ORIGINAL ARTICLE

Effects of some modulators on purine nucleoside phosphorylase activity in myocardial tissue

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Abstract

Purine nucleoside phosphorylase (PNP) in mammalian tissue is an enzyme responsible for formation of purine bases in DNA. It is also believed that PNP is crucial under energy-deprived conditions for the cell to metabolise adenosine during ATP degradation. This work describes a new method for determination of PNP activity in myocardial tissue using a commercially available substrate, 2-amino-6-mercapto-7-methylpurine riboside (MESG). The method involves the photometric assessment of the reaction between PNP (extracted from myocardial tissue) and MESG. Quantification as well as temperature- and pH-dependency for myocardial PNP activity is described. Also, the effect of some modulators has been studied. We have established the presence of PNP activity in pig myocardial tissue. Further, the results indicate a pH tolerance under slightly acid conditions and a calcium ion dependence of the enzyme.

Key Words: Analytical chemistry, myocardium, purine-nucleoside phosphorylase, enzyme activity, myocardial ischemia

Introduction

Purine nucleoside phosphorylase (PNP) catalyses the irreversible phosphorolysis of purine nucleosides, resulting in the formation of ribose 1-phosphate and purine bases. Inhibition of PNP results in no synthesis of DNA. The relation of PNP and adenosine deaminase concentrations define the degree of differentiation of T-lymphocytes. Deficiencies of these enzymes result in accumulation of deoxy-nucleosides which are highly toxic to lymphocytes. PNP knockout mice are immune deficient. Moreover, PNP seems to be a key enzyme in the ischemic preconditioning phenomenon.

PNP molecules exist as a homotrimer or a homo-hexamer. The homotrimers are present in mammals with inosine and guanosine as principal substrates. When ribose 1-phosphate is released during the phosphorolysis of purine-containing nucleosides, it could act as an energy-yielding substrate which can enter glycolysis via the pentose phosphate pathway. Methods for the determination

of PNP activity have been described earlier and include conversion of inosine by PNP and auxiliary enzymes to uric acid that can be measured by either spectrophotometry [1] or electrophoretic separation and UV detection [2]. Other techniques are radiochemical or fluorometric assays [3] as well as histochemical determination [4].

In this paper we describe a new simplified assay of PNP in pig myocardial tissue with the use of a commercial kit originally aimed at the determination of inorganic phosphate in the presence of MESG (2-amino-6-mercapto-7-methylpurine riboside, [5]). The effect of some modulators on myocardial PNP activity was also tested.

Material and methods

All experiments were performed following the approval of the Ethical Committee for Animal Research (2007-01-20 Dnr A10-07) in accordance with the 1996 Guide for the Care and Use of

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Figure 1. Reaction formula of enzymatic conversion of 2-amino-6-mercapto-7-methylpurine riboside (MESG) to ribose 1-phosphate and 2-amino-6-mercapto-7-methylpurine by purine nucleoside phosphorylase (PNP).

Laboratory animals from the National Academy of Science, USA.

Preparation of tissue samples

Myocardial tissue from healthy anesthetized pigs was taken as biopsies and immediately frozen in liquid nitrogen-cooled iso-pentane and stored in a freezer (-70°C) until analysis. These biopsies were dried in a vacuum centrifuge and homogenized in phosphate buffer (100 millimoles/L, pH 7.0). The volume of the phosphate buffer was calculated based on tissue dry weight, 1 mL to 16 mg. Homogenized samples were shaken in 4°C for 1 h before centrifugation (15 min, 10,000 g, 4°C). The supernatant (tissue extract sample) was used for the subsequent PNP assay.

PNP assay

A commercial kit originally designed for the determination of inorganic phosphate was used (EnzChek® phosphate assay kit E-6646, Invitrogen, California, USA). Our tissue extract samples were mixed with reaction buffer, 2-amino-6-mercapto-7-methylpurine riboside (MESG), phosphate buffer (25 millimoles/L, pH 7.4) and water or ethanol in a total volume of 1 mL. The assay was performed at room temperature unless otherwise indicated. MESG in the presence of phosphate in excess and PNP (rate limiting, the enzyme to be measured in our tissue extract samples), is converted by PNP to 2-amino-6-mercapto-7-methylpurine (Figure 1), which has an absorbance at 360 nm. The absorbance increase at 360 nm represents the formation rate of 2-amino-6-mercapto-7-methylpurine (catalysed by PNP) in accordance with the manufacturer's description and was followed photometrically every minute for a total of 30 min. The reaction was tested by using different PNP-containing sample volumes (10–300 µL). The volumes used (with a few exceptions) were 200 µL samples, 50 µL reaction buffer, 200 µL MESG, 350–550 µL phosphate buffer and water or ethanol in a total volume of 1 mL. Background tests of the reaction have been carried out with a mixture without MESG and also a mixture

without tissue extract sample, and the absorbance at 360 nm was read every minute.

