MYOCARDIAL PURINE METABOLISM

ASPECTS OF MYOCARDIAL ATP METABOLISM AND PHARMACOLOGICAL INTERVENTION

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Purine metabolisme in het hart

Aspecten van myocardiaal ATP metabolisme en farmacologische interventie

PROEFSCHRIFT

TER VERKRIJGING VAN DE GRAAD VAN
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Die Aufgabe ist nicht sosehr zu sehen was noch keiner gesehen hat, sondern bei das was jeder sieht, zu denken was noch keiner gedacht hat.

(Schopenhauer).

Ter nagedachtenis aan mijn moeder.
Aan mijn vader,
aan Margreet.

CONTENTS

APPENDIX PAPERS

ENZYME ABBREVIATIONS AND NOMENCLATURE

Adenine-phosphoribosyl-transferase (APRT)	-AMP:pyrophosphate phospho-ribosyltransferase (EC 2.4.2.7)
Adenosine deaminase (ADA)	-Adenosine aminohydrolase (EC 3.5.4.4)
Adenosine kinase (AK)	-ATP: adenosine 5'-phospho-transferase (EC 2.7.1.20)
Adenylate cyclase	-ATP pyrophosphate-lyase (cyclizing)(EC 4.6.1.1)
Adenylate kinase (AdK)	-ATP:AMP phosphotransferase (EC 2.7.4.3)
Adenylosuccinate lyase	-Adenylosuccinate:AMP-lyase (EC 4.3.2.2)
Adenylosuccinate synthethase	-IMP:l-aspartate ligase (EC 6.3.4.4)
AMP-deaminase (AMP-D)	-AMP aminohydrolase (EC 3.5.4.6)
Creatine kinase (CK)	-ATP:creatine phosphotrans-ferase (EC 2.7.3.2)
Glucose-6-phosphate dehydrogenase (G-6PD)	-D-glucose-6-phosphate:NADP oxidoreductase (EC 1.1.1.49)
GMP-synthethase	-xanthosine-5'-phosphate: L-amido ligase (EC 2.4.2.8)
Hypoxanthine phosphoribo-syl transferase (HPRT)	-IMP:pyrophosphate phospho-ribosyltransferase (EC 2.4.2.8)
IMP-dehydrogenase	-IMP:NAD oxidoreductase (EC 1.2.1.14)

Nucleoside phosphorylase (NP)	-Purine-nucleoside:orthophosphate transferase (EC 2.4.2.1)
5' Nucleotidase (NP)	-5'-Ribonucleotide phosphohydrolase (EC 3.1.3.5)
PRPP synthethase	-Ribosylamine-5-phosphate pyrophosphate phosphoribosyl transferase (EC 2.4.2.14)
Uricase	-Urate:oxygen oxidoreductase (EC 1.7.3.3)
Xanthine oxidase (XO)	-Xanthine:oxygen oxidoreductase (EC 1.2.3.2)
Xanthine dehydrogenase (XD)	-Xanthine:NAD oxidoreductase (EC 1.2.1.37)

OTHER NON STANDARD ABBREVIATIONS

ADP	Adenosine-5'-diphosphate
AMP	Adenosine-5'-monophosphate
ATP	Adenosine-5'-triphosphate
bpm	beats per minute
c-AMP	3',5'-Cyclic AMP
Cr	Creatine
CrP	Creatine-phosphate
GDP	Guanosine-5'-diphosphate
g dwt	gram dry weight
GMP	Guanosine-5'-monophosphate
GTP	Guanosine-5'-triphosphate
HPLC	High-performance liquid chromatography
IMP	Inosine-5'-monophosphate
K_m	Michaelis-Menten constant. The substrate concentration at which the initial reaction velocity of an enzymatic catalyzed reaction is half maximal
PP_i	Inorganic pyrophosphate
PRPP	Phosphoribosyl-5'-pyrophosphate
SAH	S-adenosyl homocysteine
XMP	Xanthosine-5'-monophosphate
SR	Sarcoplasmatic reticulum
V_{max}	Maximal initial velocity of an enzymic reaction at saturated conditions

SAMENVATTING

Ons onderzoek is gericht op de opbouw en afbraak van ATP in het hart en de mogelijkheid om deze processen medicamenteus te beinvloeden. Het hart is verplicht continu energie te leveren voor de contractie en ionenpompen. Voor de aanmaak van ATP is in het hart de oxydatieve fosforylering het meest belangrijke proces. Dit proces, dat O_2 nodig heeft, wordt verstoord, indien de O_2 vraag hoger is dan het aanbod, zoals kan ontstaan bij een onvoldoende doorbloeding (ischemie). Dan kan ATP dalen door onvoldoende aanmaak, en adenosine en zijn catabolieten inosine, hypoxanthine, xanthine en urinezuur zullen in het effluent verschijnen. Het verlaagde ATP gehalte in de cel veroorzaakt verminderde contractie en verlaagde activiteit van diverse ionenpompen. Door dit laatste proces treden dan verstoringen op van de ionensamenstelling in de cel. Als gevolg daarvan wordt de stofwisseling gestoord en kunnen membraan beschadigingen ontstaan, met eventueel celdood als gevolg.

In hoofdstuk 2 van dit proefschrift wordt een introductie gegeven met een literatuur overzicht betreffende het ATP metabolisme in het hart. Ook wordt aandacht geschonken aan de relatie ATP en celfunctie, dit met betrekking tot de Ca^{2+} huishouding.

In hoofdstuk 3 worden methoden en technieken ter bestudering van het ATP metabolisme beschreven, met relatief veel aandacht voor een nieuwe techniek, de hoge druk vloeistof chromatografie. Deze techniek maakt het mogelijk om simultaan en geautomatiseerd myocardiale nucleotiden of nucleosiden en oxypurines in hartperfusaat te bepalen, dit in tegenstelling

tot de vroeger gebruikte enzymatische bepalingen.

In hoofdstuk 4 wordt het ATP metabolisme beschreven, in relatie met de adenosinecatabolieten, die de celmembraan kunnen passeren. Bepaling van deze stoffen buiten het hart kan dus een inzicht geven in het ATP metabolisme binnen het hart. Specifieke aandacht wordt geschonken aan de enzymen AMP-deaminase en xanthine oxydase/dehydrogenase.

In hoofdstuk 5 wordt een overzicht gegeven van de werking van Ca^{2+}-antagonisten en beta-blockers op deze processen, zowel tijdens normoxie als ischemie. Het blijkt dat de Ca^{2+}-antagonisten nifedipine en diltiazem een ATP sparend effect hebben op het hart, wanneer deze toegediend worden voor of tijdens ischemie. In hoeverre dit te wijten (of te danken) is aan een negatief inotroop effect, wordt verder besproken. De beta-blokker DL-propranolol, toegediend voor ischemie in het geisoleerde rattehart, vertoont geen beschermende werking tegen ATP afbraak geinduceerd door ischemie, maar de combinatie van propranolol en nifedipine werkt nog effectiever dan nifedipine alleen.

In hoofdstuk 6 wordt de biosynthese van adenine- en guaninenucleotiden in het geisoleerde rattehart uiteengezet. Hypoxanthine wordt ingebouwd in adenine- en guanine nucleotiden en deze inbouw is versneld na ischemie en gedurende ribose infusie. Geconcludeerd wordt dat deze inbouw van hypoxanthine afhankelijk is van het fosforibosyl-pyrofosfaat (PRPP) gehalte in het hart. Inosine, de voorloper van hypoxanthine, wordt met gelijke snelheid omgezet in nucleotiden als hypoxanthine, hoewel de hoeveelheid inosine die door het hart wordt opgenomen twee maal zo groot is. Verrassend is echter het snelle herstel van de lage ischemische ATP niveaus na perfusie met inosine. Dit snelle herstel kan niet verklaard worden uit de synthese snelheid van adenine nucleotiden uit inosine. Een verklaring hiervoor is nog niet gevonden. Een relatief grote hoeveelheid purines wordt in de GTP fractie ingebouwd (ca. 25% van die in de ATP fractie), hoewel de GTP concentratie ongeveer 30 maal lager is.

Het blijkt dat de concentratie van myocardiaal ATP een belangrijke parameter is voor de functie en overlevingkansen van de cel. ATP catabolisme tijdens ischemie kan verminderd worden door (voor)behandeling met calcium antagonisten, zoals nifedipine en diltiazem, en beta-blockers w.o. DL-propranolol. Verlaagde ATP spiegels na ischemie kunnen hersteld worden door toevoeging van een van de purines hypoxanthine en inosine, of ribose met een van de purines. Vooral het gunstige effect van inosine, hoewel even snel ingebouwd in ATP en GTP als hypoxanthine, lijkt veelbelovend en verdient verdere studie in de kliniek, omdat het naast farmacologische effecten (vaatverwijdend, positief inotroop) ook snel de verlaagde ATP niveaus in het hart kan herstellen.

SUMMARY

The main points of interests of our research are the anabolism and catabolism of myocardial ATP and the possibilities to influence the processes pharmacologically. The heart cell is geared for continuous delivery of energy for contraction and ionic pumps. For the supply of myocardial ATP, oxidative phosphorylation is quantitatively the most important process. This process, which is fundamentally O_2-dependent, is disturbed, when O_2 demand is higher than O_2 supply (ischemia or hypoxia). As a result, ATP levels will decrease, and the AMP-catabolites adenosine, inosine, hypoxanthine, xanthine and uric acid appear in the coronary effluent. The lowered ATP levels in the cell cause or reflect disturbances in contraction and the action of ionic pumps. Cellular ionic homeostasis is disturbed, membrane damage can occur and ultimately the cell dies.

In chapter 2 of this thesis, a literature overview is given of the relation of ATP metabolism and cell function and survival.

In chapter 3, methods and techniques, needed for the study of myocardial ATP metabolism, are discussed. Special attention is given to a technique, developed relatively recently: high-performance liquid chromatography.

In chapter 4, ATP catabolism is discussed, in relation to adenosine and catabolite release into the coronary-venous system. Determination of the purines in the extracellular fluid gives continuously insight in intracellular ATP metabolism. Special attention is given to the enzymes AMP-deaminase and xanthine oxidase/dehydrogenase.

In chapter 5, the action of the calcium antagonists nifedipine and diltiazem is discussed. Also the role of the beta-blocker DL-propranolol on ischemic ATP catabolism and purine release in isolated perfused rat hearts is described. (Pre)treatment with the Ca^{2+}-antagonists protects the heart from ischemic ATP catabolism, while DL-propranolol shows no effect under the same conditions. The combination of DL-propranolol and nifedipine acts more effectively than nifedipine alone. Whether this protective effect of Ca^{2+}-antagonists (with or without propranolol) is due to their negative inotropic effect or to other mechanisms, is discussed.

In chapter 6, the biosynthesis of adenine- and guanine nucleotides is discussed. Hypoxanthine is incorporated into adenine- and guanine nucleotides and this incorporation is accelerated after ischemia and during ribose infusion. We conclude from these observations that the hypoxanthine incorporation into myocardial nucleotides depends on the phosphoribosyl-pyrophosphate level in the heart. Inosine, as a precursor of hypoxanthine, is incorporated at the same rate as hypoxanthine, the uptake being twice as high. Ischemic ATP levels are restored much faster during reperfusion with inosine than could be accounted for by the measured incorporation rate.

It seems that the level of myocardial ATP is an important parameter for cell function and survival. ATP catabolism during ischemia can be diminished by (pre)treatment with Ca^{2+}-antagonists such as nifedipine and diltiazem, and beta-blockers such as DL-propranolol. Lowered ATP levels after ischemia can be restored by addition of the purines hypoxanthine and inosine. In particular the beneficial effects of inosine seem promising. It is incorporated into ATP and GTP at the same rate as hypoxanthine, but seems to restore post-ischemic ATP levels at a much higher rate. We suggest a further study of this compound in the clinical setting also because of its vasodilating and positive inotropic effects.

CHAPTER 1 AIM OF THE THESIS

The heart is obliged to contract continuously. This implicates for a human with an average heart rate of 70 beats per minute in about 70 years more than 2.5 billions of contractions. For this contraction ATP is needed as the main substrate. As a consequence continuous ATP production by the heart is obligatory. This ATP is mainly produced by the oxygen-dependent oxidative phosphorylation. Disturbances of the oxygen delivery, such as that induced by partial occlusion of one or more coronary arteries can lead to a distortion of the balance between ATP production and consumption. As a result, ATP is dephosphorylated, and subsequently the products adenosine, inosine, hypoxanthine, xanthine and uric acid pass the cellular membrane and appear in the coronary effluent. Because of the importance of adequate ATP levels for the heart, the following 4 main avenues have been investigated in this study, which forms the substance of my thesis.

1. Assays have been developed for the determination of high-energy phosphates in myocardial tissue and its dephosphorylated degradation products adenosine and its catabolites inosine, hypoxanthine, xanthine and uric acid in the coronary effluent. The methods are based on a relatively new technique, high pressure liquid chromatography.
2. Since ATP breakdown can continuously be monitored by the measurement of the release of adenosine and catabolites in the coronary effluent, the appearance of these catabolites has been evaluated as a biochemical marker of ischemia.

3. Recently developed drugs have been shown to prevent or delay ischemic ATP breakdown. Studies with beta-blockers and calcium antagonists and their combination have been compared to elucidate their efficacy.
4. It has been shown that diminished ATP levels after ischemia, due to release of adenosine and catabolites, can be restored by inosine or hypoxanthine infusion. Regulatory mechanisms have been investigated.

