Biosci. Rep. (2008) / **28** / 267–273 (Printed in Great Britain) / doi 10.1042/BSR20070039

Original Paper

Bioscience Reports

AMP hydrolysis in soluble and microsomal rat cardiac cell fractions: kinetic characterization and molecular identification of 5′-nucleotidase

Daniela POCHMANN*[1], Adrine M. INNOCENTE*, Guilherme COTOMACCI†, Maria Luiza M. BARRETO-CHAVES† and João J. F. SARKIS*

*Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Porto Alegre, Rua Ramiro Barcelos, 2600 – ANEXO, 90035-003, Porto Alegre, RS, Brazil, and †Departamento de Anatomia, Instituto de Ciências Biomédicas, Universidade de São Paulo, Av. Prof. Lineu Prestes, 2145, 05508-900, São Paulo, SP, Brazil

Synopsis

The present study describes the enzymatic properties and molecular identification of 5′-nucleotidase in soluble and microsomal fractions from rat cardiac ventricles. Using AMP as a substrate, the results showed that the cation and the concentration required for maximal activity in the two fractions was magnesium at a final concentration of 1 mM. The pH optimum for both fractions was 9.5. The apparent K_m (Michaelis constant) values calculated from the Eadie–Hofstee plot were 59.7 ± 10.4 μM and 134.8 ± 32.1 μM, with V_{max} values of 6.7 ± 0.4 and 143.8 ± 23.8 nmol P_i/min/mg of protein (means ± S.D., $n = 4$) from soluble and microsomal fractions respectively. Western blotting analysis of ecto-5′-nucleotidase revealed a 70 kDa protein in both fractions, with the major proportion present in the microsomal fraction. The presence of these enzymes in the heart probably has a physiological function in adenosine signalling. Furthermore, the presence of ecto-5′-nucleotidase in the microsomal fraction could have a role in the modulation of the excitation–contraction-coupling process through involvement of the Ca^{2+} influx into the sarcoplasmic reticulum. The measurement of maximal enzyme activities in the two fractions highlights the potential capacity of the different pathways of purine metabolism in the heart.

Key words: adenosine, AMP hydrolysis, ecto-5′-nucleotidase, heart, microsome, soluble 5′-nucleotidase

Bioscience Reports

INTRODUCTION

Adenosine and adenine nucleotides (ATP, ADP and AMP) are signalling molecules that exert multiple biological actions in the heart and other tissues [1–5]. The first study on the effects of these purines on the cardiovascular system demonstrated that adenosine and AMP induced a decrease in heart rate, arterial dilatation, lowering of blood pressure and inhibition of intestinal contraction [6]. It is now clear that purines exert potent and diverse effects on the cardiovascular system and are involved, principally, in cardioprotection [7,8]. In the heart, adenosine not only plays a role in regulating growth and differentiation, angiogenesis, coronary blood flow, cardiac conduction and heart rate, substrate metabolism and sensitivity to adrenergic stimulation, but may also play a role as an endogenous determinant of ischaemic tolerance [7–10]. From a therapeutic viewpoint, adenosine-based therapies protect against ischaemic injury in a variety of animal models as well as in human cardiac tissue [2,8,9]. The effects of adenosine are mediated through the binding to P1 receptors that are expressed in the plasma membrane of cells in a variety of tissues [2,5]. Different pathophysiological processes increase the level of adenosine in the extracellular medium, and one of the major pathways of adenosine formation is the enzymatic dephosphorylation of AMP by 5′-nucleotidase both intracellularly and extracellularly [2,3,11,12].

5′-Nucleotidases are a group of enzymes that catalyse, with different specifities, the dephosphorylation of extracellular monophosphate nucleotides, resulting in the release of their corresponding nucleosides. Widely distributed in bacteria, plant cells and vertebrate tissues, 5′-nucleotidases are classified according to their cellular location and biochemical properties: five

Abbreviations used: TBST, Tris-buffered saline with Tween 20; TCA, trichloroacetic acid.

