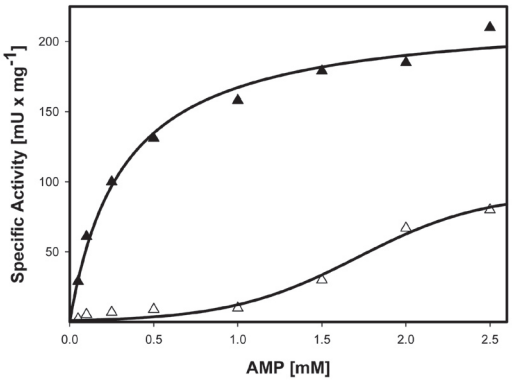

Figure 3. Influence of ADP on AMP dephosphorylation by pigeon breast muscle cN-I.
The reaction mixtures contained 100 mM TES, 10 mM $MgCl_2$, 30 mM NaCl, pH 7.0, various concentrations of AMP labeled with [2-^{3}H]AMP and the enzyme purified on phosphocellulose column. The reactions were conducted in the presence (full triangles) or absence (empty triangles) of 1 mM ADP for 10 min at 37°C and the quantity of [2-^{3}H]adenosine formed was measured by scintillation counting. Each point represents the mean for triplicate incubations. The curves were generated by a non-linear regression program.

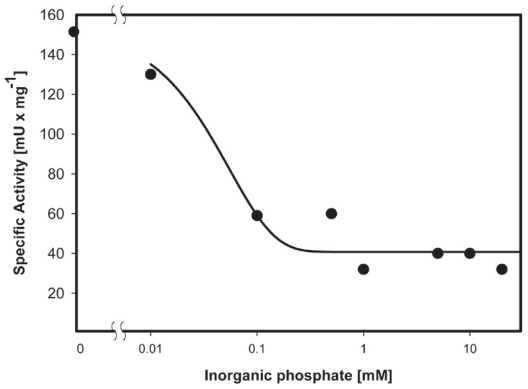

Figure 4. Inhibition by inorganic phosphate of AMP dephosphorylation catalyzed by pigeon breast muscle cN-I.
The reaction rates were measured in a mixture containing 100 mM TES, pH 7.0, 10 mM $MgCl_2$, 30 mM NaCl, 1 mM AMP labeled with [2-^{3}H]AMP, various concentration of phosphate and the enzyme purified on phosphocellulose column. Points represent averages for two parallel experiments. The curve was generated by a non-linear regression program.

Table 2. Substrate specificity of 5′-nucleotidase-I (cN-I) isolated from pigeon breast muscle.

Substrate	ADP [mM]	V_{max} [mU/mg]	Relative V_{max} [%]	Apparent $K_{0.5}$ [mM]	$V_{max}/K_{0.5}$
AMP	1	331 ± 15	100	0.29 ± 0.03	1141
	0	139 ± 35	42	1.85 ± 0.46	
dAMP	1	218 ± 29	66	5.2 ± 1.7	42
	0	30 ± 10	9	23.0 ± 6.0	
IMP	1	126 ± 9	38	1.67 ± 0.15	75
	0	20 ± 2	6	10.8 ± 1.1	
dCMP	1	63 ± 1	16	0.04 ± 0.00	1575
	0	10 ± 1	3	0.75 ± 0.07	

The V_{max} and apparent $K_{0.5}$ (defined as the concentration at half-maximum rate) of cN-I for AMP, dAMP, IMP and dCMP were calculated using non-linear regression (Marszalek *et al.*, 1989) for velocities measured at varying concentrations of substrate both in the presence and absence of ADP. Values are presented as means ± S.E. for three independent experimental sets.

Summarizing the regulatory characteristics of the 5′-nucleotidase obtained, like most of the cN-I characterized so far, it shows potent inhibition by inorganic phosphate (Fig. 4) and activation by 1 mM ADP (Fig. 3 and Table 2). It is absolutely magnesium-dependent, its residual activity in magnesium deprived medium being less than 5% (not shown). This is a widely reported feature of cN-I (Truong *et al*., 1988; Skladanowski & Newby, 1990; Yamazaki *et al.*, 1991; Darvish & Metting, 1993; Skladanowski *et al*., 1996; Hunsucker *et al*., 2001; Sala-Newby *et al*., 2003).

CONCLUDING REMARKS

The aim of this study was to identify the type of 5′-nucleotidase present in the cytosol of the main avian muscle — breast – and describe its regulatory properties in comparison to the form isolated from pigeon heart. The immunologic properties and substrate preferences of isolated enzyme allowed us to determine it as the cN-I type also named "AMP-selective". The recently discovered pronounced affinity of recombined human cN-I towards pyrimidine nucleotides and deoxynucleotides (Hunsucker *et al.*, 2001) has been confirmed by us for the avian breast muscle cN-I. The outstandingly high sensitivity towards inorganic phosphate might suggest that at a normal physiological condition (about 0.5 mM P_i, 20–30 μM ADP) the activity of breast muscle cN-I is suppressed and an increase of the activatory ADP is necessary to reverse this situation. The physiological significance of heart cN-I in adenosine production during ATP breakdown (Sala-Newby *et al*., 2003) would then still be likely. An involvement in the regulation of the physiological pyrimidine (deoxy)nucleotide pool is probable. Most reports claim, however, that *ecto*-5′-nucleotidase is the predominant adenosine producer in skeletal muscle, as it is responsible for 85–95% of total adenosine production (Cheng *et al*., 2000). Our results undermine this claim and may be of assistance in designing an experimental model which will enable estimation of the relative role of these two 5′-nucleotidases in producing adenosine in the skeletal muscle.

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