5'-NUCLEOTIDASE OF CARDIAC PLASMA MEMBRANE

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BY

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MASTER OF SCIENCE

TO

My Wife

and

My Parents

with Love

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ABSTRACT

5'-Nucleotidase was examined in sarcolemmal fractions of rat heart which had been isolated by two different methods namely, hypotonic shock-LiBr treatment and sucrose gradient separation. Enzyme activity in the two resulting fractions called HL and S respectively differed significantly in several respects. The fraction HL 5'-nucleotidase had a high affinity for AMP (K_m 35 uM) and ATP was a potent competitive inhibitor. In contrast, the 5'-nucleotidase activity displayed by fraction S showed a low substrate affinity (K_m 130 uM) and sensitivity to ATP. Trypsin and neuraminidase treatments of membranes markedly stimulated 5'-nucleotidase in fraction HL whereas only a modest effect was observed in fraction S. However, under the same experimental conditions both fractions exhibited comparable K^+-pNPPase activities. Considering the possibility that 5'-nucleotidase could be localized at the inside of the vesicular membrane of fraction S, Triton X-100 was added to the assay medium. Even though this treatment resulted in a 60% and 10% increase in enzyme activity in fractions HL and S respectively, there was no apparent change in the K_m of the enzyme in both fractions. Concanavalin A blocked almost completely (95%) 5'-nucleotidase activity in both membrane preparations at a concentration of 2 uM. Hill plots of the data of the concanavalin A inactivation process yielded a coefficient of 2.2 for fraction S which is substantially greater than the value of 1.1 obtained for fraction HL. The vectorial properties of such membrane bound enzyme systems as Na^+-K^+-ATPase, adenylate cyclase and ATP dependent Ca^{2+} binding were used to estimate the relative sidedness of the vesicles present in the fractions. The results suggested that fraction S contained a large proportion of inside-out vesicles with 5'-nucleotidase activity possibly

located at the cytoplasmic side of the membrane. Further, poor lanthanum staining of the vesicles in this preparation suggest the absence of a basement membrane thus a predominance of inside-out vesicles. The activation by trypsin and neuraminidase treatment in fraction HL is discussed in relation to the frequently reported ecto-localization of 5'-nucleotidase. Dense lanthanum staining of vesicles in the HL preparation suggest a predominance of right-side out vesicles. These findings suggest that 5'-nucleotidase can exert its action in vivo at either side of the plasma membrane. Similar evidence has been obtained by Frick and Lowenstein (9) in the perfused rat heart. In both preparations, the enzyme was active over a wide pH range. p-Hydroxymercurybenzoate did not produce any significant effect on 5'-nucleotidase thereby demonstrating that its activity is not dependent on free (-SH) groups.

In another series of experiments it was demonstrated that neither dipyridamole nor papaverine had any apparent effect on the enzyme in fractions HL and S thus suggesting that the mechanisms whereby they increase coronary blood flow do not directly involve 5'-nucleotidase. In contrast caffeine and theophylline partially inhibited the enzyme in both fractions but theophylline appeared to be a more potent inhibitor. Even though the antagonistic effect of caffeine seemed to be the same in both sarcolemmal preparations, the enzyme in fraction S appeared to be more sensitive to theophylline than that present in fraction HL. The inhibition of the enzyme by caffeine and theophylline was of the competitive type in both preparations. These results suggest that by inhibiting 5'-nucleotidase, caffeine and theophylline can influence the production of adenosine in the myocardium.

INTRODUCTION AND STATEMENT OF THE PROBLEM

Although small amounts of 5'-nucleotidase seem to be present in the cytosol (1), histochemical studies have shown that in cardiac tissue this enzyme is localized mainly in the vicinity of the transverse tubular part of the surface membrane (2, 3). Other reports have classified it as an ectoenzyme of the plasma membrane (1, 4 - 8) and it has been suggested that it can act as an adenosine translocase (9). 5'-Nucleotidase, which utilizes 5'-AMP as a substrate, is believed to be responsible for the physiological production of adenosine in cardiac tissue (10). Since the concentration of this metabolite increases in the heart during ischemia, hypoxia, and increased cardiac work (11, 12), adenosine has been proposed to be a link between myocardial energy metabolism and coronary blood flow (10, 11, 13). ATP and ADP inhibit this enzyme, and because of their low Ki, it would appear that 5'-nucleotidase is inhibited in both the hypoxic and adequately oxygenated hearts (14). Hence, it seems that the origin of adenosine in the ischemic or hypoxic heart cannot be explained by considering a change in adenine nucleotide levels.