[1]To whom correspondence should be addressed (email danipochmann@hotmail.com).

cytoplasmic forms, one in the mitochondrial matrix and a membrane-anchored ecto-5′-nucleotidase (also known as CD73, EC 3.1.3.5), [13]. All members of this family (except for ecto-5′-nucleotidase) are absolutely dependent on Mg^{2+} for their maximal activity [13]. The molecular mass, apparent K_m and pH of 5′-nucleotidases are variable and depend on the type of 5′-nucleotidase [11,13].

The physiological function of 5′-nucleotidases probably differs in various organisms and tissues, and possibly extends beyond its catalytic activity. Ecto-5′-nucleotidase anchored to the plasma membrane by GPI (glycosylphosphatidylinositol) is involved in cell–cell and cell–matrix interactions and in transmembrane signalling, as well as in the control of cell growth, maturation and differentiation processes [11,13]. Acting with the E-NTPDases (ecto-NTP diphosphohydrolases), which catalyse ATP and ADP hydrolysis, the activity of 5′-nucleotidases controls the intracellular and extracellular levels of AMP and adenosine. Seven human 5′-nucleotidases with different subcellular localization have been cloned, suggesting that these enzymes perform important metabolic functions [13]. Furthermore, alterations in 5′-nucleotidase levels have been observed in a considerable number of diseases [11,13].

Although the actions of adenosine and adenine nucleotides have been characterized in great detail, the contribution of the different subcellular fragments to cardiac adenosine production are not well understood to date. A study by Kiviluoma et al. [14] on heart muscle demonstrated that cytosol, lysosomes and plasma membrane were particularly rich in 5′-nucleotidase activity, whereas mitochondria and sarcoplasmic reticulum were devoid of activity. A mitochondrial 5′-nucleotidase has also been cloned and characterized previously [13].

In the present study, the aim was to demonstrate 5′-nucleotidase activity in the microsomal fraction from rat cardiac cells, which is derived from sarcoplasmic reticulum. Furthermore, we describe the characteristics of 5′-nucleotidase in a soluble fraction, investigate the biochemical and kinetic properties and demonstrate the presence of this enzyme in both fractions.

MATERIALS AND METHODS

Chemicals

AMP, EDTA, Trizma base, levamisole, tetramisole, aprotinin, leupeptin and pepstatin were obtained from Sigma–Aldrich. PMSF was obtained from Gibco. Nitrocellulose membrane was from Bio-Rad. A polyclonal antibody against ecto-5′-nucleotidase (SC-14684) was purchased from Santa Cruz Biotechnology. A horseradish-peroxidase-conjugated goat anti-IgG secondary antibody was from Jackson ImmunoResearch. ECL® (enhanced chemiluminescence) detection reagents were from Amersham Biosciences. X-ray film (T-MAT G/RA Film) was purchased from Kodak. All other reagents were of analytical grade.

Animals

Male Wistar rats weighing 200–280 g were used in the present study. All the animals were housed in cages with food and water available *ad libitum*. They were maintained under a 12 h light/12 h dark cycle at a constant temperature of $23 \pm 2^{\circ}C$. Procedures for the care and use of animals were performed according to the regulations of COBEA (Colégio Brasileiro de Experimentação Animal), based on the Guide for the Care and Use of Laboratory Animals (National Institutes of Health).

Isolation of cardiac soluble and microsomal fractions

Rats were killed by decapitation, the hearts were carefully removed and the ventricles were isolated. The fractions were prepared as described previously [15], with minor modifications. Briefly, cardiac ventricles of two animals were minced and homogenized for 3 min in 1:23 (w/v) of 0.25 M sucrose/10 mM Tris/HCl (pH 7.4), using a tissue homogenizer (Sorvall Omni-Mixer 17105, setting 4). The homogenate was centrifuged at 10000 ***g*** for 30 min. The pellet was discarded and the supernatant was centrifuged at 21400 rev./min for 60 min (Hitachi P28S rotor). The supernatant obtained represented the soluble fraction, whereas the pellet, resuspended in homogenization buffer, represented the microsomal fraction. The soluble and microsomal fractions were prepared fresh daily and kept at $4^{\circ}C$ throughout the process.