Quantification with reference samples

In order to quantify the PNP activity, reference samples were made in different ways. Different concentrations of PNP (0.6–80 units/L) from the kit were used together with reaction buffer (50 µL), MESG (200 µL), phosphate buffer (210 µL, 25 millimoles/L, pH 7.4) and water. The absorbance at 360 nm was read after 30 min. Absorbance was plotted against PNP concentration and linearity curves were calculated. Reference samples were also made by using different amounts of MESG (5–200 nanomoles/L) together with a given amount of PNP from the kit (10 µL, 100,000 units/L), reaction buffer (50 µL), phosphate buffer (210 µL, 25 millimoles/L, pH 7.4) and water. The absorbance at 360 nm was read and plotted against MESG concentration, and linearity curves were calculated.

Reference samples with different concentrations of PNP showed linearity ($R^2 = 0.9983$) in the range between 0.6–5 units/L, and this covered the absorbance interval between 0.400–0.900. Reference samples with different concentrations of MESG were linear in the interval between 5–200 nanomoles/L, which corresponded to 5–200 µL added MESG to the reaction mixture, and covered an absorbance interval from 0.006–1.200 (Figure 2).

Figure 2. Absorbance at 360 nm at 30 min reaction with different concentrations (nanomoles/L) of MESG with a given amount of PNP from the kit.

Precision of the assay

The overall precision of the assay was tested by using dry homogenized myocardial tissue which was pooled and then subdivided into 6 tubes. The tubes were mixed with phosphate buffer, shaken and centrifuged. The reaction with MESG was performed, and the absorbance at 360 nm was read at 30 minutes.

Temperature dependence

Myocardial tissue extract samples were pre-incubated for 30 min at the following temperatures: 4°C, 23°C, 37°C, 50°C and 80°C. This preincubation was followed by reaction with MESG for 30 min at the respective temperature, after which the absorbance was read.

pH dependence

Myocardial tissue was homogenized under dry conditions to a powder, pooled and divided into 12 samples. Phosphate buffer (100 millimoles/L) with different pH (6.0, 6.8, 7.4 and 7.8) was added to each sample, 3 replicates for each pH, and all samples were shaken at 4°C and centrifuged. The supernatant was mixed with water (instead of reaction buffer), MESG and phosphate buffer (25 millimoles/L) with different pH (6.0, 6.8, 7.4 and 7.8) and the absorbance was read at 360 nm after incubating for 30 min.

Ischemic myocardial tissue

Myocardium in 6 animals was made ischemic for 40 min by coronary artery ligation, at the end of which myocardial tissue was biopsied both from the ischemic area and from the non ischemic area. The biopsies were stored and prepared in the same way as described in the section 'preparation of tissue

samples' and analysed as described in the section 'PNP assay'.

Incubation with modulators

Myocardial tissue from non-ischemic pig hearts were incubated in phosphate buffer (1 mL to 16 mg) with different modulators as listed in Table I. Some modulators were allowed to react with MESG, only, and not with homogenized tissue extract. For each tested modulator, controls were concomitantly run in absence of the modulator in question.

EGTA, EDTA. Twelve tissue extract samples from 12 hearts were used. Each extract was subdivided into 4 samples and to each sample EDTA (2.25 millimoles/L), EGTA (2.25 millimoles/L) or a mixture of EDTA and EGTA in phosphate buffer (25 millimoles/L) or only phosphate buffer (25 millimoles/L) was added prior to the reaction with MESG. Absorbance was read at 30 min. From a pooled tissue extract sample, EDTA in different concentrations (1.3–6.6 millimoles/L) was added together with MESG and reaction buffer. The absorbance was read at 30 minutes.

cAMP, cGMP. Thirty tissue extract samples from pooled tissues from three different hearts were used. The phosphate buffer added after homogenization consisted of either buffer or buffer with 10 micromoles/L cAMP, 50 micromoles/L cAMP, 10 micromoles/L cGMP or 50 micromoles/L cGMP. Six replicates were made for each solution. Samples were shaken, centrifuged and the reaction was started with MESG. Absorbance was read at 30 min. From another pooled sample, higher concentrations of cGMP and cAMP were compared with addition of only buffer. Furthermore, dry homogenized

Table I. Modulators incubated with homogenized tissue (I) or added with tissue extract sample to the reaction mixture (R) with MESG. The effects of the modulators, the tested concentrations as well as number of tests per modulator (*n*) are listed in the table.