In addition to its coronary vasodilatory action, adenosine also inhibits the release of norepinephrine in the heart (56), and antagonizes the catecholamine-induced stimulation of cardiac adenylate cyclase (50, 64). Furthermore, there is evidence to show that adrenergic nerve stimulation leads to the formation and release of adenosine in the heart (96). Since this metabolite is utilized in the organ in which it is produced, it can be classified as a local hormone (10). But for adenosine to be effective in this capacity it must be released from the cell. The simplest hypothesis for the biochemical mechanism of release is that adenosine is produced intracellularly and

is then transported across the cell membrane by a carrier-mediated process (facilitated diffusion) according to its concentration gradient. However, the major problem with this hypothesis is the localization of 5'-nucleotidase which has been described as an ectoenzyme. Consequently, adenosine may be produced directly into the extracellular space (10). There is little doubt that in many tissues a considerable proportion of the nucleotidase has access to extracellular 5'-AMP as substrate, but it is not clear if the enzyme has access to only extracellular or to both intra and extracellular AMP (10). Most of the studies on the properties of 5'-nucleotidase in the heart have been studied on the solubilized and purified enzyme (14, 15). But solubilization and purification can influence the behavior of an enzyme relative to its unsolubilized and unpurified form. Hence, the present investigation was undertaken to study the intrinsic properties of 5'-nucleotidase in heart sarcolemmal fractions. Special attention was placed on the possible non-identical orientation of the highly purified membrane vesicles from two different preparations as this can be responsible for the observed difference in the behavior of the enzyme in these preparations.

Recently theophylline has been reported to inhibit the activity of 5'-nucleotidase in the brain and kidney (53, 65) and attenuated the adenosine-induced vasodilation in the brain and heart (45, 47, 49, 63). It was also found to antagonize the inhibition of cardiac adenylate cyclase and myocardial contractile force development by adenosine (64). Caffeine inhibited 5'-nucleotidase in the brain (65) and attenuated the catecholamine-induced stimulation of cardiac adenylate cyclase (50). The mechanisms by which methylxanthines antagonize the effects of

adenosine in the heart are not known. These may partially reside in the inhibition of cyclic nucleotide phosphodiesterase with a subsequent increase of intracellular cAMP levels. Another possible explanation might be a direct influence on 5'-nucleotidase activity as already observed in the brain and kidney. In view of the possibility that such a mechanism could also be operative in the heart, the effects of caffeine and theophylline on cardiac sarcolemmal 5'-nucleotidase were therefore investigated. Furthermore, some experiments were designed to determine whether the elevation of adenosine levels in the extracellular environment by dipyridamole (10, 46) could be partly correlated with changes in 5'-nucleotidase activity. The effect of papaverine on this enzyme was also investigated since this drug is known as an exogenous vasodilator and, like methylxanthines and dipyridamole, is a phosphodiesterase inhibitor (51). The interaction of 5'-nucleotidase with these drugs was investigated by employing heart sarcolemmal preparations obtained by different methods.

REVIEW OF THE LITERATURE

Adenosine exerts important functional and metabolic actions on the myocardium (68) in addition to its well known effect on coronary blood flow (11, 94). The cardiac effects of adenosine include negative chronotropic (69, 94) and inotropic actions (64, 94), increase in glucose uptake by cardiac cells (13, 97) and reduction of myocardial oxygen consumption (70). The first hint that adenosine modifies physiological processes came in 1929 when Drury and Szent-Gyorgi observed that injection of adenosine into mammals lowered the arterial blood pressure, dilated the coronary arterioles, induced sleep and inhibited movements of the small intestine (10, 95). During the following thirty years more attention was given to the pharmacological effects of adenine nucleotides than those of adenosine. In particular, studies were focussed upon ATP, which had not been available in large quantities at the time of Drury's work. Nonetheless, perhaps the most investigated effect of adenosine is on the rate of blood flow. In 1936, Drury suggested that adenosine may be involved in the control of blood flow but this possibility has only been intensively investigated since 1960 (10).