5′-Nucleotidase assay

Unless otherwise stated, enzyme activity was determined in incubation medium [50 mM glycine buffer (pH 9.5) and 1 mM $MgCl_2$] in a final volume of 200 μl. Soluble and microsomal proteins (90 μg and 50 μg respectively) were added to the reaction medium and pre-incubated for 10 min at $37^{\circ}C$. The reaction was started by the addition of AMP to a final concentration of 2 mM. After 40 min incubation (soluble fraction) or 10 min incubation (microsomal fraction), the reactions were stopped by the addition of 200 μl of 10% (v/v) TCA (trichloroacetic acid). The samples were chilled on ice and the P_i released was measured following the method of Chan et al. [16]. For all enzyme assays, incubation times and protein concentrations were chosen to ensure the linearity of the reactions. All experiments were performed in triplicate. Controls with the enzyme preparation added after mixing with TCA were used to correct for non-enzymatic substrate hydrolysis. Enzyme activity is expressed as nmol P_i released/min/mg of protein. For analysis of pH dependence, AMP hydrolysis was determined as described above, except that the glycine buffer was substituted for a buffer solution reaction mixture containing 50 mM Tris, 50 mM Hepes and 50 mM glycine buffers (pH 6–10.5).

Western blotting analysis

After isolation of cardiac subcellular fractions, a solution containing protease inhibitors (5 mM aprotinin, 1 mM leupeptin, 1 mM pepstatin and 100 mM PMSF) was added (1:30 dilution) to maintain the sample integrity. Total protein (75 μg) was resolved by SDS/PAGE (15% gels), and the resolved proteins were transferred on to a nitrocellulose membrane. The membrane was stained with Ponceau solution to demonstrate that the protein

concentration was similar in the different lanes. The membrane was then washed with TBST [Tris-buffered saline with Tween 20; 50 mM Tris/HCl (pH 7.5), 150 mM NaCl and 2% (v/v) Tween 20] for 20 min at room temperature (23 ± 2°C). After this, the membrane was incubated at 37°C for 2 h with a polyclonal anti-ecto-5′-nucleotidase antibody (1:500 dilution) in TBST with 1% non-fat dried skimmed milk powder. After washing the membrane, a horseradish-peroxidase-conjugated-goat anti-IgG antibody (1:1000 dilution) was incubated with the membrane for 1 h at room temperature. The membrane was washed again with TBST and incubated with ECL detection reagents, which produced a chemiluminescence signal that was detected by exposure to X-ray film. The protein bands were quantified by densitometry and the band density was then calculated and expressed as arbitrary units (Gel Pro Imager; Media Cybernetics).

Protein determination

Protein concentrations were measured by the Coomassie Blue method of Bradford [17], using bovine serum albumin as a standard.

RESULTS

Time course and protein concentration

We first investigated AMP hydrolysis in soluble and microsomal fractions from rat cardiac cells as a function of time and protein concentration in order to determine the optimum assay conditions. The fractions were incubated as described in the Materials and methods section. The results indicated that the time course of AMP hydrolysis was linear up to 50 min (for the soluble fraction) and up to 15 min (for the microsomal fraction) (results not shown). In order to ensure that the incubation time was within the linear portion of the reaction, we choose 40 min and 10 min as the assay times for the soluble and microsomal fractions respectively.

With regards to protein concentration, the results demonstrated that AMP hydrolysis was linear up to 110 μg (for the soluble fraction) and up to 70 μg (for the microsomal fraction) (results not shown). Thus in the subsequent experiments we used 90 μg of the soluble protein fraction and 50 μg of the microsomal protein fraction.

Cation dependence

To further optimize assay conditions, the effects of different Ca^{2+} and Mg^{2+} concentrations on AMP hydrolysis were investigated. In both fractions, the results showed a gradual decrease in enzyme activity in the presence of different concentrations of Ca^{2+} when compared with the respective control groups [3.51 ± 0.446 nmol P_i/min/mg of protein (mean ± S.D., $n = 4$) from the soluble fraction and 105.29 ± 8.43 nmol P_i/min/mg of protein (mean ± S.D., $n = 3$) from the microsomal fraction]. A significant increase in AMP hydrolysis was observed with all concentrations of Mg^{2+} tested when compared with the respective control groups, and the pattern was similar for both fractions (Figure 1). In subsequent experiments, a final concentration of 1 mM $MgCl_2$ was used.