Modulator	I/R	Effects	Concentrations	n
EDTA	R	Mg ²⁺ and Ca ²⁺ chelator	1.3, 2.25, 2.6, 4.0, 5.3, 6.6 millimoles/L	29
EGTA	R	Ca ²⁺ chelator	2.25 millimoles/L	12
EDTA & EGTA	R		1.13 & 1.13 millimoles/L	12
cAMP	I	second messenger in many systems	10, 50, 140, 520 micromoles/L	14
cGMP	I	second messenger in many systems	10, 50, 570 micromoles/L	13
cGMP + MgCl	I		50 µM + 2 millimoles/L	4
cGMP + ZnCl	I		50 µM + 10 micromoles/L	4
Thapsigargin	R	releases intracellular Ca ²⁺	1, 0.5, 0.25, 0.125 micromoles/L	9
Wortmannin	R	inhibition of PI3-kinase	1, 0.5, 0.25, 0.1, 0.05, 0.025 millimoles/L	10
D-myo-Inositol-trisphosphate hexasodium salt	R	stimulates intracellular Ca ²⁺ mobilisation	0.87, 0.65 millimoles/L	2
Staurosporine aglycone	R	inhibition of protein kinase C	1 millimoles/L	2
Adenosine	I, R	substrate for PNP	1 millimoles/L	9

myocardial tissue was pooled and mixed with cGMP + MgCl in phosphate buffer, cGMP + ZnCl in phosphate buffer or only phosphate buffer. The tissues were shaken at 4°C and centrifuged. The reaction with MESG followed and the absorbance read at 360 nm.

Adenosine. Homogenized, pooled myocardial tissue was divided into 12 samples and mixed with either adenosine (1 millimoles/L, $n = 6$) in phosphate buffer or only phosphate buffer ($n = 6$) before being shaken at 4°C and centrifuged. The reaction with MESG was performed with tissue extract, reaction buffer, MESG, and phosphate buffer (100 millimoles/L, pH 7.0) with or without adenosine, and the absorbance was read at 360 nm and 30 min. From a pooled tissue extract sample, adenosine was added, only, for reaction with MESG ($n = 3$) and compared with controls ($n = 3$).

Thapsigargin, wortmannin, D-myo-inositol-trisphosphate hexasodium salt and staurosporine. Two different myocardial tissues were prepared and divided into 3 tissue extract samples. To each sample, either ethanol or staurosporine in ethanol or thapsigargin in ethanol was added together with MESG and the other components in the reaction mixture. The absorbance was read before MESG was added as well as after the addition every minute for a total of 30 min. Tissue was also homogenized and pooled. This pool was used to add D-myo-inositol-trisphosphate hexasodium salt, wortmannin and thapsigargin in different concentrations. D-myo-inositol-trisphosphate hexasodium salt, prepared in water was compared with controls in water whereas the other two substances were prepared in ethanol and compared with controls in the same amount of ethanol. Both thapsigargin and wortmannin were tested in the reaction without addition of tissue extract sample, i.e. without PNP.

Statistical analyses

Statistical calculations were made using Student's *t*-test for group or pair-wise measurements, and a *p*-value < 0.05 was considered significant.

Results

Time and enzyme dependence

In three different myocardial extract samples analysed for PNP activity, the absorbance at 360 nm rose over time after the addition of MESG (Figure 3). When increasing volumes of extract samples were used in the reaction mixture, the absorbance at 30 min was proportionally augmented. Hence, there was a linear relationship between sample volume and

Figure 3. Time dependence for the conversion of MESG to 2-amino-6-mercapto-7-methylpurine by myocardial PNP. The three graphs represent three different myocardial tissue extract samples from three pigs. 2-amino-6-mercapto-7-methylpurine, absorbance was read at 360 nm every minute for 30 min. Absorbance of the incubation mixture without MESG was also given. The three representative experiments demonstrate variation in background absorbance in the tissue extract samples.

absorbance at 360 nm (Figure 4). When MESG or tissue extract sample was excluded there was no increase of absorbance over time. The starting level of the absorbance before MESG varied depending on the colour of the tissue extract sample (data not given). When comparing different tissue preparations the background absorbance was withdrawn before addition of MESG. For pooled samples this was not necessary, since all tissue extract samples had approximately the same background absorbance.