In the course of studying the deamination of nucleotides in heart and skeletal muscle in 1934, Reis discovered that adenosine is produced by the hydrolysis of 5'-AMP (71) and deduced that the reaction was catalysed by an enzyme specific for 5'-nucleotidases now known as 5'-nucleotidase (10). This enzyme catalyses the dephosphorylation of 5'-AMP according to the reaction (31):

$$\text{5'-AMP} \xrightarrow{\text{5'-nucleotidase}} \text{adenosine and orthophosphate}$$

Subsequent to his deduction of the presence of 5'-nucleotidase in

animal tissue, Reis then undertook the task of characterizing this enzyme. His experiments however, were performed with impure preparations of the enzyme thus it is difficult to draw any meaningful conclusions from such studies (71). Since then, the advent of better techniques for studying enzymes have greatly improved the accuracy in the characterization of 5'-nucleotidase.

Adenosine is produced, released, taken up and metabolized by most animal tissues. A number of pathways could be involved in the metabolism of this nucleoside; however, some of these are of little or no quantitive significance (10). Acid or alkaline phosphatases could be involved in the formation of adenosine since they catalyse the hydrolysis of 5'-AMP to adenosine. However, these enzymes show no specificity for 5'-AMP and their activities at neutral pH are too low to be of quantitive physiological importance in adenosine production (10, 73). Similarly, purine nucleotide phosphorylase which could produce adenosine from adenine has a very low activity (10, 74). On the other hand, 5'-nucleotidase is specific for 5'-nucleotides (10, 71); its pH optimum is close to the physiological pH and its maximal activity can more than adequately account for known rates of adenosine formation (10, 76).

A. Cellular Localization and Characteristics of 5'-Nucleotidase:

Perhaps the earliest attempt to determine the intracellular localization of 5'-nucleotidase was that of Novikoff and his associates in 1953 (77). By using an elaborate scheme of centrifugation and separation of rat liver suspensions into different subcellular fractions, these workers reported that most of the enzyme was associated with their nuclear fraction. The distribution of 5'-nucleotidase was estimated from charts and expressed as a fraction of the activity in the homogenate

(71). Electron microscopic studies however, have demonstrated the presence of 5'-nucleotidase in the plasma membranes of practically all mammalian cells (71, 78). Because of this finding this enzyme is now widely used as an enzymatic marker during purification of these membranes (78). 5'-Nucleotidase is a glycoprotein (61, 79, 80). In the mouse liver, it has a molecular weight of 140-150,000 daltons, and is composed of two probably identical subunits (61, 79), but the molecular weight and structure of the cardiac enzyme are unknown (61).

There are marked species differences with respect to the cardiac 5'-nucleotidase activity. Experiments performed by Baer et al (35) have demonstrated that rat hearts contain 100 times the activity found in rabbit heart. Their experiments also revealed that the activity of 5'-nucleotidase in the hearts of dog, sheep and guinea pig is considerably lower than the activity found in rat heart. Further, Nakatsu and Drummond (15) have reported that turtle and pigeon ventricles contain no detectable 5'-nucleotidase activity. These findings clearly demonstrate that the activity of this enzyme is neither related to the size of the animal nor to animals that experience large and rapid changes in heart work load, for example birds as compared to animals like reptiles that maintain constant loads on their hearts (15).

Rat ventricular 5'-nucleotidase possesses a broad substrate specificity for nucleoside 5'-monophosphates. However, 5'-AMP is the preferred substrate (14). According to Nakatsu and Drummond (15), phosphate esters not bearing base moieties were not attacked by this enzyme. Thus, ribose-5-phosphate, glucose-6-phosphate, fructose-1-phosphate, ribulose-5-phosphate and galactose-6-phosphate do not appear to serve as substrates. Phosphate is not a substrate; neither is p-nitrophenylphosphate (15) which serves as a substrate for non-specific

phosphatases.