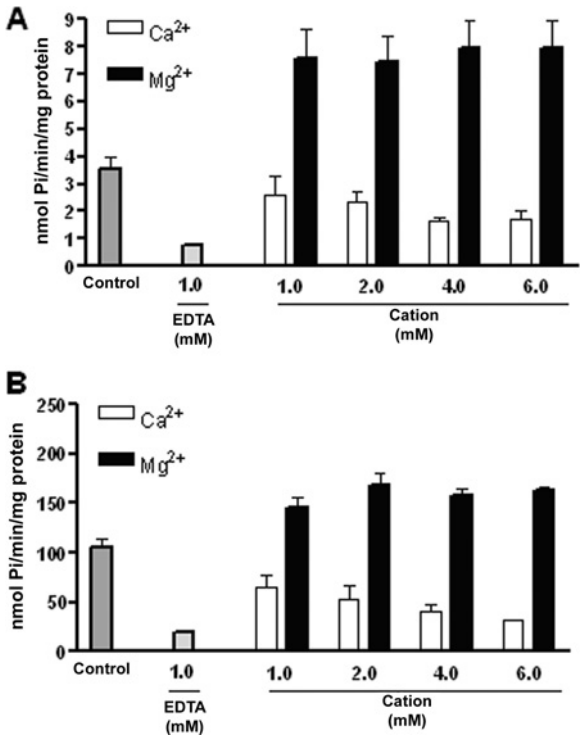

Figure 1 Dependence of AMP hydrolysis on bivalent cations

Hydrolysis of AMP by soluble (A) and microsomal (B) rat cardiac cell fractions was analysed in the absence of cations (Control), in the presence of EDTA (1 mM) and in the presence of 1–6 mM Ca^{2+} (white bars) or Mg^{2+} (black bars). Results are expressed as nmol P_i/min/mg of protein and are means ± S.D. ($n \geqslant 3$).

An activity of approx. 46% and 72% of the total measurable activity in the presence of 1 mM of Mg^{2+} from soluble and microsomal fractions respectively, was still seen in the absence of added bivalent cations. However, this endogenous activity could be removed almost completely by the addition of 1 mM EDTA. This result indicates the presence of endogenous bivalent cations in the soluble and microsomal fractions and the dependence on cations for enzyme activity in both fractions.

Effect of pH

The optimum pH value for AMP hydrolysis was determined in a mixture containing 50 mM Tris, 50 mM Hepes and 50 mM glycine buffers (pH 6–10.5). A parallel profile was obtained for both fractions and the maximal rate of AMP hydrolysis was observed at pH 9.5 (Figure 2). In parallel experiments, we used 1 mM levamisole or tetramisole, classical alkaline phosphatase inhibitors, to exclude the effect of alkaline phosphatase on AMP hydrolysis. The inhibitors studied did not have any effect on AMP hydrolysis, excluding the participation

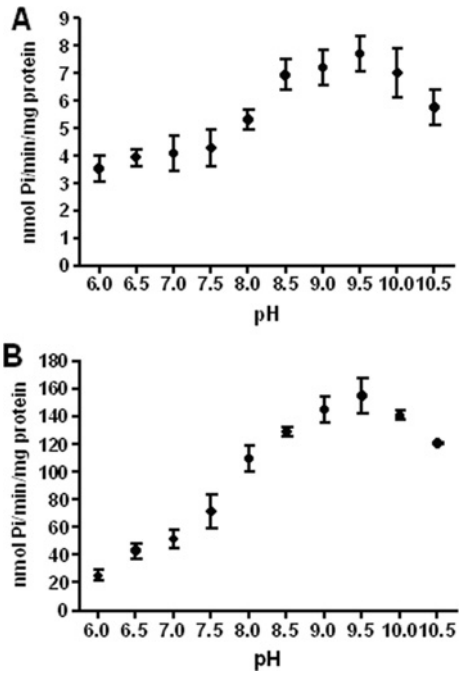

Figure 2 Effect of pH on AMP hydrolysis in soluble (A) and microsomal (B) rat cardiac cell fractions

Enzyme activity was determined as described in the Materials and methods section using a mixture of Tris, Hepes and glycine buffers (pH 6–10.5). Results are expressed as nmol P_i/min/mg of protein and are means ± S.D. ($n = 3$).

of alkaline phosphatase in substrate hydrolysis (results not shown).