Temperature dependence

The highest absorbance and therefore the highest enzyme activity were observed at 37°C, with lower activities both at higher and lower temperatures (Figure 5).

pH dependence

PNP exhibited enzyme activity over a rather broad pH interval. An acidic condition with pH down to pH 6 was well tolerated while a pH above 6.8 and higher (up to pH 7.8) showed decreased enzyme activity (Figure 6).

Figure 4. Enzyme dependence of PNP reaction illustrated by increasing amount of enzyme from extracted pig heart tissue. Absorbance was read at 360 nm at 30 min incubation.

Figure 5. Temperature dependence of myocardial PNP activity. Absorbance was read at 360 nm at 30 min incubation.

Precision

The overall coefficient of variation (CV) for the PNP assay was 3.7% (6 pooled samples).

Ischemic tissue

The ischemic tissue biopsies showed the same PNP activity as the biopsies from the normal tissue of the same hearts. Paired Student's *t*-testing showed no difference between ischemic and normal heart tissue. The mean absorbances $\pm$ SEM for the groups were 1.09 ± 0.10 for normal tissues and 0.99 ± 0.11 for ischemic tissues.

Effect of different modulators

EGTA, EDTA. The addition of EDTA and/or EGTA showed that EDTA activated PNP ($p < 0.001$, paired measurements). When grouping the measurements, mean absorbance $\pm$ SEM was 1.31 ± 0.06 with the addition of EDTA and 1.20 ± 0.06 for controls. EGTA on the other hand inactivated the enzyme ($p < 0.001$, paired measurements), absorbances 0.99 ± 0.04 with addition of EGTA and 1.14 ± 0.04 without. The mixture of both EDTA and EGTA had no influence on PNP activity (Figure 7). There was a linear relationship between increased EDTA concentrations and PNP activity ($y = 0.054x + 1.4316$,

Figure 6. pH dependence of myocardial PNP activity, pH range 6.0–7.8. Absorbance was read at 360 nm at 30 min incubation. Each point represents mean value $\pm$ SD of 3 replicates.

Figure 7. Positive and negative differences in absorbance at 360 nm between controls on one hand and stimulators and inhibitors on the other, respectively after 30 minutes' incubation. Twelve different samples (extracted pig heart tissue) were used.

where y is the absorbance after 30 min reaction and x is the EDTA concentration in millimoles/L) in the cuvette; the correlation coefficient was 0.9877.

cAMP, cGMP. Addition of cAMP or cGMP to the incubation medium did not influence the PNP activity. This was also true for incubation with cGMP in presence of either Mg^{2+} or Zn^{2+} .

Adenosine. Addition of adenosine to both the homogenized tissue (prior to assay) and to the reaction mixture with MESG resulted in a decreased PNP activity by about 23%, $p < 0.001$, grouped measurements, shown in Figure 8. Mean absorbance (all given as mean $\pm$ SEM) with addition of adenosine was 1.32 ± 0.02 compared to controls that had a mean absorbance of 1.72 ± 0.03 . When adenosine was added to the reaction mixture with MESG and compared with controls, the activity was 7% lower in adenosine samples compared to controls ($p < 0.001$) for grouped measurements. Mean absorbance with addition of adenosine was 1.30 ± 0.005

Figure 8. Inhibitory effect of adenosine (1 millimole/L) on myocardial PNP activity. The bars represent mean values $\pm$ SD for 6 replicates. There was a significant difference between the groups, $p < 0.001$.

Figure 9. Stimulatory effects of thapsigargin and staurosporine on myocardial PNP. Note that an absorbance of 2.0 represents a net maximal value. A representative experiment out of two experiments is shown.

compared to controls of 1.21 ± 0.004 . The inactivating effect of adenosine was in other words smaller when adenosine was added to the reaction mixture with MESG compared with adenosine being added prior to assay.