5'-Nucleotidase activity in the heart appears to be influenced by divalent cations. As indicated by Nakatsu and Drummond (15), the activity of this enzyme was greatly increased in the presence of Mg^{2+}, Ni^{2+} and Mn^{2-} at concentrations of 16, 2 and 1 mM respectively. Of these elements, Mn^{2+} appears to be the more potent stimulator; however, it was inhibitory at concentrations above 2 mM. Ca^{2+} had only slight stimulatory effect but does not appear to affect the activity of the enzyme in the presence of Mg^{2+}. Sullivan and Alpers (14) have confirmed the stimulatory effect of Mg^{2+} and Ca^{2+} on 5'-nucleotidase activity. In contrast to these findings are the earlier observations of Edwards and Maguire (81) who reported that both Mg^{2+} and Ca^{2+} inhibited the activity of 5'-nucleotidase in the heart. The general agreement is however, that Mg^{2+} is required for the maximal activity of cardiac 5'-nucleotidase.

The inhibition of 5'-nucleotidase by ATP and ADP has been well documented by several groups of investigators. The type of inhibition exerted by these nucleoside di-and-triphosphates is uncertain. ATP inhibition has been reported to be competitive (35), mixed (61), or noncompetitive (61, 80), while ADP inhibition is said to be competitive (14, 80, 81) or noncompetitive (15). According to Olsson and Patterson (61), this conflict is probably due to differences in experimental conditions, particularly pH and Mg^{2+} concentration. Other known inhibitors of 5'-nucleotidase activity are methylxanthines (53, 65), orthophosphate (one of the products of the dephosphorylation of 5'-AMP by this enzyme (15)) and the synthetic compound γ, β-methylene adenosine diphosphate (AOPCP). The type of inhibition exerted by AOPCP, an ADP analog, is strongly dependent on pH, being of the mixed type below pH 7 and competitive at pH 7 and above (61).

B. Regulation of 5'-Nucleotidase in vivo:

One approach to understanding the regulation of adenosine production is to define how 5'-nucleotidase is regulated in vivo. Cardiac tissue appears to contain much more 5'-nucleotidase than is required to account for the observed rates of adenosine production in beating hearts. Dog heart homogenates for example contain enough of this enzyme to produce about 130 nmoles adenosine/g per min (61), yet oxygenated hearts produce less than 1 nmole/g per min (61, 80) and this figure increases only about 6-fold during coronary occlusion (61, 90). This disparity between the actual and potential rates of adenosine production suggest that 5'-nucleotidase is markedly inhibited in vivo (61). Several factors that can contribute to this relatively low apparent activity have been suggested by different investigators.

When Baer, Drummond, and Duncan (35) found that ATP inhibited 5'-nucleotidase, they proposed that this inhibition accounted for the in vivo control of the enzyme. According to this postulate, hypoxia should lead to an increase in the production of adenosine because it causes ATP levels to fall, thereby relieving the inhibition of the enzyme. Further, the conversion of ATP to AMP in hypoxic heart would provide more substrate for the enzyme. However, current evidence does not appear to support this hypothesis (61). ATP levels in heart do not change for at least 15 seconds after coronary artery ligation (61, 91), whereas adenosine levels increase after as little as 5 seconds of coronary artery occlusion (61, 90). Further, a decrease in ATP necessarily implies an increase in ADP, which has been observed within 15 seconds after coronary occlusion (61, 92). ADP inhibits 5'-nucleotidase even more strongly than ATP (14, 80) which should increase rather than decrease the inhibition of the enzyme (61).

According to Olsson and Patterson (61), the ionic composition of the microenvironment of the enzyme may also influence in vivo activity of 5'-nucleotidase. Because the enzyme is located in a membrane through which there are very important ion flexes which ultimately determine cardiac performance, it may be very susceptible to this sort of local chemical control. Sullivan and Alpers (14) have proposed that the regulation of 5'-nucleotidase in vivo may depend on the deinhibition by magnesium. In this regard it was demonstrated that the inhibition imposed by either ATP or ADP was not relieved by a number of metabolic intermediates known to accumulate in the hypoxic heart. However, magnesium, one of the many divalent cations required for the activity of this enzyme relieved the nucleotide-induced inhibition completely. This relief was in part caused by the formation of nucleotide-magnesium complex which was less inhibitory. Further increases in magnesium relieved the inhibition imposed by the complexes (14).