In as much as the results of the experiments have mostly been published in the relevant literature, reprints of these papers are bundled in the appendix. In the following chapters of this thesis, these results are discussed and placed in a more general context.

CHAPTER 2 GENERAL INTRODUCTION: ATP METABOLISM IN THE MYOCYTE

The function of the heart is to pump blood through the organs of the body. The heart has to do this without a moment of rest. To achieve its task, a double-chambered hollow muscle propels the blood into the vascular system by means of contraction. The power needed for this function is evoked by shortening of striated muscle cells, the myocytes. The substrates and oxygen for these cells are provided by the coronary arteries, which are the first branches of the aorta, under a pressure of about 120 mm Hg in systole and about 70 mm Hg during diastole. Removal of waste products occurs by way of the coronary venous system, which ends in the right atrium, a low pressure system. The vasculature contains smooth muscle cells, whose tonus is regulated and which in turn determines the coronary flow. This is the most important mechanism for substrate provision to the myocytes.

The substrates in the myocyte are oxidized to form ATP. Hydrolysis of this compound liberates and transforms chemical energy into mechanical energy, shortening of the fibers via actin/myosin interactions, as well as into electro-chemical energy for the ionic pumps, or for the synthesis of other chemical bonds.

2.1 Energy metabolism of the myocyte

In this paragraph a short review is given on ATP metabolism in the myocyte during three clinically important conditions, namely normoxia, ischemia and reperfusion. Special attention is

given to the significance of maintaining proper ATP levels in the heart.

2.1.1 Normoxic metabolism

In normoxic myocytes a balance exists between the production of ATP and its usage. About 90 % of the ATP is synthetized by oxidative phosphorylation in the mitochondria (see for a review [89]), and about 10% by anaerobic glycolysis [62]. Mitochondria are strictly dependent on O_2 [92, 93]; they mainly oxidize fatty acids and pyruvate, the latter arising from the anaerobic glycolysis of glucose, which takes place in the cytosol. A complex regulatory mechanism exists between fatty acid and carbohydrate metabolism [59, 60, 62, 63, 65, 83], to ensure adequate ATP supply.

ATP is used by the contractile apparatus; ionic pumps like Na^+-K^+ATPase and Ca^{2+}ATPase in sarcolemma and Ca^{2+}ATPase in the sarcoplasmatic reticulum (SR) and biosynthetic processes. About 80% of the ATP synthetized is used by the contractile apparatus. This is about 1 µmol per gram dry weight (µmol/g dwt) ATP per beat [23, 61] or about 5% of the ATP content in each cell. It has been shown, by [^{31}P]-nuclear magnetic resonance techniques, that ATP and creatine phosphate (CrP) levels fluctuate about 20% during the systole-diastole cycle in isolated, perfused rat hearts [18]. It has been postulated that the energy produced by the mitochondria is predominantly used by the contractile apparatus [34, 67, 72, 77]. This energy is transported from mitochondria to the contractile apparatus by CrP (for a review see [5]). The energy produced by anaerobic glycolysis is thought to be used by ionic pumps and membrane phosphorylation reactions [65, 83]. In fig. 2.1 this is schematically presented.

In the normoxic cell, the respiratory rate of the mitochondria is regulated by the ADP and P_i availability, i.e., the ADP and P_i generated during contraction regulates the respiratory rate of the cell. As a consequence ATP synthesis is in balance with its consumption [82].

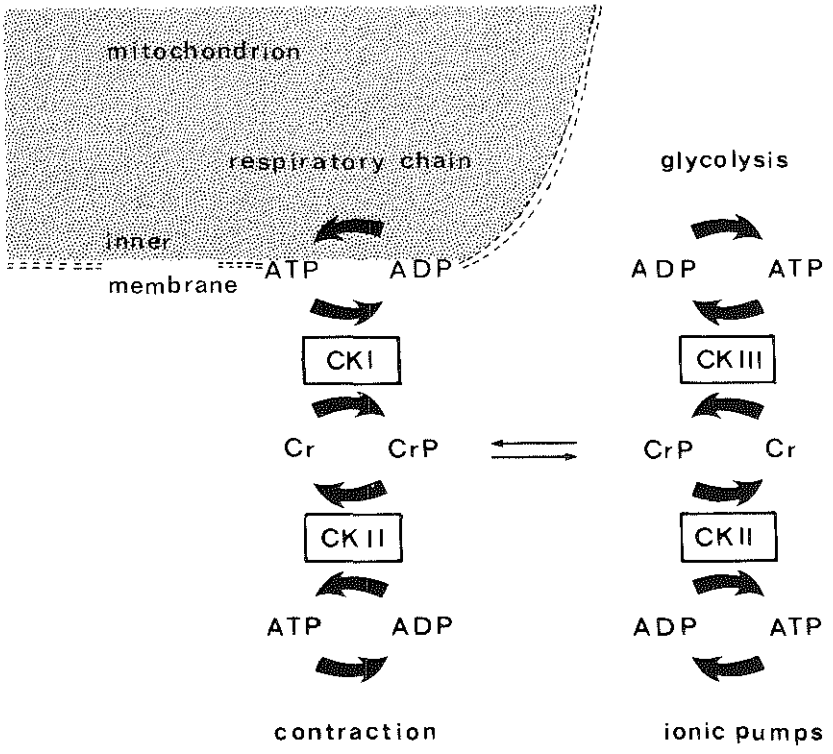

Fig 2.1. "Energy flows" in the myocyte during normoxia.
In the left part of the figure, energy derived from the oxidative phosphorylation in the mitochondria is shown to be transported to the myofibrils, while the energy derived from the glycolysis is used by ionic pumps. CK-I, CK-II and CK-III are different forms of creatine kinase.

Contraction is evoked by the amount of free Ca^{2+} in the cell. This amount is regulated by a voltage dependent Ca^{2+}-flux through the sarcolemma, a redistribution of intracellular Ca^{2+}, and a removal of Ca^{2+} by several Ca^{2+} pumps in sarcolemma and SR [14, 47, 56]. This Ca^{2+}-dependent contraction is mediated by c-AMP through changed Ca^{2+} sensitivity of the contractile apparatus [54, 75] and changed Ca^{2+} influx by phosphorylation of several membrane proteins. Whether this last process is mediated by changes in the glycocalix, the glycoprotein coat of the sarcolemma, is still a matter of debate [48].

2.1.2 Ischemic metabolism

Ischemia can be defined as a situation, where coronary blood flow (and hence oxygen and substrate supply, and carbon dioxide and metabolite removal) cannot meet the tissue demand [30, 50]. As a consequence of this lower O_2 supply vs demand, mitochondrial function is restricted [93]. The balance between ATP production and usage is disturbed, CrP levels fall, followed by a decline in ATP [8, 10, 32, 35, 44, 68, 78]. Mitochondrial ATP production (fig. 2.2) is impaired, the turnover rate declines and contractility decreases. Creatine (Cr), ADP, phosphate and H^+ levels increase [21, 25, 27], glycolysis rate is enhanced [1, 61, 74] and lactate levels rise.

The anaerobic ATP production, however, is insufficient to meet the amount of ATP needed for contraction [25]. Whether the fall in contractility is caused by a fall in ATP [25], an increase of H^+ [6, 13, 41, 61] or a decrease in the phosphate potential (=[ATP]/[ADP][P_i]) [40] is unknown at the moment. One of the problems hampering this investigation relates to the compartmentalization of ATP and the interdependence of several parameters. For example, a fall in ATP or CrP would cause a decrease in phosphate potential and an increase of H^+, therefore rendering the interpretation difficult.

When ATP levels decrease, cellular ADP levels increase. ADP is converted to ATP and AMP, by the action of adenylate kinase. AMP is deaminated to IMP, or dephosphorylated to adenosine, which is further catabolized to inosine and hypoxanthine. These components pass the cell membrane (a.o [9, 11, 12, 36, 66, 80, 84]), where adenosine acts as a vasodilator [4, 64, 80]. A slight decrease of ATP therefore results in an immediate rise in AMP-catabolites [86, 93]. This release can be used to monitor myocardial ATP-breakdown; this aspect will be discussed in more detail in chapter 4.

As a result of, or paralleled by, the fall of ATP, intracellular ionic homeostasis is disturbed by impairment of ionic pumps. Cellular Na^+ increases and K^+ decreases [52, 71]. Oedema occurs, membranes are damaged and enzymes leak from the

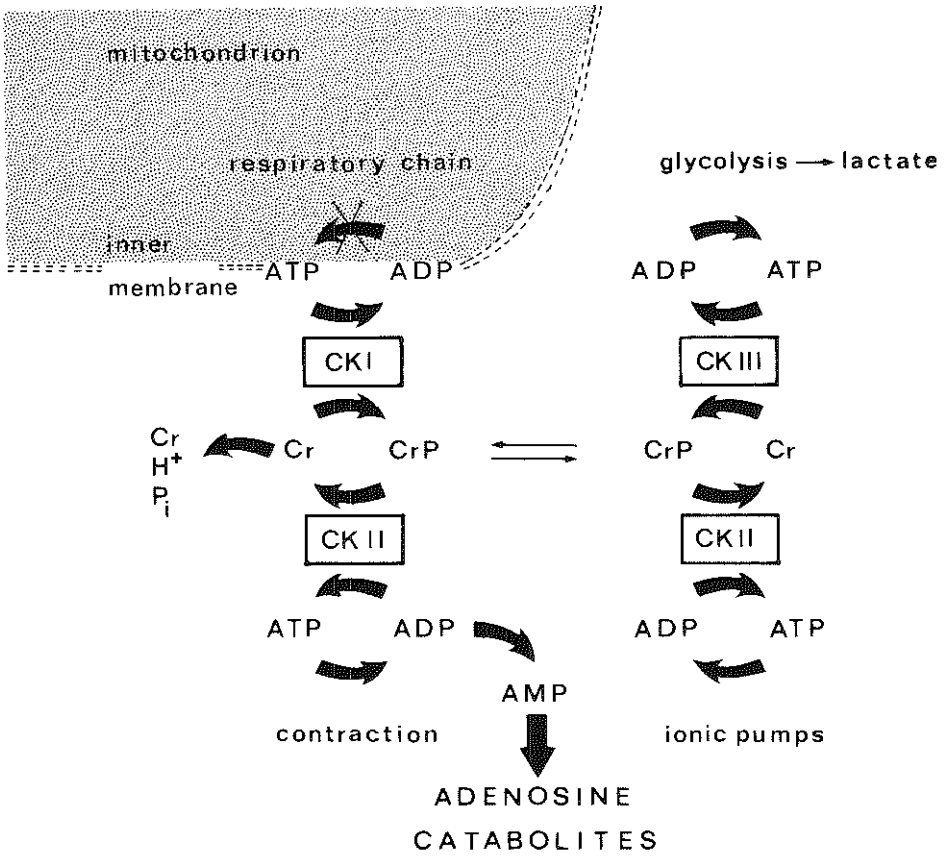

Fig. 2.2. A schemetic view of an ischemic myocyte.
Due to impaired O_2 supply, mitochondrial function is disturbed. CrP levels fall, and Cr and H^+ and P_i raise, since ATP demand of the myofibrils continues. Subsequently ATP decreases and ADP and AMP increase. The latter is broken down to adenosine and catabolites. Glycolysis can be enhanced, but will be slowed due to increased lactate or H^+ levels (see also legends to fig. 2.1).

cytosol and enter the blood stream. A slight increase in intracellular Ca^{2+} is seen [58, 81]. Also the long-chain fatty acid ester [60] and the acyl-carnitine ester [68] concentration increase, which can further deteriorate cellular function, with distortion of membrane structures [31, 42, 51] or inhibition of enzyme systems [46, 60, 65]. At a certain point the cell is irreversibly damaged, i.e., cellular functions are not restored during reperfusion and the cell dies. The onset of irreversible ischemic damage is associated with markedly depleted ATP

levels, cessation of glycolysis, depletion of glycogen and ultrastructural changes such as gaps in the sarcolemma, severely distorted mitochondria and a disorganized contractile apparatus [1, 20, 37, 44, 55, 71, 78].