Kinetic constants

AMP hydrolysis in the cardiac soluble fraction was determined at different concentrations of AMP (10–2000 μM). For the microsomal fraction, AMP hydrolysis was determined at various concentrations of AMP (75–2000 μM). The results (insets in Figure 3) indicated that enzyme activity increased with increasing concentrations of nucleotide. The Michaelis constant (K_m) and V_{max} values were estimated from the Eadie–Hofstee plot (Figure 3). The apparent K_m values calculated from this plot were 59.7 ± 10.4 μM and 134.8 ± 32.1 μM, with V_{max} values of 6.7 ± 0.4 nmol P_i/min/mg of protein and 143.8 ± 23.8 nmol P_i/min/mg of protein for soluble and microsomal fractions respectively (means ± S.D., $n = 4$).

Western blotting analysis

A polyclonal antibody against ecto-5′-nucleotidase was used for Western blotting analysis. As seen in Figure 4, the antibody reacted specifically with a protein with a molecular mass of approx. 70 kDa in both soluble and microsomal rat cardiac cell fractions. The band densities were quantified by densitometry and expressed as arbitrary units. The microsomal fraction contains a large quantity of ecto-5′-nucleotidase (approx. 143.72%) in comparison with the soluble fraction.

DISCUSSION

Adenine nucleotides and adenosine are continually present in variable amounts in both the intracellular and extracellular spaces of the heart. Adenosine is the final product of the breakdown of ATP, ADP and AMP by the ectonucleotidase cascade. This nucleoside has an important role in the modulation of cardiac functions and its possible cardioprotective effects have been emphasized [1–3,5,7,9]. In the present study, we demonstrate the kinetic and biochemical properties of a 5′-nucleotidase with respect to AMP hydrolysis in soluble and microsomal fractions from rat cardiac cells. At the same time we identified and quantified the presence of ecto-5′-nucleotidase in these fractions.

It is known that Mg^{2+} optimizes assay conditions for a great number of 5′-nucleotidases [11,13] and, in the present study, both the soluble and microsomal fractions presented an elevation in AMP hydrolysis in the presence of this bivalent cation. The Mg^{2+} concentration required for maximal activity was near to the millimolar range for the two fractions. On the other hand, the addition of calcium decreased AMP hydrolysis when compared with the control group without cation addition. When EDTA (1 mM), a metal chelator, was added to the reaction mixture, this resulted in a decrease in the 5′-nucleotidase activity in both fractions, demonstrating that these enzymes are dependent on bivalent cations for activity.

The optimum pH for 5′-nucleotidases is variable and depends on the location of the enzyme. Generally, this pH is in the range of pH 6–8 [11]. In the present study, using a mixture of Tris, Hepes and glycine buffers, we observed the greatest enzyme activity at pH 9.5 for both fractions. A study by Naito and Lowenstein [18] with purified soluble 5′-nucleotidase from rat heart demonstrated the complex behaviour of this enzyme in the presence of various buffers with a pH above pH 7.5. They suggest that some buffers, such as glycine buffers, interact with the enzyme, possibly by interacting with a metal ion bound to the enzyme. Furthermore, they demonstrated that Mg^{2+} ions modify this behaviour, since they activate the enzyme above pH 8 in the presence of some buffers. They also observed that in the absence of Mg^{2+} ions, glycine buffer yields an optimum pH at 9.5 and the activity of 5′-nucleotidase in this buffer was increased approx. twice by the addition of 10 mM Mg^{2+} ions. The optimum pH values observed in the results of the present study with a buffering system containing glycine in the presence of Mg^{2+} are in accordance with the previous study, which described pH values for heart purified 5′-nucleotidase [18]. Furthermore, we excluded the participation of alkaline phosphatases because the classical alkaline phosphatase inhibitors, levamisole and tetramisole, did not have any effect on AMP hydrolysis at pH 9.5 (results not shown).