As indicated by Arch and Newsholme (76) one mechanism for lowering the activity of 5'-nucleotidase in vivo maybe that the concentration of substrate (5'-AMP) is well below the K_m value of the enzyme. Thus, the concentration of AMP in the tissue is similar to or below the K_m value of the nucleotidase. Moreover, much of this AMP may be bound to proteins and/or localized within the mitochondria (76, 93). Such conditions could play an important role not only in decreasing the activity of 5'-nucleotidase in vivo but also in modifying the activity of the enzyme under certain physiological conditions in order to produce a change in the steady-state concentration of adenosine (76).

C. Physiological Role of 5'-Nucleotidase:

A physiological role has not been firmly established for

5'-nucleotidase although a function in adenosine production has been proposed. Physiological concentrations of adenosine cause vasodilation in a number of mammalian organs including the heart, skeletal muscle, brain, intestine and adipose tissue (82, 83, 84). In contrast, adenosine produces vasoconstriction in the kidney (53) and liver (10). Further, adenosine inhibits lipolysis in adipose tissue (85) and has been implicated as neurohumoral agent in the brain (5). Adenosine also influences myocardial contractility in several mammalian species (10) and low concentrations of this nucleoside inhibit platelet aggregation (10, 86).

It has been shown by several investigators that the concentration of 5'-AMP influences the activity of at least three important enzymes in the glycolytic pathway and citric acid (71, 87, 88). This has been termed the adenylate control hypothesis, and some of the most important enzymes this control are glycogen phosphorylase, phosphofructokinase and fructose-1-6-diphosphatase. It is conceivable that the intracellular activity of 5'-nucleotidase may influence the concentration of 5'-AMP, and thus the activity of these enzymes (71).

There have been several observations that various hormones alter 5'-nucleotidase activity in various tissues, particularly those tissues that are target organs. For example, Gepts and Toussaint reported that the histochemical staining reaction for 5'-nucleotidase was intensified in the pancreatic islets of cortisone-treated rats (71). In 1958, Reid and Stevens (89) demonstrated that hypophysectomy in rats resulted in a decrease in liver nucleotidase activity. Although the hormonal control of the myocardial 5'-nucleotidase is poorly understood, it must be emphasized that further investigations about the behavior of 5'-nucleotidase in myocardium is greatly warranted mainly because it

plays a significant role in adenosine production which influences myocardial contractility. The information thus gathered can be of some importance in assigning a specific physiological role for this seemingly important enzyme in the heart.

METHODS

A. Animals

Male Sprague-Dawley rats (300-400 grams) were used for all experiments in this study. After decapitation, the hearts were quickly excised and washed free of blood with ice-cold saline solution.

B. Preparation of Plasma Membranes

i) Hypotonic shock-LiBr treatment method

Rat heart ventricles were washed thoroughly, diced with a pair of scissors, and homogenized in 10 volumes of 10 mM Tris-HCl, pH 7.4 containing 1 mM EDTA in a Waring Blender for 1 min. (30 sec. x 2), with an interval of 1 min. The homogenate was filtered through gauze and centrifuged at 1,000xg for 10 min. The sediment was suspended in 25 volumes of the above Tris-buffer and stirred in a cold room for 15 min. and centrifuged at 1,000xg for 10 min. The residue was then resuspended in 25 volumes of 10 mM Tris-HCl, pH 8.0, stirred for 15 minutes and centrifuged at 1,000xg for 10 min. The sediment was suspended in 10 mM Tris-HCl, pH 7.4, stirred for 15 min. and centrifuged at 1,000xg for 10 min.; this step was repeated again. The sediment was suspended in 25 volumes of 10 mM Tris-HCl, pH 7.4, extracted with 0.4 M Libr for 30 min. and centrifuged at 1,000xg for 10 min. The sediment was resuspended in 25 volumes of 10 mM Tris-HCl, pH 7.4, stirred for 10 min., then centrifuged for 10 min. The residue was suspended in 0.6 M KCl, 10 mM Tris-HCl, pH 7.4 and stirred for 15 minutes and centrifuged at 1,000xg for 10 min. The sediment was again washed with 10 mM Tris-HCl, pH 7.4, stirred for 10 min. and centrifuged for 10 mins. This sediment was suspended in 1 mM Tris-HCl, pH 7.4 and used. All the