2.1.3 Metabolism during reperfusion

During reperfusion, cells are reoxygenated and waste products removed. After reversible ischemia, reperfusion induces an enhanced Ca^{2+} influx, mitochondria are activated and ATP and CrP are again produced [26, 33, 53]. The latter compound is transported to the myofibrils (fig. 2.3). Because

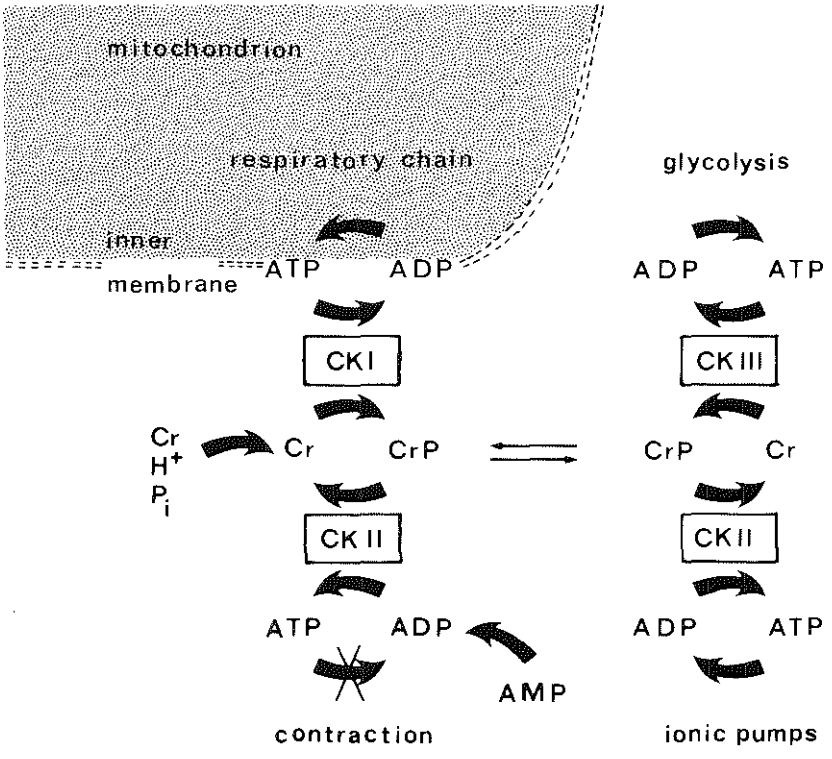

Fig 2.3. A schematic view of a reperfused myocyte.
During reperfusion the O_2 supply is restored, and the mitochondria start to produce ATP. Because myofibrillar ATP consumption is still disturbed, CrP may increase to levels above normal (see also legend to fig. 2.1).

of ionogenic disturbances in the cell, contraction is still decreased at this stage probably due to disturbed Ca^{2+} concentrations in the cell [69]. This can be demonstrated by an increased ventricular wall tension [2, 24, 49], indicating an increased Ca^{2+} level, and an increased CrP even to levels higher than the normal range [3, 17, 78 and appendix papers 6 and 8]. This indicates that ATP consumption by contraction is at this state below ATP production. After activation of the ionic pumps, cellular homeostasis is restored and the cell starts beating again. ATP levels will remain subnormal, because of washout of the AMP-catabolites during ischemia and the early phase of reperfusion (as indicated in chapter 4 and demonstrated by others [9, 11, 12, 36, 70, 84, 85, 88, 90]). Although the cell is able to function, it seems that these low ATP levels cause an extra risk in as much as a critically low ATP level will be reached earlier during the next ischemic attack [7, 22, 45, 73].

After irreversible ischemia, a massive Ca^{2+} influx parallels or causes an extension of ultrastructural damage, involving the development or intensification of contracture, activation of proteases and other hydrolases, disruption of myofibrils and sarcolemma, explosive cell swelling, rupture and loss of mitochondrial structure and the appearance of intra-mitochondrial calciumphosphate particles [20, 29, 37, 78].

The amount of Ca^{2+} influx during reperfusion is an important determinant for myocardial cell survival [15, 16, 43, 56, 57]. This influx is coupled to mitochondrial function [28, 76]. Reactivation of mitochondrial function restores the normal existing proton gradient over the inner membrane. It is likely that this triggers a cellular Ca^{2+} movement, which causes sarcolemmal membrane damage. The precise mechanism(s) however, remain(s) to be elucidated. There is a striking correlation between the cytosol Ca^{2+} during reperfusion and the ATP levels at the end of the ischemic period [15, 57]. Whether changes in the basement membrane play a role as they do during the Ca-paradox [95], is also still unknown [19, 28].

2.2 The significance of ATP in the myocyte

The importance of maintaining adequate ATP levels is indicated in several papers [29, 30, 38, 57, 74, 78, 87, 88, 91], where a good correlation was found between myocardial ATP levels at the end of an ischemic period and recovery of cell function during the subsequent reperfusion phase.

Ischemic ATP breakdown is a time-dependent process [27]. It is therefore of utmost importance to reperfuse the myocardium as fast as possible after ischemia. If the cell is completely devoid of ATP, cell death is inevitable. Because ischemia is a condition in which ATP levels are decreased, an important mechanism of maintaining ATP levels is to reduce ATP demand. Ca^{2+}-antagonists in particular, but also beta-blockers or their combination, seem suitable for this purpose. This will be further discussed in chapter 5. Another way of reducing ATP consumption can be achieved by lowering the myocardial temperature [57], thereby slowing down all biochemical processes, including ATP breakdown.
At present there is no substantial evidence, that calcium antagonists restore cell survival by lowering the Ca^{2+}-influx during reperfusion; this has been incompletely studied [79].

After reperfusion, reduced levels of ATP are restored by its biosynthesis. Several mechanisms are found to be involved as discussed in chapter 6. It is possible to enhance ATP synthesis with inosine [94 and appendix paper 8]. Current knowledge encourages future investigation on this topic in a clinical setting.

2.3 Conclusions

From this literature review, the following conclusions can be drawn:

It is beyond doubt, that the concentration of ATP (and its catabolites) is an important regulatory factor in the myocyte. For instance, a lot of enzymes needed in the sequence of ATP

catabolism are inhibited by high levels of ATP. Due to ischemia, a fall in ATP causes or parallels several undesirable effects, such as a fall in contractility and disturbed ionic homeostasis. The direct correlation between ATP concentration and disturbed cellular function is hampered by ATP compartmentalization. However, linear correlations between ATP concentration at the end of the ischemic period and the possibility to restore the cellular function during reperfusion indicate its significance. As a consequence of the importance to maintain proper ATP levels in the heart, measures must be taken to reduce its catabolism and low ATP levels after ischemia must be restored as quickly as possible.

CrP is thought to be the "energy carrier" from the mitochondria to the myofibrils. A fall in CrP and a rise in P_i indicate a disturbance of this transfer and probably precedes the fall in contractility. During reperfusion, it seems to indicate the restoration of the mitochondrial function and can therefore be used as a marker for the function and survival of the heart during the reperfusion phase.

Because ATP and CrP have to be measured in the heart, a process which is difficult to realize in the clinical setting, any reflection of these myocardial compounds at the outside of the heart is desirable. Measurements of the dephosphorylated ATP catabolites adenosine, inosine, (hypo)xanthine and uric acid seem to fullfill this criterion.

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CHAPTER 3 METHODS

3.1. The Langendorff preparation

To investigate myocardial ATP metabolism, the isolated rat heart has been studied. In the preparation, first described by Langendorff in 1895 [10], the isolated rat heart was attached to the perfusion apparatus at the aorta (see fig. 3.1) [3,12]. In our set up, the aorta was perfused retrogradely with a pressure of 72 mm Hg. This pressure ensured sufficient perfusion of the coronary system in a normal direction. The perfusate fluid consisted of a salt buffer [13], enriched with 10 mmol/l glucose. The buffer was kept at a temperature of 37 ^{o}C and was ventilated with an oxygen/carbon dioxide (95:5, v:v) mixture. The coronary effluent dripped from the heart and was collected continuously. In the perfusate, adenosine, inosine, hypoxanthine, xanthine, Uric acid and lactate were assayed [appendix paper 1]. The hearts beated at a spontaneous frequency between 240 and 320 bpm. As a standard procedure, the bundle of His was severed, and as a result the frequency fell to about 60 bpm. Thereafter the hearts were paced at a constant rate of 300 or 360 bpm.

In this preparation the aortic valve was closed, so the left ventricle was (nearly) empty and did not deliver external pump work. According to Neely [16] and Opie [17], glucose metabolism and contractility is proportional with perfusion pressure, i.e., work delivered by this preparation is dependent on perfusion pressure. As an indicator of the mechanical behaviour of the heart, apex displacement was measured [14, 21]. In this preparation, the heart contracts isotonically. Stam and De Jong

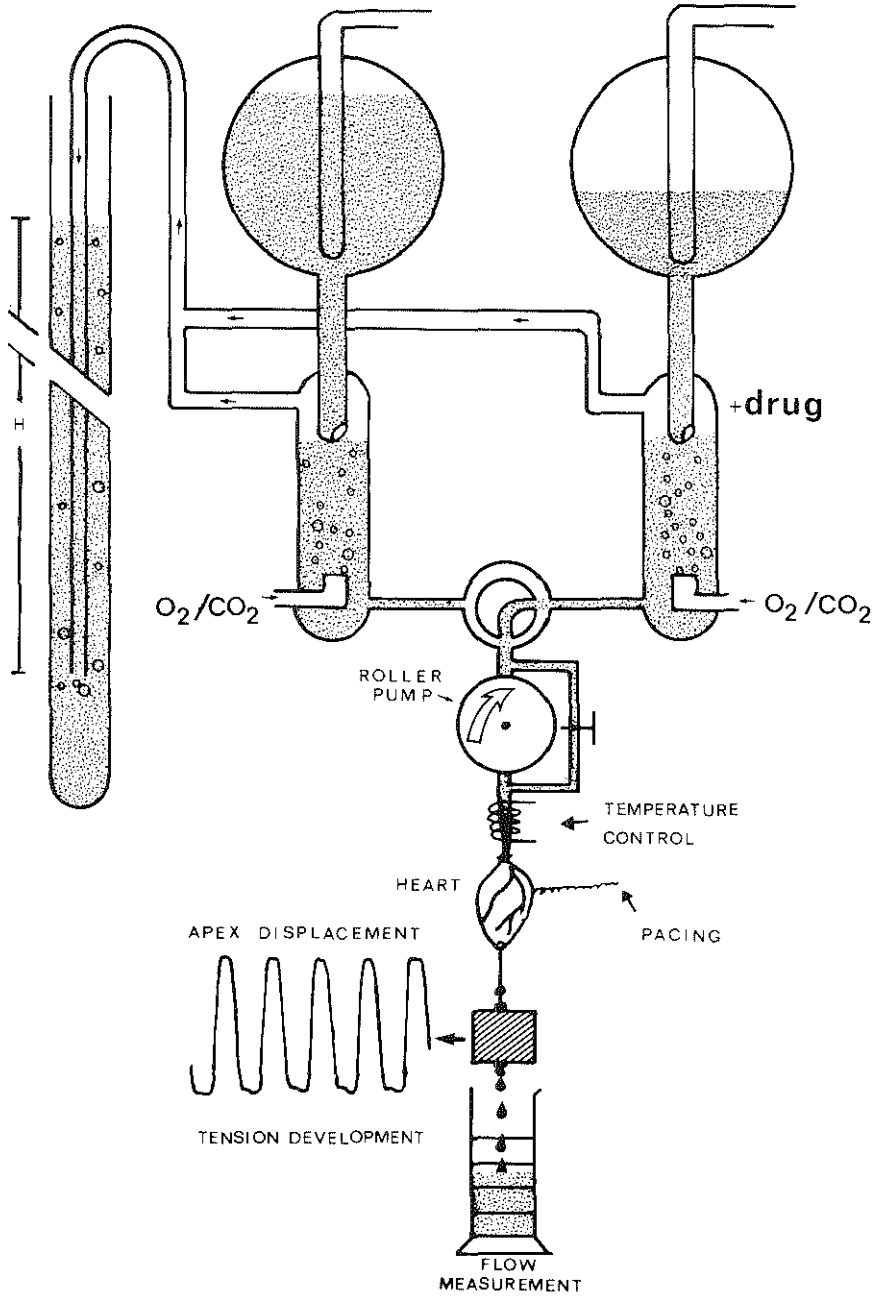

Fig 3.1. Schematic diagram of a perfusion apparatus.
The rat heart is attached to the canula via the aorta. H = perfusion height.

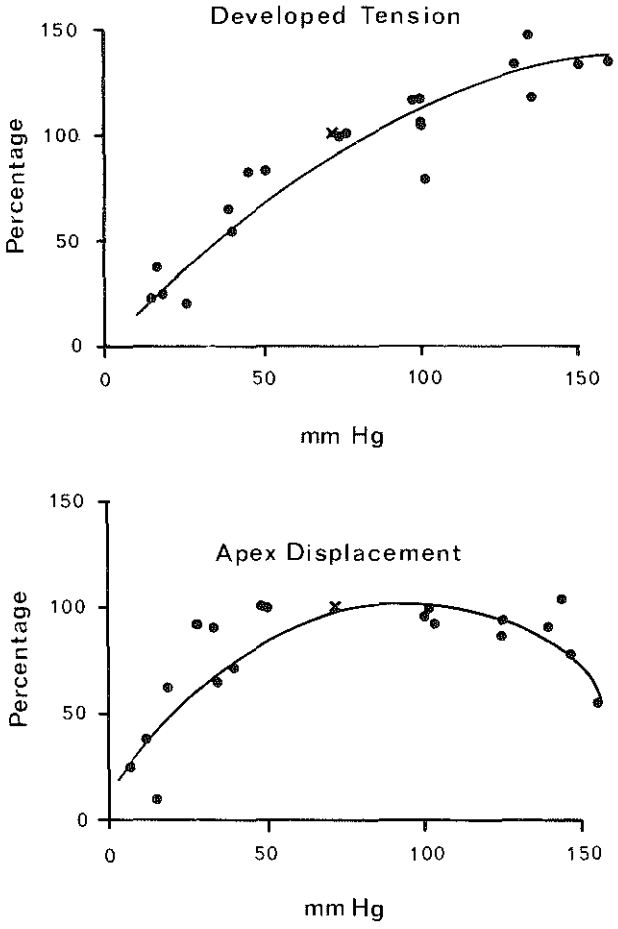

Fig 3.2. The influence of the perfusion pressure on developed tension and apex displacement.
Isolated rat hearts were perfused with a modified Tyrode solution, equilibrated with 95% O_2 and 5% CO_2. The hearts were paced at 300 bpm. Developed tension (upper panel) was measured with a pressure transducer, connected to the apex of the heart and monitored with a Brush recorder. Before ischemia the hearts were adjusted to a resting tension of about 12 g. Apex displacement (lower panel) was measured with a displacement transducer, fitted with a steel tampon, hooked to the apex of the ventricle and monitored on a Brush recorder. The values found at 72 mm Hg were taken as 100% (x), n = 3.
(Experiments were performed by Dr. J.W. de Jong and Mr. P.Ph. de Tombe.)