The comparison of enzyme activities in cardiac soluble and microsomal fractions showed different kinetic properties. The 5′-nucleotidase from the soluble fraction presented a low K_m in comparison with the enzyme from the microsomal fraction, but in both fractions the values were in the micromolar range. In relation to the V_{max} values, the enzyme from the soluble fraction demonstrated a value approx. 21 times lower when compared

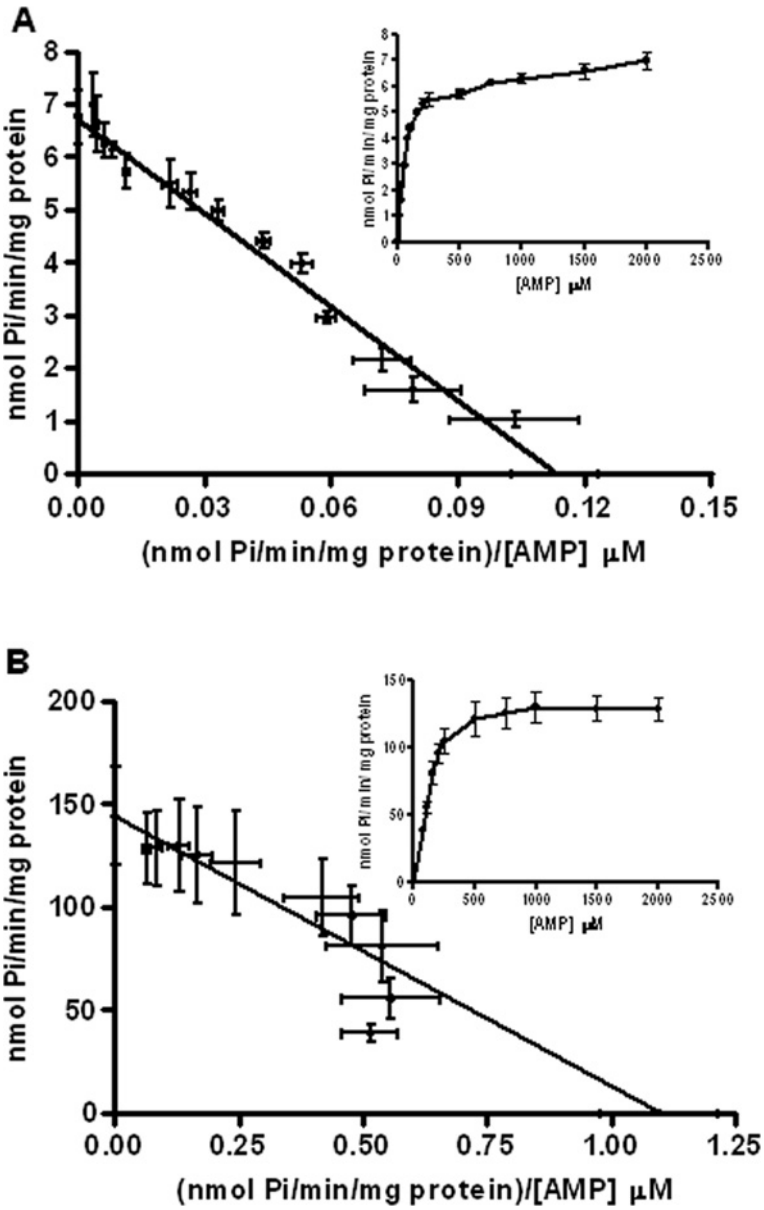

Figure 3 Eadie–Hofstee plot for AMP hydrolysis

Nucleotide hydrolysis as a function of substrate concentration from soluble (A) and microsomal (B) rat cardiac cell fractions is shown (insets). The mean K_m values calculated for AMP hydrolysis were 59.7 ± 10.4 μM and 134.8 ± 32.1 μM with V_{max} values of 6.7 ± 0.4 nmol P_i/min/mg of protein and 143.8 ± 23.8 nmol P_i/min/mg of protein for soluble and microsomal fractions respectively. Results are means $\pm$ S.D. ($n = 4$).

with that observed for the microsomal fraction enzyme. These differences are related in the literature and can be attributed to the type of 5′-nucleotidase and the location of the enzyme [11,13].