above steps were performed at 0^{o} - 4^{o} C. For convenience of discussion these membrane vesicles will be called fraction HL. This isolation procedure was carried out essentially according to the method of McNamara et al (16) except that stirring times were reduced by 15 min. and an additional 0.6 M KCl 10 mM Tris-HCl pH 7.4 extraction step was performed before the last washing with 10 mM Tris-HCl, pH 7.4 (17).

ii) Discontinuous sucrose density gradient centrifugation

Rat heart ventricles were washed thoroughly; diced with a pair of scissors and homogenized in 0.25M sucrose by a 15 sec. burst at a setting 9 of polytron PT 10. The homogenate was layered on top of a gradient 39%, 49% and 80% (W/W) sucrose prepared in 50 ml tubes. These were then centrifuged in an SB-116 rotor for 90 min. at 55,000xg. The plasma membranes appeared mainly at the 0.25M and 39% interphase. Microsomes were located in the vicinity of interphase 39%/49%, and the dense mitochondrial fraction in the area of the 45%/80% interphase. Each fraction was collected with a Pasteur pipette, diluted with 0.25M sucrose then centrifuged for 20 min. at 90,000xg in an A-211 rotor. The final suspension of each particular set of pellets was in a small volume of 0.25M sucrose. All of the above steps were performed at 0^{o} - 4^{o} C. For convenience, this sarcolemmal preparation will be referred to as fraction S. This isolation procedure was carried out in principle according to the method previously described by Kidwai et al (18, 24). The modifications included the use of a discontinuous gradient, and the omission of the 100,000xg centrifugation of the homogenate. Both fractions (HL and S) could be stored at -80^{o}C for about 3 weeks without loss of 5'-nucleotidase activity.

C. Isolation of Mitochondria

Thoroughly washed rat heart ventricles were minced with a pair of scissors. The tissue was homogenized in 10 volumes of 0.18 M KCl-10mM EDTA-0.5% albumin. pH 7.4 in a Waring Blender for 2 x 10 sec with a 1 min. interval. The homogenate was filtered through 4 layers of gauze and centrifuged at 1,000xg for 10 min. The pellets were discarded and the supernatant was centrifuged at 10,000xg for 20 min. The pellets were washed in 10 volumes of the above mentioned KCl-buffer and centrifuged at 1,000xg for 10 min. The residue was again discarded and the supernatant further centrifuged at 8,000xg for 10 min. The pellets were thoroughly washed and suspended in a small volume of 50 mM KCl-20mM Tris-HCl, pH 6.8 and used. This isolation procedure was carried out according to the method of Sordahl et al (39) at 0^{o} - 4^{o}C. A teflon-glass homogenizer was used for homogenization of pellets.

D. Isolation of Microsomes

After the rat heart ventricles were thoroughly washed, they were minced with a pair of scissors. The tissue was homogenized in 10 volumes of 10 mM $NaHCO_3$-5mM NaN_3-15mM Tris-HCl, pH 6.8 in a Waring Blender for 45 sec. The homogenate was filtered through 4 layers of gauze then centrifuged at 10,000xg for 20 min. The residue was discarded and the supernatant further centrifuged at 40,000xg for 45 min. The pellets were suspended in 10 volumes of 0.6 M KCl-20mM Tris-HCl, pH 6.8 and centrifuged at 40,000xg for 45 min. Finally, the pellets were suspended in 0.25 sucrose-20mM Tris, pH 6.8 and used. This method is essentially the same as that described by Sulakhe and Dhalla (40). Isolation procedures were performed at 0^{o} - 4^{o} C.