[21] had shown, that at a fixed perfusion pressure, a good correlation exists between apex displacement and left ventricular pressure. Apex displacement during normoxia, however, was slightly influenced by the perfusion pressure (fig. 3.2). Increased perfusion pressure resulted in a decrease in apex displacement, while contractility is known to increase during this condition [16, 17].

In some experiments, developed tension was considered to reflect myocardial function [15]. In this situation the heart contracts isometrically. Resting tension is set at about 10 g; a normoxic heart develops about 35 g. In fig. 3.2 is shown that during normoxia an increase in perfusion pressure gives an enhancement of developed tension. It seemed therefore that in experiments in which perfusion pressure had to be changed, developed tension is a more suitable measure for contractility than apex displacement.

Ischemia in the Langendorff heart was induced by lowering the flow to about 20% of control flow, either by decreasing the perfusion pressure or by restriction of the flow. To ensure a good temperature control, temperature of the perfusion buffer was measured in the canula. The temperature of the perfusion buffer was regulated by means of a feed-back control with a electric heating coil (fig. 3.1). This device gave us the possibility to keep the temperature fixed at 37 ^{o}C, even at a low flow rate.

Coronary flow was determined by timed collection of the perfusate. At the end of the perfusion experiment, hearts were clamped between two alumina blocks (Wollenberger clamps), precooled in liquid nitrogen. Nucleotides and CrP were assayed as described in appendix paper 2.

3.2 High-performance liquid chromatography

Chromatography is a separation technique, developed by Tswett in 1903, who used a chalk column to separate pigments in green leaves. The principle of chromatography is based upon

dynamic distribution of compounds (from a sample) between a moving phase (the column eluent) and a stationary phase (the column packing material). When flow rates, necessary to elute the column, produce a pressure exceeding 20 barr, it is called high-performance liquid chromatography (HPLC). This increase in pressure was needed because the particle size of the column material was decreased to obtain better separation characteristics. An HPLC system consists of one or more pumps to force the mobile phase through the column, an injector to apply the sample to the column, the stationary phase, positioned in a column, one or more detectors, recorders and electronic integrators (see fig. 3.3). The type of column, in combination with the buffer(s), used for elution, determines the separation of the compounds in the sample. The separation is a function of the particle size of the column packing, the composition of the elution buffer, and the flow.

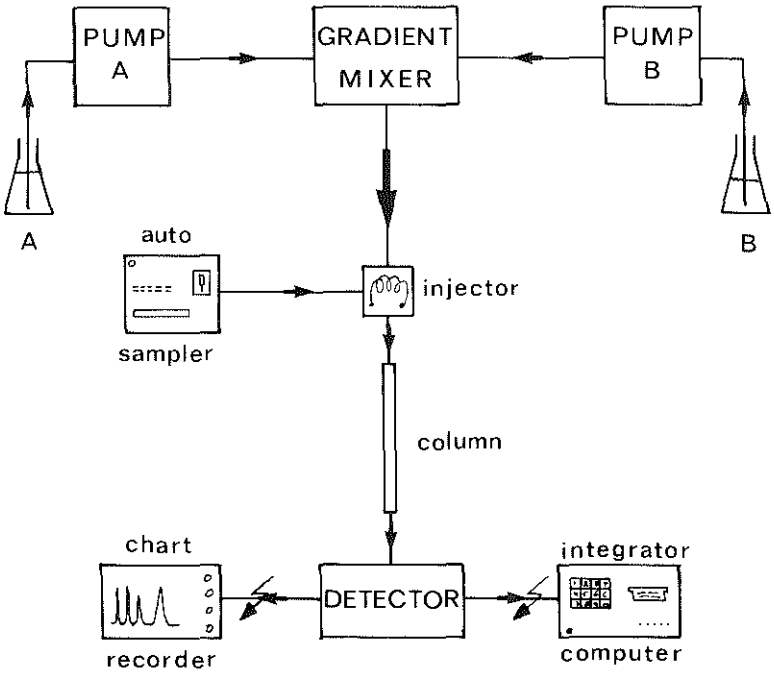

Fig. 3.3. Schematic diagram of a high-pressure liquid chromatograph. A and B are elution buffers of different composition.

We have used two types of columns. The first column is filled with apolar material (particles with a diameter of about 10 μm). This type of column is suitable for separations of apolar compounds such as adenosine and other catabolites. The other type of column is filled with polar particles of a strong anion exchanger for the separation of the polar high-energy phosphates in the myocardium.

3.2.1 Adenosine(catabolites) determination

Adenosine, inosine, hypoxanthine, xanthine and uric acid were separated on a C_{18}-μBondapak reversed phase column. The eluent contained 3 to 10% methanol, with flow rates between 1 and 3 ml/min [see appendix papers 1 and 5]. Samples must be deproteinized to maintain a proper column function [4]. Rat heart perfusates were directly injected onto the HPLC system (200 μl) and peaks were detected at 254 nm. The minimal detectable concentration of the adenosine(catabolites) was about 10 nM. Analysis time was 25 min and the whole system was automated.

For determination of these compounds in blood, the procedure was more complicated. In appendix paper 1 we have shown, that during the preparation of plasma, hypoxanthine and xanthine levels increase 2 to 5 times. When serum was prepared, adenosine, inosine, hypoxanthine and xanthine levels increased up to 20 times (fig 3.4). This implies catabolism of ATP and GTP from erythrocytes and platelets during sample preparation [7,25]. This has recently been confirmed [2, 6]. We decided to deproteinize whole blood with cold perchloric acid as described by Remme et al. [19] to stop nucleotide breakdown. Nucleotides interfered with the HPLC separation and were removed by alumina oxide (appendix paper 1). Minimal detectable concentrations of purines in whole blood are 100 nM, with a sample loop of 200 μl; HPLC analysis time is about 1 h. Per column, 200-500 analyses can be performed.

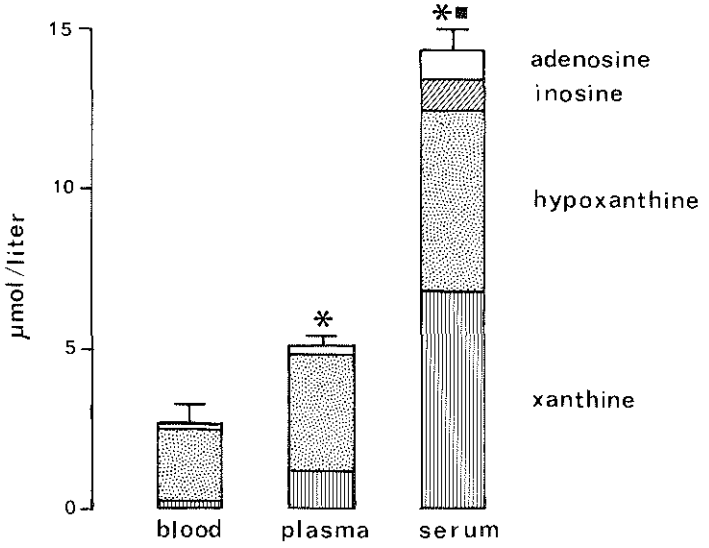

Fig 3.4. Concentration of adenosine and catabolites in human blood, plasma and serum.
The purines were measured by HPLC as described in appendix paper 1. Data represent mean values ± SE of 5-6 non-resting volunteers * P<0.01 vs blood, ■P<0.05 vs plasma [21].

3.2.2 High-energy phosphate determination

Myocardial high-energy phosphates were determined on a Partisil SAX-10 column. Because of large differences in polarity between AMP and ATP, gradient elution was necessary. Within 30 min AMP, ADP, ATP, GMP, GDP, GTP, Cr, CrP, and NAD^+ can be separated and quantitated in heart extract as described in appendix paper 2. Fifty to 250 analyses can be performed per column. We have also used this technique for the determination of nucleotides in liver, kidney and adipose tissue. We adapted this system for determination of adenine nucleotides and CrP in heart biopsies as small as 5 mg wet weight(= about 1 mg protein). In this case about 1 µmol nucleotide/ g protein can be detected, detection limits were about 1 µmol nucleotide/g protein. Control values of ATP in pig heart biopsies were found to be comparable with the values found in isolated rat hearts, which were freeze clamped [22].

3.2.3 Special applications of HPLC

1. Radiolabeled compounds have been injected onto the HPLC system, subsequently the separated products were collected after detection. [20 and appendix paper 1].

2. Enzyme reactions have been followed by monitoring decreasing substrate concentration and increased product concentration [9, 20].

3. It proved possible to collect an eluent fraction after ion-exchange HPLC and inject it onto a reverse phase column. If this fraction was collected in a sample loop, it could directly be injected to an other column ("column cutting"). An example for this technique is the assay of S-adenosyl-homocysteine [1].

4. Application of the fluorimeter as a detector gave the possibility to selectively detect compounds in a complex mixture [5, 8].
For example, direct injection of 100 µl urine onto a reversed phase column, with fluorescence detection at 440 nm (excitation 260 nm) resulted in only 2 peaks within 5 min, one being riboflavine. Concentrations of 1 nM could easily be detected [De Tombe, personal communication]. Detection of riboflavine in urine is important, for instance when riboflavine is used as a tracer compound in drugs to test the compliance.

5. Detection of drugs in plasma can easily be achieved with minor sample preparation [8]. For example, HPLC assays for verapamil [24], nifedipine [18], diltiazem [23], propranolol and catabolites [11] in plasma have been published.

3.3. Conclusions

The isolated rat heart, perfused according to Langendorff, provides a system to study metabolic processes. Although no

pump work is performed, the preparation contracts and therefore ATP is used by myofibrils. Contractility, however, is difficult to measure. Apex displacement can easily be obtained and during constant perfusion pressure a good correlation was shown between apex displacement and left ventricular pressure. When differences in contractility during changes of the perfusion pressure must be established, the measurement of developed tension seems more appropriate.

HPLC is a flexible technique to determine several compounds in biological matrices. The system is easily automated, several compounds can be detected within one run, and also parts of the chromatogram which are not used for compound quantitation, can be used as a visual check in the course of the analysis. Commonly samples must be purified, in which case one makes sure that the compounds to be determined are indeed recovered.

3.4. References

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CHAPTER 4 MYOCARDIAL ATP CATABOLISM

In this chapter, the literature about the enzymes which are involved with the catabolism of ATP in the heart is reviewed. Furthermore, some experiments are discussed, which show the relation between ischemia and the dephosphorylated ATP catabolites, recovered in the perfusate. Subsequently, the adequacy of purine release as a marker of ischemia in humans is discussed.

4.1 Introduction

During ischemia the balance which exists between ATP formation and ATP breakdown is disturbed. Mitochondria consume oxygen. When the pO_2 is lowered, mitochondria may conserve all O_2 available, and oxidative phosphorylation stops. Anaerobic glycolysis is enhanced and lactate is produced, but this pathway does not produce enough ATP. As a consequence, a fast decline in CrP is observed, concomitant with a slower decline of ATP. Simultaneously, P_I, ADP, AMP and IMP accumulate in the heart [1, 9, 17, 33, 41, 42, 86]. This process is schematically depicted in fig. 4.1. The mononucleotides AMP and IMP are dephosphorylated to adenosine and inosine, respectively [9, 28, 63, 84]. These purines are released into the venous coronary system [10, 19-22, 31, 44, 46-48, 53, 77-79]. Further breakdown of these purines to hypoxanthine, xanthine and uric acid can also take place in the heart [9, 20, 58, 67, 68, 76, 78, 81, 82 and appendix papers 5-8]. In the next paragraph a discussion of the enzymes is presented, which are intimately involved in the catabolism of ATP up to uric acid or allantoin.

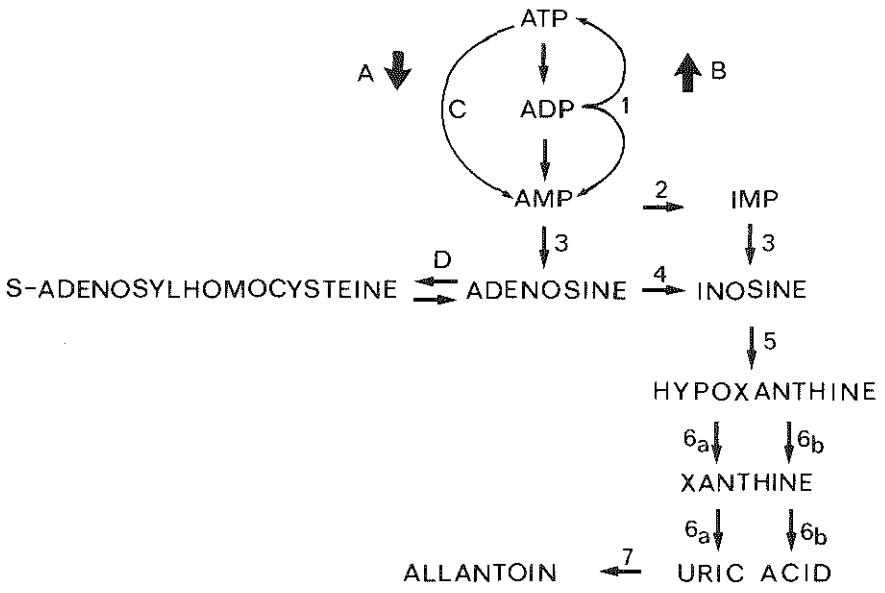


Fig. 4.1. Catabolic pathways of ATP.
1 = adenylate kinase, 2 = AMP-deaminase, 3 = 5' nucleotidase, 4 = adenosine deaminase, 5 = nucleoside phosphorylase, 6^a = xanthine oxidase, 6^b = xanthine dehydrogenase, 7 = uricase. These enzymes are discussed in chapter 4. A = ATP-consuming processes, such as contraction and ionic pumps. B = ATP-forming processes, such as oxidative phosphorylation and glycolysis. C = PP_i-forming enzymes (for instance, PRPP synthetase or adenylate cyclase). D = SAH-hydrolase.