Thapsigargin, wortmannin, D-myo-inositol-trisphosphate hexasodium salt and staurosporine. A representative test with thapsigargin and staurosporine in the presence of controls are shown in Figure 9, with absorbance measured each minute. Both thapsigargin and staurosporine showed a stimulatory effect on PNP activity. Even though there was a relatively large variation in the measurements, the absorbance was higher for thapsigargin and staurosporine in all measurements made at 20 min reaction compared to controls. We have no explanation for this large variation; further studies need to be done in order to investigate this. A separate tissue extract sample resulted in similar results (data not shown). A maximal absorbance value of 4.5 was the upper limit for detection and for samples with thapsigargin or staurosporine in the reaction mixture, this value was reached during the reaction. The absorbance before MESG was added was withdrawn from all samples giving a maximal absorbance net value of approximately 2.0 (Figure 9). Thapsigargin in different concentrations showed a strong linear relationship to absorbance at 30 min. However, when the absorbance before MESG was withdrawn, linear correlation was weak ($R^2 = 0.30$). Thapsigargin together with MESG in absence of PNP did not influence the absorbance value.

Wortmannin in the sample showed a higher increase in absorbance at 30 min compared with controls (data not shown). The same problem as for thapsigargin appeared with weaker linear correlation between concentration of wortmannin and absorbance value when the absorbance before MESG was subtracted ($R^2 = 0.8486$). Wortmannin per se gave an increase of absorbance at 360 nm even without PNP. The sum of this increase and the increase of a control sample was lower than when wortmannin

was added to the sample. Regarding addition of D-myo-inositol-trisphosphate hexasodium salt, no change in PNP activity was noted.

Discussion

The present method to measure PNP activity in a tissue extract by using MESG as the substrate is new and turned out to be stable and reliable. The method was simple, straightforward and did not involve any complicated equipment. In other methods dialysis of samples has been necessary [5], or use of HPLC [6] or capillary electrophoresis [2]. The inter assay imprecision was low with a CV of 3.7%. This was in agreement with another report on CV for PNP assayed differently being 3.2% [2]. The reaction between PNP in our tissue extract sample and MESG seems to be an accurate way of determining PNP activity. The reaction gives an increase in absorbance over time only when both tissue extract sample, i.e. PNP, and MESG are present. Further proof that the increase of absorbance is a reliable measure of PNP activity is the fact that increasing volumes of tissue extract sample, and thereby also increasing amount of PNP, resulted in proportionally augmented absorbances, that is, a dose/response relationship existed. Furthermore, adenosine, as a substrate of PNP, displayed an inhibitory effect in our assay.

In the pH interval used in this study (6.0–7.8), the absorbance for the product 2-amino-6-mercapto-7-methylpurine was not pH-dependent [7]. The myocardial PNP activity was higher at a slightly acid pH compared to that at a slightly alkaline pH. This might be functional when considering the rescue role of PNP under energy-deprived ischemic conditions [8] meaning that lactic acidosis (lowered pH) is prevailing. Energy deprivation on a cellular level also means a disturbed calcium homeostasis leading to an elevated intracellular calcium pool [9]. In this context, it is interesting to note the inhibitory effect of EGTA (a calcium ion chelator) while thapsigargin (releases calcium ions from intracellular stores) clearly stimulated PNP activity, hence demonstrating a calcium dependence for the myocardial form of PNP. The activation with EDTA, which is a chelating agent for calcium, manganese, zinc and other divalent cations, could mean that a divalent cation other than Ca^{2+} might be involved in inactivation of the enzyme.

Staurosporine activated myocardial PNP. Staurosporine has great influences on Ca^{2+} homeostasis in intact cellular systems by interfering with both Ca^{2+} influx across the plasma membrane and with intracellular Ca^{2+} mobilization. Our assay comprised a myocardial cell extract and still showed this staurosporine activation effect on PNP. The exact activating mechanism was however not studied in detail. Our results from modulators chosen indicate that Ca^{2+} is important in the regulation of PNP which might have

a pathophysiological impact on the mechanism of the preconditioning phenomena [10]. It would seem physiologically that intracellular calcium overload (as in energy deprived cells) would increase PNP activity and thus tend to restore intracellular ATP via the PNP route (adenosine conversion to inosine and further on to lactate forming two ATP).

During ischemia the PNP activity was similar to that in normal tissue. This is interesting since the glycogenolysis is minimal after such a long period of ischemia [11], 40 min in this case. These results support the idea that PNP and its possibility to enhance energy metabolism is important during ischemia.

In conclusion, these results demonstrate a simple and reliable method to determine PNP activity in myocardial tissue samples. Our study of effects of some modulators showed calcium dependence for PNP activity.

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