As well as the biochemical and kinetic analysis, Western blotting was used to quantify the levels of ecto-5′-nucleotidase present in the two fractions. The method used revealed a protein of approx. 70 kDa in both fractions. The microsomal fraction showed a strong signal, indicating that the presence of ecto-5′-nucleotidase is greater in this fraction compared with the soluble fraction. The presence of an ecto-enzyme in the soluble fraction is not surprising. Zimmermann [11] previously described that up to 50% of the enzyme may be associated with intracellular membranes and released during homogenization, forming a soluble protein. The detection of individual nucleotidases by enzymatic assays in cell lysates is problematic because different nucleotidases can be co-expressed in the same tissue or cell type. For this reason, we cannot exclude the presence of a soluble

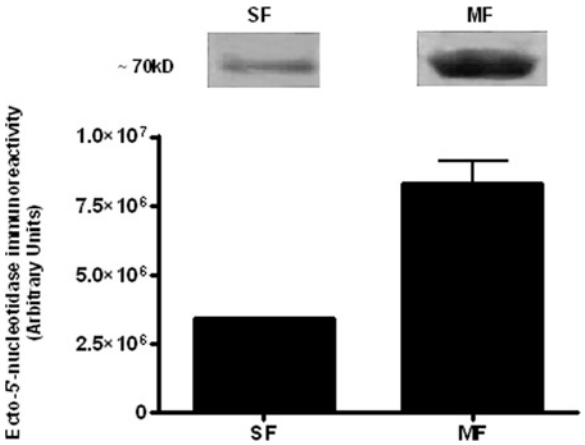

Figure 4 Ecto-5′-nucleotidase identification in soluble (SF) and microsomal (MF) rat cardiac cell fractions measured by Western blotting

The protein bands were quantified by densitometry and the density was calculated and expressed as arbitrary units. The image is representative of three experiments and results are means ± S.D. ($n = 3$). kD, mass in kDa.

5′-nucleotidase since different kinetic properties were found in both fractions. The characteristics of soluble 5′-nucleotidases derived from various tissues have been demonstrated in a great number of studies, principally using the purified enzyme [11,13,18,19]. In heart muscle, the expression of these enzymes has a physiological function in the generation of adenosine signalling during ischaemia and in the regulation of nucleotide pools [13].

The presence of 5′-nucleotidase producing adenosine in the subcellular fragments of the heart may have other important roles beyond those well established roles. The microsomal fraction is derived from the sarcoplasmic reticulum. This is the major intracellular organelle that sequesters intracellular Ca^{2+} and regulates the relaxation and tension development of the myocardium. Previous studies have demonstrated that adenine nucleotides and adenosine affect the release of Ca^{2+} from the sarcoplasmic reticulum both in skeletal muscle and in cardiac fibres [7,20–23]. Furthermore, the work of Duke and Steele [22] utilizing frog skeletal-muscle fibres suggests that adenosine could exert opposite effects to adenine nucleotides in this mechanism. Therefore the presence of a 5′-nucleotidase in the microsomal fraction controlling AMP hydrolysis and adenosine levels could be important for the regulation of Ca^{2+} influx throughout the sarcoplasmic reticulum membrane, modulating the excitation–contraction coupling process.

In conclusion, the great variety of actions of adenosine emphasizes the importance of studying the enzymes involved in its production, and the aim of this work was to characterize AMP hydrolysis in a soluble cellular fraction and to compare this activity with that in a microsomal fraction in order to try and determine the contribution of each fraction to adenosine production in the heart.

ACKNOWLEDGEMENTS

This work was supported by grants from CNPq-Brasil and CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superio).