4.2.1 Adenylate kinase

The enzyme adenylate kinase (AdK) catalyzes the following equilibrium:

$$2\ ADP \Longleftrightarrow ATP + AMP$$

A rise in ADP levels during ischemia or hypoxia will shift the equilibrium towards the formation of ATP and AMP. Two AdK isoenzymes have been reported for heart, one in the mitochondria, the other in the cytosol [61, 92]. Disturbances of oxidative phosphorylation will increase ADP levels near the mitochondrion, where the first AdK might function to lower the raised ADP concentration [29]. This reaction is thought to be one of the major cellular reactions which stabilize and

maximize the so-called energy charge, (ATP + 0.5 ADP)/(ATP + ADP + AMP) [7]. Arch and Newsholme [6] attribute to AdK the role of biological amplifier, because a relatively small ATP decline will bring about a manifold rise in AMP concentration. This is important in, for example, the regulation of glycolysis.

The cytosolic AdK form is thought to play a role in the reverse reaction, to transfer AMP into ADP. This isoenzyme is inhibited by high AMP concentrations (> 1 mM) and thereby spares the use of ATP during this condition. Only after severe ischemia or hypoxia, can high AMP concentration be expected [39 and appendix paper 2].

4.2.2 AMP-deaminase

AMP which is formed by way of AdK can be deaminated to IMP by the enzyme AMP-deaminase [AMP-D]. NH_3 is released in this reaction.

$$AMP \longrightarrow IMP + NH_3$$

IMP is the common point between the de novo nucleotide synthesis and hypoxanthine salvage; both reactions are involved in ATP and GTP biosynthesis (see chapter 6) and may have important regulatory properties [30]. The NH_3 formed may stimulate the glycolysis [45]. AMP-D is another enzyme which elevates the energy charge of the cell. Although by this reaction total adenine nucleotide levels are decreased, the IMP formed is not lost from the heart and can eventually be reincorporated into the adenine nucleotide pool.

Currently, controversy exists about the exact mechanism which regulates AMP-D activity. ATP, ADP, K^+, Mg^{2+} and CrP may all play a role in this context [14, 43].

We have purified the enzyme AMP-D from pig skeletal and heart muscle, to get an insight into the importance of this enzyme, especially in heart tissue [appendix paper 3]. In skeletal muscle we found AMP-D to be present in a larger amount, more active and with a higher V_{max} and a lower K_m than

the pig heart enzyme. In skeletal muscle a low molecular weight inhibitor is present, which can be removed by Sephadex column chromatography. This may indicate the relative importance of the enzyme in skeletal muscle compared to myocardium. Both purified enzymes are found to be regulated by the energy charge [see appendix paper 3]. From this study we conclude, that in the heart the enzyme is present, but during normoxia it will not play an important role. During ischemia or anoxia, however, this enzyme is activated and a substantial part of the AMP can be deaminated to form IMP, which in turn can be dephosphorylated to inosine. This is in accordance with findings of Achterberg and De Jong [2], who stated that part of the inosine released by the heart is not derived from adenosine.

4.2.3 5'-Nucleotidase

Apart from deamination to IMP, AMP can be broken down to adenosine by dephosphorylation according to the following equation:

$$AMP \longrightarrow adenosine + P_i$$

Dephosphorylation of AMP by aspecific phosphatase plays a minor role in this process [79]. This process also increases the energy charge, but in this case the end product can leave the heart. Two forms of 5' nucleotidase (5'N) exist, namely ecto- and endo-5'N. Ecto-5'N is a membrane-bound enzyme. AMP is the preferred substrate; the enzyme exerts its action on the outside of the cell. Extracellular AMP probably is produced by breakdown of extracellular c-AMP and/or ATP, the last compound being released by the heart in minute concentrations (< 50 nM) as a physiological vasodilator [16, 69, 79, 83]. The extracellular adenosine formed by ecto-5'N is thought to be taken up by the heart and rephosphorylated by adenosine kinase into intracellular AMP [25, 32, but see 73].

Endo-5'N is a cytosolic and soluble enzyme. It shows less substrate specificity than the ecto-form. It dephosphorylates

AMP, IMP, GMP and XMP to their nucleosides. Endo-5'N has a low K_m for AMP (between 20 and 30 μM), is Mg^{2+} dependent and inhibited by ATP [79]. It is thought that AMP formed by anoxic ATP breakdown, is predominantly broken down by endo-5'N [51, 55, 79].

Recently, Lamers et al. [50] purified two 5'N isoenzymes from rat heart sarcolemma, one having the characteristics of the ecto-5'N, the other having endo-5'N characteristics.

The metabolic fate of cytosolic AMP is dependent on the relative activity of endo-5'N and AMP-D. In the myocardium from several species, a tremendous variation between the activities of these two enzymes can be found [55]. Burger and Lowenstein [14] stated that in rat heart AMP is predominantly dephosphorylated. However, studies in our laboratory seem to indicate the importance of deamination [2]. In control and ischemic rat hearts IMP concentrations remain low [38, 66, 84]. It is likely that IMP formed from AMP is directly converted to AMP or GMP, or dephosphorylated to inosine.

Adenosine, formed by AMP dephosphorylation, is transported from the cytosol to the extracellular space and the coronary venous system. Adenosine is a strong vasodilator and is thought to be involved in the autoregulation of the heart [10, 44, 54, 62, 79]. Autoregulation is a process, by which a larger O_2 demand of the heart is reflected by an increase in coronary flow. In this respect, the papers of Nutinen et al. [60] and Thompson et al. [85] are interesting. They describe the relation between the O_2 need of the mitochondria and the adenosine production in the heart. However, recently conditions were found, in which increased flow is not correlated with increased adenosine concentration [23, 49]. These authors conclude from their studies that adenosine is not the only metabolic factor responsible for coronary vasodilation.

Adenosine influences c-AMP dependent processes, either by direct modulation of the adenylate cyclase system or by way of purinergic receptors. Adenosine also inhibits the voltage-dependent Ca^{2+}-influx, which could explain the cardio-depressant effects of high doses of adenosine [27, 71].

4.2.4 Adenosine deaminase

The enzyme adenosine deaminase (ADA) is widely distributed. It is found in myocytes, interstitium, epithelial cells, blood and blood cells and catalyzes the following reaction [18, 57, 70, 72, 88]:

$$\text{adenosine} \longrightarrow \text{inosine} + NH_3$$

Because of of ADA activity in the above mentioned compartments, adenosine concentrations are low. Olsson et al. [64] speculate that in normoxic hearts, most of the cellular adenosine is bound to the enzyme SAH-hydrolase.

4.2.5 Nucleoside phosphorylase

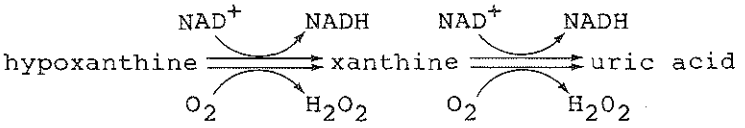
Nucleoside phosphorylase (NP) catalyzes the following equilibrium:

$$\text{inosine} \xrightarrow{P_i} \text{hypoxanthine} + \text{ribose-1-phosphate}$$

NP is found in epithelial cells, blood and blood cells [57, 70, 72, 88]. It is not found in myocardial cells. Therefore the production of hypoxanthine mainly occurs in the vessel walls and the blood. Hypoxanthine can be reutilized by the cells to form IMP, while inosine cannot (see chapter 6).

4.2.6 Xanthine oxidase/dehydrogenase

The enzyme wich catalyzes the conversion of hypoxanthine to uric acid appears in two different forms. One form uses O_2 as an electron acceptor and produces radicals (xanthine oxidase (XO)), the other form uses NAD^+ as electron acceptor and forms NADH (xanthine dehydrogenase (XD) [8, 76]).



The enzyme is found in large amounts in the liver but also in small amounts in the heart [51, 76], probably situated in or on the epithelial cells [12]. It exists mainly in the dehydrogenase form [8, 76], but can be converted to its oxidase form under certain conditions [74].

Surprisingly, XO is also found in large amounts in bovine milk. In 1950 Oster claimed that drinking of homogenized milk increases blood XO activity. These increased levels of blood XO are thought to cause epithelial damage in the coronary arteries, which could start the atherosclerotic process [for a review, 15]. Although this theory is highly speculative, we found in our laboratory that milk XO can be absorbed from the intestinal tract in the blood. Also, incubation of heart homogenates with XO produces depletion of plasmalogen, a phospholipid constituent of cell membrane [75]. Production of superoxide radicals by XO, either derived from milk or from conversion of heart XD, may also lead to membrane damage [74].

Most investigators ignore the existence of XO/XD in heart tissue, and assume that hypoxanthine is the end product of myocardial ATP breakdown [10, 66, 77]. However, already in 1961, Benson et al. [9] detected xanthine in the myocardium. Furthermore myocardial xanthine and uric acid release is shown by several authors [1, 20, 38, 68, 76 and appendix papers 5-8]. We found that in a normoxic rat heart over 70% of the purine release consists of uric acid. During ischemia this percentage falls to about 20% (see fig 4.2).

4.2.7 Uricase

In primates, including man, and in the dalmatian dog, uric acid is the end product of nucleotide catabolism [26]. It is excreted by the kidneys [35]. In all other mammals uric acid is converted to allantoin. In rat heart no allantoin release is

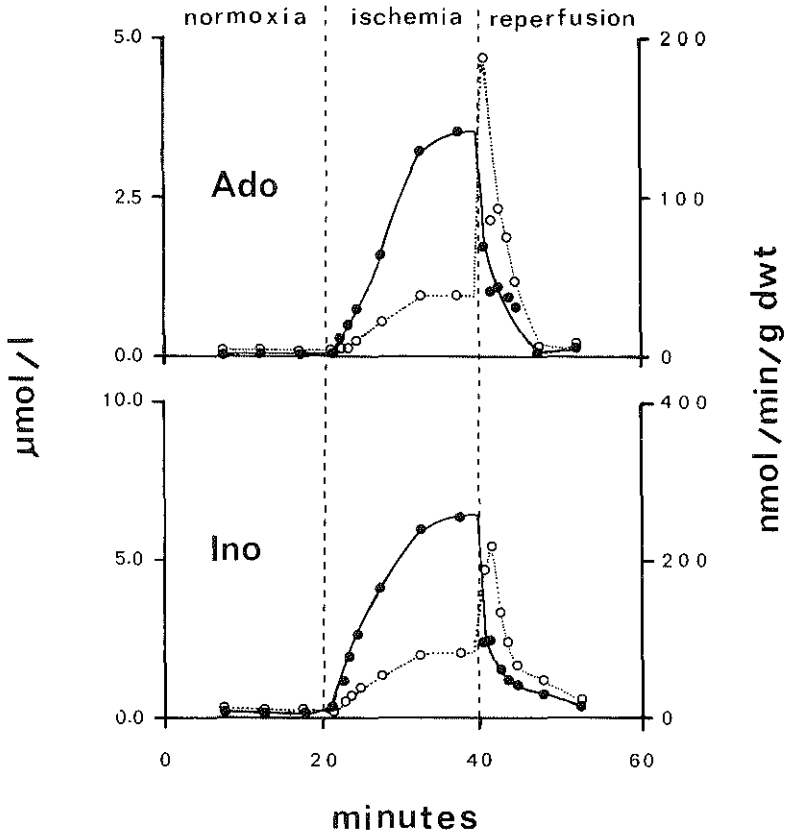
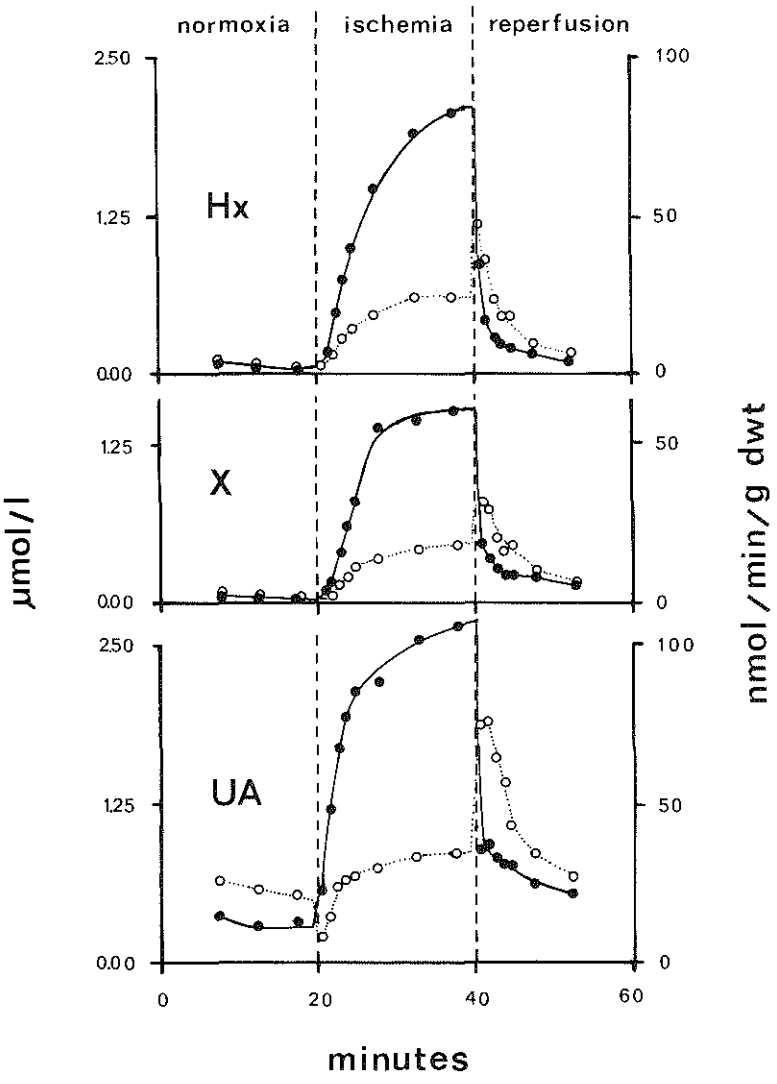


Fig. 4.2. Myocardial nucleoside release during normoxia, ischemia and reperfusion.
Isolated rat hearts were retrogradely perfused with a modified Tyrode solution, gassed with 95% O_2 and 5% CO_2, pH = 7.4. Heartrate = 300 bpm, perfusion pressure during normoxia and reperfusion = 70 mm Hg, and during ischemia = 15 mm Hg. Nucleosides were determined in the coronary effluent by HPLC. ●---● = concentration in the coronary effluent (µmol/l). o---o = amount released by the heart (nmol/min/g dwt). In the right part of the figure are plotted the oxypurines hypoxanthine (Hx), xanthine (X) and uric acid (UA) and in the left part of the figure the nucleosides adenosine (Ado) and inosine (Ino).



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detected, even after perfusion with 20 μM ^{14}C-inosine or -hypoxanthine [38 and appendix paper 8]. We conclude that this enzyme is either absent or inactive in rat heart.

4.3.1. Purine release as a marker for ischemia

In the previous paragraphs it is described that catabolism of ATP ultimately results in the appearance of adenosine, inosine, hypoxanthine, xanthine and uric acid (see fig. 4.1 and 4.2). It was also established that a decrease of myocardial adenine nucleotides during ischemia is recovered as the amount of AMP-catabolites in heart and coronary effluent [1, 41, 72]. Because of the fast transport mechanisms between intra- and extracellular nucleosides and oxypurines [65], it seems plausible that adenosine catabolites in the coronary effluent reflect the myocardial ATP breakdown [see also 17, 20, 82 and appendix paper 7]. In fig. 4.2 an increase of perfusate adenosine and catabolites is demonstrated within the first minutes of ischemia. Lactate, the end product of anaerobic glycolysis, is also used as a biochemical marker for ischemia [3-5, 17, 19, 40, 46-48, 52, 90, 91 and appendix papers 1 and 5].

4.3.2 The use of purine release as a marker for ischemia in the clinical setting

From 1960 on, several studies have discussed the release of purine components during ischemia or anoxia (see table 4.1). A close correlation has been found between purine- and lactate release from animal and human hearts. [18, 19, 22, 46-48, 67, 91]. Lactate as a marker of ischemia, however, has several disadvantages. During normoxia, lactate is preferentially taken up by the heart [24]. In fact, lactate released from a local ischemic area can be metabolized by the surrounding normoxic tissue [5]. The formation and removal of lactate is also influenced by blood fatty acid levels, acidosis and by a

Table 4.1.
AMP-catabolites in blood and urine as marker for myocardial ischemia in patients.

Purine	Body fluid	Clinical situation	Ref.
Adenosine	A-CS plasma	APST	[31]
Uric acid	Serum	AMI, IHD	[37, 53]
Hypoxanthine	A-CS blood	APST	[app. 1, 67]
Hypoxanthine	Peripheral venous plasma	ischemia, AMI,AP	[34, 56]
Adenosine, inosine, hypoxanthine	A-CS plasma	CABS	[31]
Inosine, hypoxanthine	A-CS plasma	APST	[46-48]
Hypoxanthine, xanthine, uric acid	Urine	Cardiac arrest AP, AMI	[13]
Adenosine, inosine, hypoxanthine, xanthine	A blood	CABS	[11]
Hypoxanthine,	A-CS blood	2 x APST	[22]

A = arterial; CS = coronary sinus; APST = atrial pacing stress test; AMI = acute myocardial infarction; IHD = ischemic heart disease; CABS = coronary-aorta bypass surgery; AP = angina pectoris; app. 1 = appendix paper 1.

diabetic condition [for a review, see 89]. In appendix paper 1, fig. 4, the myocardial hypoxanthine and lactate release are plotted from patients with a coronary stenosis larger than 50%, during an atrial pacing stress test. Thirty min after the end of the pacing period, hypoxanthine is still being released by the heart, while lactate release is converted to uptake. Selwyn et al. [80] have shown, that even 30 minutes after an atrial pacing stress test, the Na^+K^+-ATPase activity is still disturbed. They conclude that during that post-pacing period the heart has still not recovered from the provoked ischemia. This observation is in agreement with the prolonged hypoxanthine release found after the pacing period.

4.3.3 Problems with the use of purine release as a marker of ischemia

1. It is important to determine those purines in blood which are released by the heart and not those originating from other tissues. Therefore, paired arterial and coronary-venous samples must be drawn.

2. When one ischemic event is relatively quickly followed by a second one, purine production during the latter period is substantially lower. Therefore, an experimental animal or a patient cannot serve as its/his own control during a study of drug efficacy under these conditions [81].

4.4 Conclusions

Myocardial purine production is a direct result of ischemia and therefore a promising biochemical indicator of ischemic heart disease. Because purine production can take place in many other tissues, the arterio-venous differences across the heart must be assessed, to assure that indeed purine release from the heart is measured.

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CHAPTER 5 PHARMACOLOGICAL PREVENTION OF ISCHEMIC ATP BREAKDOWN

5.1 Introduction

During ischemia, intracellular ATP levels decline as a result of the disturbed balance between production of ATP and its usage as detailed in chapter 4. As a result of low ATP levels, the ionic pumps are disturbed [54, 100], ionic homeostasis will be changed and ultimately, perhaps via action of activated lipases and proteases [49], membranes are damaged and finally the cell dies [54, 63]. This process is depicted in fig. 5.1. Whether the decrease in ATP is the main cause in this sequence to cell death is still a matter of debate [9, 23, 42-44], but ATP levels at the end of the ischemic period are inverse related to the damage during reperfusion [33, 43, 45, 63, 79]. From any point of view, it seems advisable to maintain high ATP levels by preventing or delaying ATP catabolism during ischemia.

In principle, 4 different methods can be used to achieve this:

1. Myocardial flow can be increased. Higher flow rates will result in a higher substrate and oxygen supply and hence accelerate myocardial ATP production. Ca^{2+}-antagonists can achieve this, provided that a stenosis is not the permanently restricting factor [17, 36, 37, 104, 114].
2. Cardiac work can be decreased. When ATP production is limited by flow, reduced usage of ATP can restore the disturbed ATP balance. This is achieved by negative inotropic agents such as beta-blockers, Ca^{2+}-antagonists [10, 11, 29, 82], or by

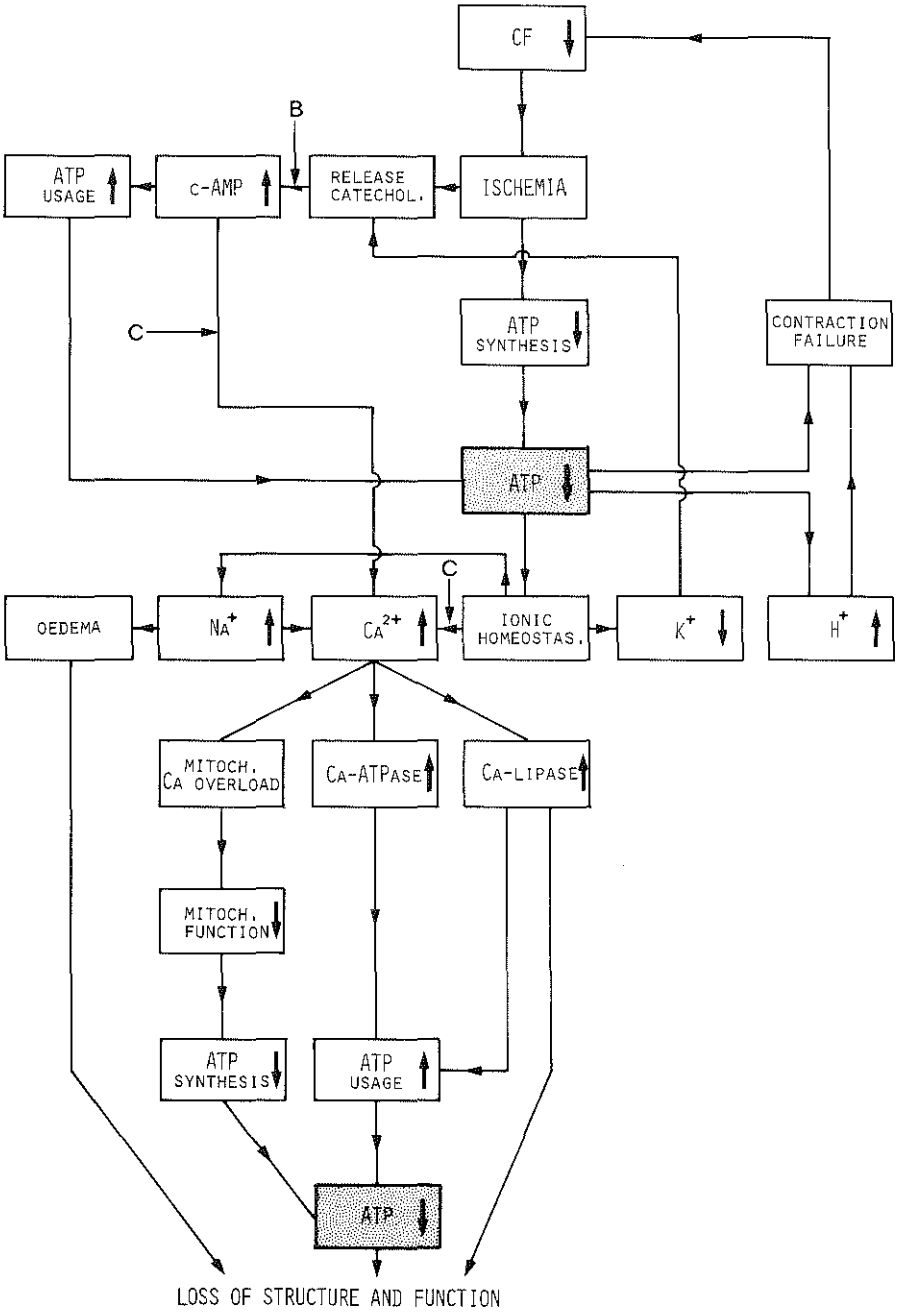

Fig. 5.1. Schematic representation of some events, precipitated by inadequate flow (ischemia).

The inhibition of certain pathways by Ca^{2+}-antagonists and beta-blockers (B) is indicated.

CF = coronary flow, cathechol. = cathecholamine, mitoch. = mitochondrial.

peripheral vasodilators (Ca^{2+}-antagonists and adenosine, cf. [8, 29]). In the clinic its usefulness is limited as the heart has to continue beating and contractile power can be reduced only to a certain degree.
3. Stimulation of ATP-producing processes. "Extra" ATP can be produced by stimulation of the anaerobic glycolysis (with a mixture of glucose-insuline-potassium, see for a review [99], but with the restrictions described by Apstein et al. [3]).
4. The rate at which ATP is hydrolysed can be slowed down by hypothermia [6, 44, 79].

In the context of this thesis, treatments 1 and 2 are discussed. Especially the ATP-sparing effects of the Ca^{2+}-antagonists nifedipine and diltiazem and the beta-blocker propranolol have been investigated. A short introduction on the cellular activators Ca^{2+} and c-AMP is now given, since these are centrally placed regulators in this context.