REFERENCES

1 Mubagwa, K., Mullane, K. and Flameng, W. (1996) Role of adenosine in the heart and circulation. Cardiovasc. Res. 32, 797–813
2 Shryock, J. C. and Belardinelli, L. (1997) Adenosine and adenosine receptors in the cardiovascular system: biochemistry, physiology, and pharmacology. Am. J. Cardiol. 79, 2–10
3 Obata, T. (2002) Adenosine production and its interaction with protection of ischemic and reperfusion injury of the myocardium. Life Sci. 71, 2083–2103
4 Borowiec, A., Lechward, K., Tkacz-Stachowska, K. and Składanowski, A. C. (2006) Adenosine as a metabolic regulator of tissue function: production of adenosine by cytoplasmic 5′-nucleotidases. Acta Biochim. Pol. 53, 269–278
5 Burnstock, G. (2006) Pathophysiology and therapeutic potential of purinergic signaling. Pharmacol. Rev. 58, 58–86
6 Drury, A. N. and Szent-Györgi, A. (1929) The physiological activity of adenine compounds with special reference to their action upon the mammalian heart. J. Physiol. 68, 213–237
7 Headrick, J. P., Hack, B. and Ashton, K. J. (2003) Acute adenosinergic cardioprotection in ischemic-reperfused hearts. Am. J. Physiol. Heart Circ. Physiol. 285, H1797–H1818
8 Ralevic, V. and Burnstock, G. (2003) Involvement of purinergic signaling in cardiovascular diseases. Drug. News Perspect. 16, 133–140
9 Kitakaze, M., Minamino, T., Node, K., Takashima, S., Funaya, H., Kuzuya, T. and Hori, M. (1999) Adenosine and cardioprotection in the diseased heart. Jpn. Circ. J. 63, 231–243
10 Xu, Z., Mueller, R. A., Park, S., Boysen, P. G., Cohen, M. V. and Downey, J. M. (2005) Cardioprotection with adenosine A2 receptor activation at reperfusion. J. Cardiovasc. Pharmacol. 46, 794–802
11 Zimmermann, H. (1992) 5′-nucleotidase: molecular structure and functional aspects. Biochem. J. 285, 345–365
12 Deussen, A. (2000) Metabolic flux rates of adenosine in the heart. Naunyn Schmiedebergs Arch. Pharmacol. 362, 351–363
13 Bianchi, V. and Spychala, J. (2003) Mammalian 5′-nucleotidases. J. Biol. Chem. 278, 46195–46198
14 Kiviluoma, K. T., Hiltunen, J. K., Hassinen, I. E. and Peuhkurinen, K. J. (1990) Subcellular distribution of myocardial 5′-nucleotidase. J. Mol. Cell. Cardiol. 22, 827–835
15 Floreani, M., Napoli, E., Quintieri, L. and Palatini, P. (2003) Oral administration of trans-resveratrol to guinea pigs increases cardiac DT-diaphorase and catalase activities, and protects isolated atria from menadione toxicity. Life Sci. 72, 2741–2750
16 Chan, K., Delfert, D. and Junger, K. D. (1986) A direct colorimetric assay for Ca^{2+}-ATPase activity. Anal. Biochem. 157, 375–380
17 Bradford, M. M. (1976) A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254
18 Naito, Y. and Lowenstein, J. M. (1981) 5′-Nucleotidase from rat heart. Biochemistry 20, 5188–5194
19 Truong, V. L., Collinson, A. R. and Lowenstein, J. M. (1988) 5′-nucleotidases in rat heart: evidence for the occurrence of two soluble enzymes with different substrate specifities. Biochem. J. 268, 117–122

20 Smith, J. S., Coronado, R. and Meissner, G. (1986) Single channel measurements of the calcium release channel from skeletal muscle sarcoplasmic reticulum – activation by Ca^{2+} and ATP and modulation by Mg^{2+}. J. Gen. Physiol. 88, 573–588
21 Rousseau, E., Ladine, J., Liu, Q. and Meissner, G. (1988) Activation of the Ca^{2+} release channel of skeletal muscle sarcoplasmic reticulum by caffeine and related compounds. Arch. Biochem. Biophys. 267, 75–86
22 Duke, A. M. and Steele, D. S. (1998) Effects of caffeine and adenine nucleotides on Ca^{2+} release by the sarcoplasmic reticulum in saponin-permeabilized frog skeletal muscle fibres. J. Physiol. 513, 43–53
23 Hleihel, W., Lafoux, A., Ouaini, N., Divet, A. and Huchet-Cadiou, C. (2006) Adenosine affects the release of Ca^{2+} from the sarcoplasmic reticulum via A_{2A} receptors in ferret skinned cardiac fibres. Exp. Physiol. 91, 681–691

Received 15 August 2007/1 August 2008; accepted 6 August 2008
Published as Immediate Publication 6 August 2008, doi 10.1042/BSR20070039