5.1.1 The influence of Ca^{2+} on the myocyte

The coupling between the voltage-dependent membrane changes and mechanical contraction is called the exitation contraction coupling (for a review see, a.o., [10, 29]). Contraction is a process, which results from the interaction of the contractile proteins myosin and actin. In the absence of Ca^{2+}, this interaction is inhibited by the configuration of the actin/troponin/tropomyosin complex. Myosin/actin interaction occurs by the configuration change induced by the binding of Ca^{2+} to troponin. To achieve this, a relatively high intracellular Ca^{2+} concentration is needed, an increase from 10^{-7} to 10^{-5} M, [65]. This Ca^{2+} increase is primarily caused by a (voltage-dependent) Ca^{2+}-influx through the sarcolemma (fig. 5.2), which is estimated to be only 10% of the total amount needed for contraction. It is currently thought that this voltage-dependent Ca^{2+}-influx triggers an intracellular Ca^{2+} pool which resides in the sarcoplasmatic reticulum (SR), from

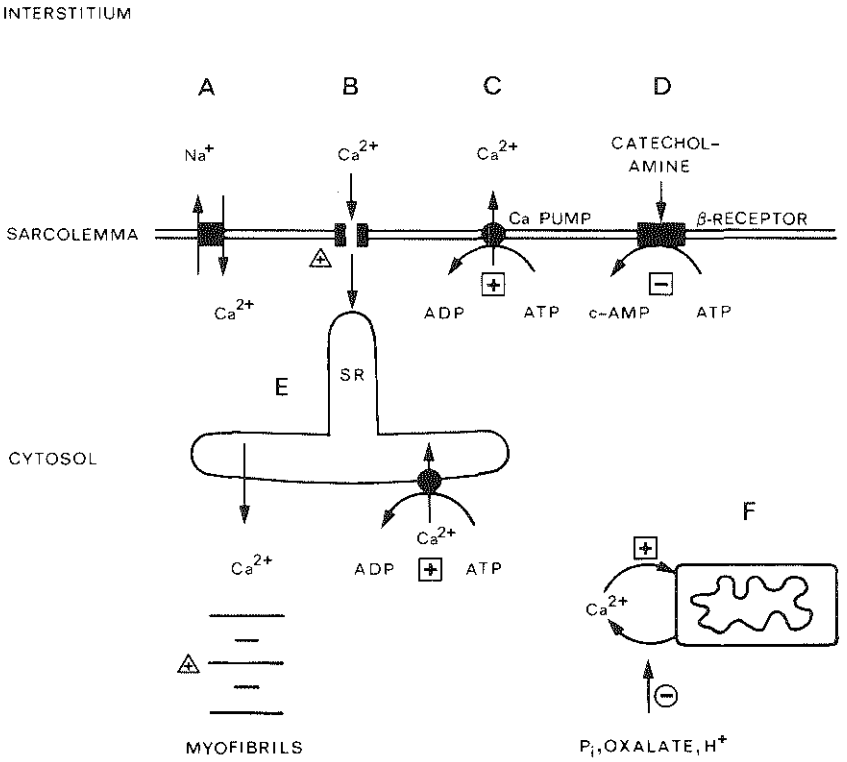

Fig. 5.2. Function of Ca^{2+} and c-AMP in the myocyte.
A = sarcolemmal Na^{+}/Ca^{2+} antiporter. B = Voltage dependent slow Ca^{2+} channel. C = Ca^{2+} pump, D = beta-adrenergic receptor, E = Ca^{2+} uptake and release by the sarcoplasmatic reticulum. F = Ca^{2+}-uptake and release by the mitochondria. SR = sarcoplasmatic reticulum, ⊞ = Ca^{2+} stimulated, ⊟ = Ca^{2+} inhibited, ⚠ = c-AMP stimulated, ⊖ = inhibition.

which additional Ca^{2+} is liberated. Cytosolic free Ca^{2+} not only causes actin and myosin to interact but also stimulates the myosin-ATPase [65, 82]. Under experimental conditions the Ca^{2+} influx is dependent on the extracellular Ca^{2+} concentrations [29, 30].

Relaxation of the myocyte occurs, when the cytosolic Ca^{2+}-levels are reduced. Part of this Ca^{2+} is removed by the sarcoplasmatic reticulum (via a Ca^{2+}- and Mg^{2+}-dependent Ca^{2+} pump); remaining Ca^{2+} is removed from the cell by a Ca^{2+}-ATPase in the sarcolemma. Other pumps may play additional roles. As a consequence of the lowered cytosolic Ca^{2+}, Ca^{2+}-troponin dissociates, the binding between actine and myosin is disconnected and relaxation takes place [10, 65].

ATP is needed as substrate for the various Ca^{2+} pumps, involved in the removal of the cytosolic free Ca^{2+}, but it is also necessary for relaxation [56]. When the ATP levels are below a certain level, no relaxation can take place and the heart remains in a contractile state (hypoxic contracture or rigor, [42, 43, 70]). When, during reperfusion, an excessive Ca^{2+} overload occurs, it may cause an abrupt contracture. This inability of the cell to adequately remove the excessive Ca^{2+} leads to reperfusion contracture [40, 41, 59, 60, 70, 78].

In the sarcolemma a Na^{+}/Ca^{2+} antiporter (which exchanges extracellular Na^{+} for intracellular Ca^{2+} or vice versa) is situated together with the Ca^{2+}-ATPase. The former also plays a role in the maintenance of intracellular Ca^{2+} levels [10, 56, 58, 122]. This antiporter is responsible for the coupling between intra- and extracellular Ca^{2+} and Na^{+}. No ATP is needed for that process. The distribution of the two ions across the sarcolemma is dictated by Nernst's Law.

The role of mitochondria in the maintenance of Ca^{2+} homeostasis is still a matter of debate. The transfer of Ca^{2+} across the inner membrane of the mitochondria consists of separate uptake and release processes [13, 14, 27, 66, 91, 95, 108]. The influx process is driven by the energy of the respiratory chain and is modulated by, a.o., ATP, P_i and Mg^{2+}. Ca^{2+} lowers the mitochondrial ATP production. The mitochondrial

Ca^{2+} efflux is mediated by a Ca^{2+}/Na^{+} exchange. Increased Ca^{2+} levels cause mitochondrial swelling and impairment of function. It is thought that the mitochondrial Ca^{2+} functions as a buffer for Ca^{2+} in the cell, and "tunes" the free Ca^{2+} in the cytosol [27]. Whether this process is important for Ca^{2+} variations to regulate contraction is still a matter of debate [13].

In conclusion, Ca^{2+} is the main regulating factor of contraction and it plays a critical role in the activation of phosphorylases, phosphokinases, lipases and proteases (fig. 5.1). The phosphokinase reactions are of utmost importance for enzymic activations and membrane processes. Finally Ca^{2+} per se plays an important role in stabilizing the membrane structure [19, 32].

5.1.2 The influence of c-AMP in the myocyte

Ca^{2+} is considered as the "beat to beat" regulator in the myocardial cell, while c-AMP can be regarded as the global cellular activator, which reacts to influences from outside the cell via several receptors in the sarcolemma. Stimulation of beta-adrenoceptors activates adenylate cyclase and as a result c-AMP is formed [1, 23, 24] (fig 5.2). Increased c-AMP levels in turn stimulate several phosphorylation reactions (phosphokinases). This causes activation of enzymes needed for glycogenolysis [56]. Also the proteins myosine and/or troponine are phosphorylated, which makes the system more sensitive to Ca^{2+} [74, 101]. Several membrane structures are phosphorylated, which lead to activation of the voltage-dependent Ca^{2+} influx [2, 89] concomitant with augmented Ca^{2+} binding of the sarcolemma [110]. C-AMP causes a shift from glycolysis to fatty acid oxidation and the cell becomes more dependent on O_2 [87]. Higher c-AMP levels, as a result, increase the contractility of the myocyte.

5.2.1. Calcium antagonists

In 1964, Fleckenstein and Kronenberg discovered that

verapamil, a vasodilator which has cardiodepressant activities, selectively inhibits the cardiac exitation-contraction coupling without a major change in the action potential [10, 29, 81]. This effect could be counteracted by increasing the extracellular Ca^{2+} concentration, beta-adrenergic catecholamines or cardiac glycosides. They suggested the name "Ca^{2+}-antagonists" for drugs with this action. These compounds cause negative inotropy in the myocardium, while the vascular cells are relaxed and vasodilation occurs (but see ref [113]). In 1969, it was found that Ca^{2+}-antagonists specifically interfere with the slow trans-sarcolemmal Ca^{2+} inward current, without affecting the fast-channel mediated transmembrane Na^{+} influx, hence the name "slow-channel blockers" [30]. Since skeletal muscle does not need extracellular Ca^{2+} for its contraction, Ca^{2+}-antagonists do not seem to affect this type of muscle [80].

If one follows Fleckenstein's definition of Ca^{2+}-antagonists (inhibitors of the voltage-dependent transmembraneous Ca^{2+}-influx), a great variety of this type of compounds exists [28, 29, 64, 80]. Nayler [80] has tried to classify them. She distinguishes two main groups, the relatively unspecific group of inorganic compounds and the more specific group of organic compounds. The organic compounds are divided into three classes on the basis of their organ specificity: Class I consists of compounds, which primarily act on cardiac tissue (for example, diltiazem). Class II compounds mainly influence vascular cells (nifedipine, niludipine, nitrendipine, nimodipine), while class III compounds mainly act upon cardiac nerve tissue (verapamil, gallopamil). Compounds in the last class also seem to exhibit some influence on the fast Na^{+}-current. However other classifications are also feasable. Rodenkirchen et al. [100a] suggest a classification on their specificity of modes of action.

The binding characteristics of these compounds to sarcolemmal- or sarcoplasmatic reticular membranes differ considerably [22, 78, 80, 84, 92, 94].

It is important to note, that the action of (at least some)

Ca^{2+}-antagonists can be pluriform [46]. For example, diltiazem at low concentrations blocks the voltage-dependent slow Ca^{2+} channel, while at higher concentration it also halts the Ca^{2+}-pump in the sarcolemma [15, 102]. However, general agreement exists about the dominant inhibitory action of Ca^{2+}-antagonists on the voltage-dependent channel. The nature of this channel is not clearly defined at this moment, although some speculation about models exists [10, 57]. Organ to organ diversity seems likely, which could explain the different specificity of the various compounds. At this moment, other actions of Ca^{2+}-antagonists apart from inhibition of the slow Ca^{2+}-channel cannot be excluded.

We became interested in the ATP-sparing effect of Ca^{2+}-antagonists as claimed by Fleckenstein [28] and subsequently (partly) documented by Nayler et al. [83], and not found by Geary et al. [34]. We have investigated the ATP-sparing effect of the Ca^{2+}-antagonists nifedipine and diltiazem in the isolated rat heart [appendix papers 5-7].

In normoxic hearts, addition of nifedipine or diltiazem causes vasodilatation and negative inotropy. The maximal vasodilatory effect for both drugs is found at a lower concentration than that which is necessary for the maximal negative inotropy, which could indicate a higher sensitivity of the vascular cell than the myocytes [28, 35, 84]. To produce equipotent effects, about 30 times more diltiazem than nifedipine must be used.

During ischemia two observations have been made:

1. In hearts which have been pretreated with nifedipine (up to 0.3 μM [appendix paper 5]) or diltiazem (up to 100 μM, [appendix paper 7]), ischemic purine release was found to be depressed by both drugs in a dose-dependent manner. In the non-treated hearts, apex displacement fell 60-80% during ischemia, due to the flow reduction. When hearts were pretreated with the Ca^{2+}-antagonist, the apex displacement during ischemia was comparable to that of the non-treated hearts (with the exception of the 100 μM diltiazem dosage,

which completely arrests the heart during normoxia, ischemia and reperfusion). Ischemic purine release, however, was diminished in a dose-dependent way with a concomitant better preservation of myocardial ATP levels. The ischemic purine release correlated with the negative inotropic effect of the drug before the ischemic period [appendix papers 5-7].

2. When Ca^{2+}-antagonists are presented to the heart during ischemia (diltiazem [appendix paper 7]), the reduction of purine release is paralled by a reduction in apex displacement (negative inotropy).

The ATP-sparing effects of the Ca^{2+}-antagonists administered before or during ischemia confirm the results claimed earlier [28, 46-48, 83, 96, 119-121]. The explanation for this effect is not yet known. Fleckenstein [28] stated that the ATP sparing effect of Ca^{2+}-antagonists is only due to their negative inotropic properties. Nayler et al. [83] postulated enhanced preservation of mitochondrial function during ischemia, by preventing mitochondrial Ca^{2+}-overload. Ichihara et al. [53] found a nifedipine induced shift from fatty acid to glucose oxidation, suggesting another intracellular action, while Church and Zsoter [15] and Henry and Wahl [48] did not find diminished myocardial Ca^{2+}-influx during ischemia, when they treated hearts with Ca^{2+}-antagonists.

In apppendix papers 5 and 7 we speculated about an intracellular action of nifedipine and diltiazem. This speculation, however, cannot be fully proven yet, and the following mode of action of the two drugs appears to be the most likely at present:

In the isolated rat heart, treatment with diltiazem during ischemia, showed a correlation between the negative inotropic effect of the drug and the ATP sparing effect. This could be due by a reduction in cardiac work. Hearts pretreated with nifedipine and diltiazem, however, do not show any relation between purine release and apex displacement during ischemia. On the other hand the correlation between purine release during ischemia and the change in apex displacement during normoxia is striking. In the diltiazem-treated hearts, a correlation

coefficient of 0.84 is found between these parameters for the concentration range used. Pretreatment of the heart with Ca^{2+}-antagonists will reduce cardiac work and therefore ATP and O_2 demand before ischemia. Subsequent flow reduction will induce ischemia of which the severity is dependent of the work of a heart before ischemia. In other words, Ca^{2+}-antagonists can reduce the severity of ischemia by pre-ischemic action. This effect is dose-dependent and is directly achieved by inhibition of the voltage-dependent Ca^{2+}-influx. Although this explanation seems satisfactory in our experimental set-up, an additional intracellular action of these drugs cannot be excluded [38, 48, 76, 93, 116, 119].

An important question which remains is the effect of Ca^{2+}-antagonists when applied after ischemia. In the early phase of reperfusion, an excessive Ca^{2+}-influx takes place, particularly when ischemia has been present long enough to lead to low ATP levels at the end of the ischemic period [98, 109]. This Ca^{2+}-influx is probably mediated by Na^+/Ca^{2+} exchange, and Ca^{2+} leaks across the damaged sarcolemma [35, 66]. In this case, no effect of Ca^{2+}-antagonists can be expected [112, 118]. Schols et al. [103], however, have shown that the so-called Ca^{2+}-antagonist lidoflazine applied within the first 5 min of reperfusion can still protect the myocardium. This compound exhibits besides Ca^{2+}-antagonistic properties, also an effect on the sarcolemmal transport of adenosine. Part of the beneficial effect of adenosine might be explained by increased adenosine phosphorylation (see chapter 6). Also after the Ca^{2+}-paradox [44, 123], some protection by Ca^{2+}-antagonists was found [5].

5.2.2. Beta-blockers

Beta-blockers protect the heart against the deteriorating effects of ischemia [11, 26, 68, 69] primarily by blockade of the beta-adrenoreceptors. As a result, the normal augmentation of c-AMP by beta-adrenergic stimulation is inhibited, which has a beneficial effect via reduction of tissue energy demand [62].

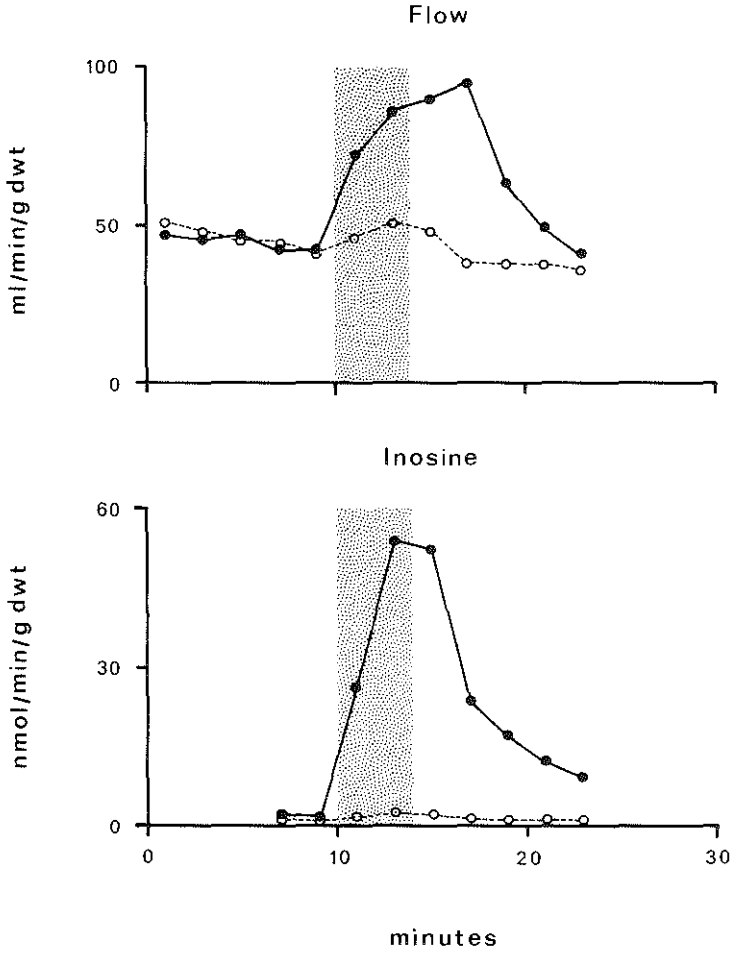

Fig 5.3. Influence of D- and L-propranolol on beta-adrenergic stimulation. Rat hearts were perfused with a modified Tyrode solution, equilibrated with 95% O_2 and 5% CO_2, pH = 7.4. Five min after the start of the perfusion pressure, apex displacement was set at 100 %. D (●---●) and L-propranolol (O---O) were solved in the perfusion medium at a concentration of 150 µg/l. Isoproterenol (Isuprel) is added between t = 10 and t = 14 min (shaded area) at a concentration of 15 µg/l.
Values are means of 4 perfusion experiments, performed by Dr. J.W. de Jong and Mr. P.Ph de Tombe.

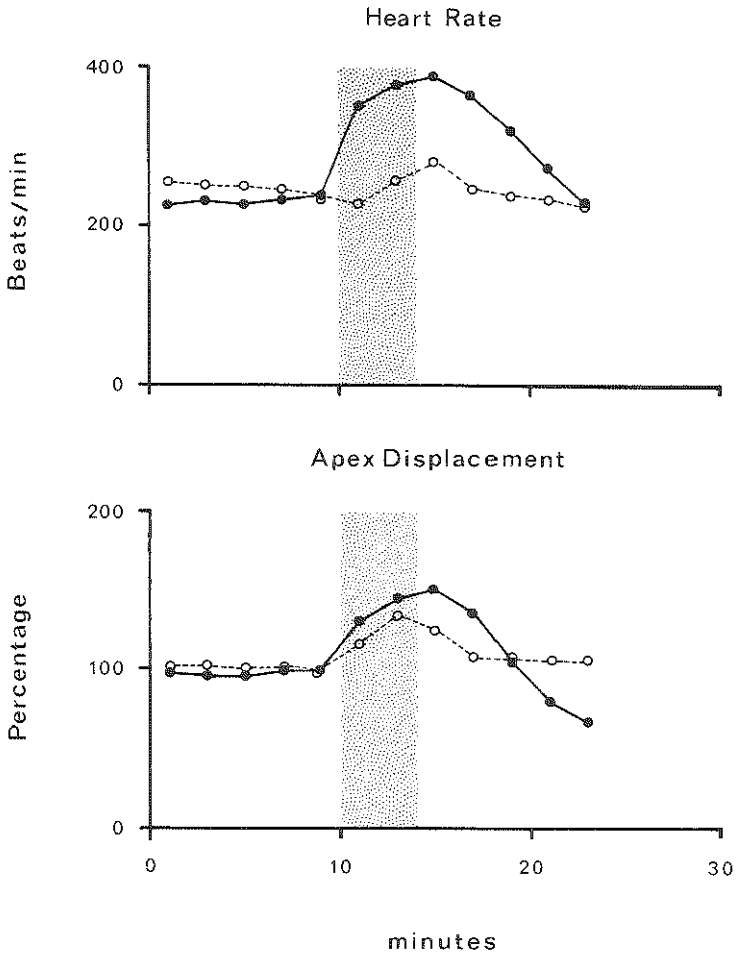

Fig 5.3. Influence of D- and L-propranolol on beta-adrenergic stimulation. Rat hearts were perfused with a modified Tyrode solution, equilibrated with 95% O_2 and 5% CO_2, pH = 7.4. Five min after the start of the perfusion pressure, apex displacement was set at 100 %. D (●---●) and L-propranolol (O---O) were solved in the perfusion medium at a concentration of 150 µg/l. Isoproterenol (Isuprel) is added between t = 10 and t = 14 min (shaded area) at a concentration of 15 µg/l.
Values are means of 4 perfusion experiments, performed by Dr. J.W. de Jong and Mr. P.Ph de Tombe.

Propranolol, as one of the most commonly used beta-blockers in the clinic, has also a "membrane stabilizing" effect [4]. However, this last effect is of no clinical importance.

As is shown in appendix paper 6, DL-propranolol, added to the perfusion medium in concentrations of 30 or 150 µg/l, does not show any protective action on ischemic ATP levels and purine release in an isolated heart. However, preliminary studies with D- and L-propranolol indicate that in this concentration range, L-propranolol effectively inhibits the beta-adrenergic stimulation of isoproterenol (see fig. 5.3). D-propranolol, during the same stimulation has no beta-adrenergic blocking properties. Probably, the catecholamine release in an isolated rat heart is of minor importance during ischemia. This is in accordance with the experiments of Edoute et al. [25] and Lubbe et al. [71]. However, Nakazawa et al. [77] found in isolated rat hearts a normalization of the ischemic myocardial pH during DL-propranolol treatment. They concluded that DL-propranolol had a protective effect. Nayler et al. [85], Conway and Weiss [18] and Manning et al. [72] had found, after application of propranolol in the intact animal, a protective effect when these hearts were perfused and then made ischemic. In intact animals, however, circulating catecholamines are expected to play a more important role than in isolated, perfused hearts, so that the effect of these drugs would be more likely to show up.

5.2.3. Combined effects of Ca^{2+}-antagonists and beta-blockers

In the previous paragraphs, the ATP-sparing effect of Ca^{2+}-antagonists during ischemia was demonstrated, while propranolol under the same conditions showed no protective effects. In contrast, the combination of these drugs showed a better protection than either drug alone [20, 21, 117 and appendix paper 6]. Apex displacement and flow were not different before or during ischemia during the combined therapy in our experiments [appendix paper 6], compared with the action

of nifedipine alone. Therefore it seems that this synergistic effect is independent of contractility and flow and some intracellular action of the combination must be assumed. Nayler et al. [85] suggest an effect of propranolol on Ca^{2+}-transport, while Katz et al. [58] found an influence of propranolol on microsomal Ca^{2+}-binding. Several ATP-consuming processes are both Ca^{2+} and c-AMP regulated. Whether a (molecular) compensatory mechanism exists in the myocyte comparable with the baroreflex activation in the intact system [86], is not known at the present. If so, it is conceivable that Ca^{2+}-antagonists in their action alter the Ca^{2+}-balance in the cell which leads to a compensatory stimulation of c-AMP, which in turn could be blocked by propranolol. However, no increase in c-AMP is found after addition of nifedipine or verapamil in isolated rat hearts [39, 111], which makes this explanation less plausible. Clearly, much further work on the efficacy of this combination must be done to elucidate its mechanism(s).

5.3. Clinical importance of Ca^{2+}-antagonists and beta-blockers

The main action of Ca^{2+}-antagonists administered intravenously to patients, is coronary and peripheral arterial vasodilation [37, 104]. The peripheral vasodilatory effect results in reduction of the systemic resistance, thereby relieving the workload of the heart [29, 67]. Intracoronary application of Ca^{2+}-antagonists results in coronary vasodilatation and negative inotropy [55, 106, 107, 115]. These drugs are therefore used as powerful anti-anginal drugs [50, 51] and are especially recommended when angina pectoris is caused by increased vasomotor tone of the coronary arteries [7]. Protection is also found during cardiac arrest induced for open heart surgery [12, 16] and during transluminal angioplasty [105].

The protective effect of beta-blockers has been recognized for years, both for the reduction in the ischemic sequela of myocardial infarction and for treatment of anginal attacks

[11]. They act mainly by inhibition of the catecholamine response. Beta-blockers are therefore well suited for the treatment of patients with stable angina pectoris, as they reduce cardiac frequency.

Since several years the combination of the two drugs is employed and an additive protective response is found [20, 52, 90]. Beta-blockers block the baroreflex response which induces tachycardia [67]. In many cases of angina pectoris, both drugs are indicated instead of either of the drugs alone [51]. Due to their additive (or even synergistic) effect, the dosis of each drug can be lowered, thereby lowering the side effects [20, 31, 97].

However, one serious side effect of this combination deserves special attention [75, 88]: Through blockade of the baroreflex induced catecholamine release, severe bradycardia and excessive fall in blood pressure may lead to shock, underperfusion of the heart, acute myocardial infarction and even death. In particular the combination of verapamil (which slows the conductive system and leads to A-V block) with propranolol is dangerous [88]. However, Kieval et al. [61] found no negative side effects with this combination in patients with ischemic heart disease, provided initial cardiac function was normal and cardiomegaly absent.

5.4. Conclusions

From studies in isolated perfused rat hearts it can be concluded that:

1. Administration of nifedipine or diltiazem to the heart before ischemia, prevents or delays ATP breakdown. We propose that this effect is in a large part due to the negative inotropic properties of the drugs during normoxia by diminishing the normoxic ATP demand and therefore decreasing the severity of the ischemia due to the subsequent (fixed) flow reduction. As a consequence of this lower ATP demand, ATP

levels remain normal, ionic homeostasis is longer preserved and mitochondrial function is less disturbed. However, additional intracellular actions of Ca^{2+}-antagonists are not excluded by our experiments.

2. Administration of diltiazem during ischemia decreases ATP breakdown, which parallels the negative inotropic effect, observed in the isolated rat hearts.

3. No apparent difference is found between the action of nifedipine and diltiazem. However, for a given dosis of nifedipine, 30 times more diltiazem must be given to achieve the same protective effect.

4. DL-propranolol is an effective beta-adrenergic blocker, but it does not protect the (isolated) heart from ATP depletion. However, in combination with nifedipine it reinforces the ATP-sparing effect of nifedipine. In this case, no correlation between contractility and purine release is found, compared with nifedipine alone. Therefore this effect may have some intracellular mechanism.

5.5. References

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CHAPTER 6 MYOCARDIAL ATP BIOSYNTHESIS

6.1. Introduction

In chapter 2, the importance of adequate myocardial ATP levels is indicated, while in chapter 4 it can be read that due to mitochondrial impairment (caused by lack of oxygen) ATP levels decrease. Dephosphorylated catabolites such as adenosine and inosine pass the cell membrane and leave the heart. Restocation of O_2 supply after reversible ischemia is followed by restoration of mitochondrial function. ATP levels, however, remain low because of washout of the adenosine and its catabolites [a.o., 1, 6, 21, 27, 55 and appendix papers 2, 6 and 8].

Low ATP levels have two important consequences:

1. A reperfused heart cell with a lowered ATP level is more vulnerable during a next ischemic period, because the critical limit between reversible and irreversible damage is sooner reached (supposedly in patients with frequent anginal attacks and subsequent purine release) [5].

2. Recovery of heart function after an ischemic period is thought to be proportional to recovery of ATP levels. This has been shown in patients with acute myocardial infarction and those undergoing cardiac surgery. Fast recovery of ATP levels is followed or paralleled by a fast recovery period [27, 55].

In this chapter, several pathways are described for the biosynthesis of AMP. The AMP formed can be used by adenylate kinase and transferred to ADP (see chapter 3). ADP can be

rephosphorylated to ATP by oxidative phosphorylation or anaerobic glycolysis.

6.2 Nucleotide biosynthesis

The different biochemical pathways for cellular ATP and GTP biosynthesis are summarized in fig. 6.1.

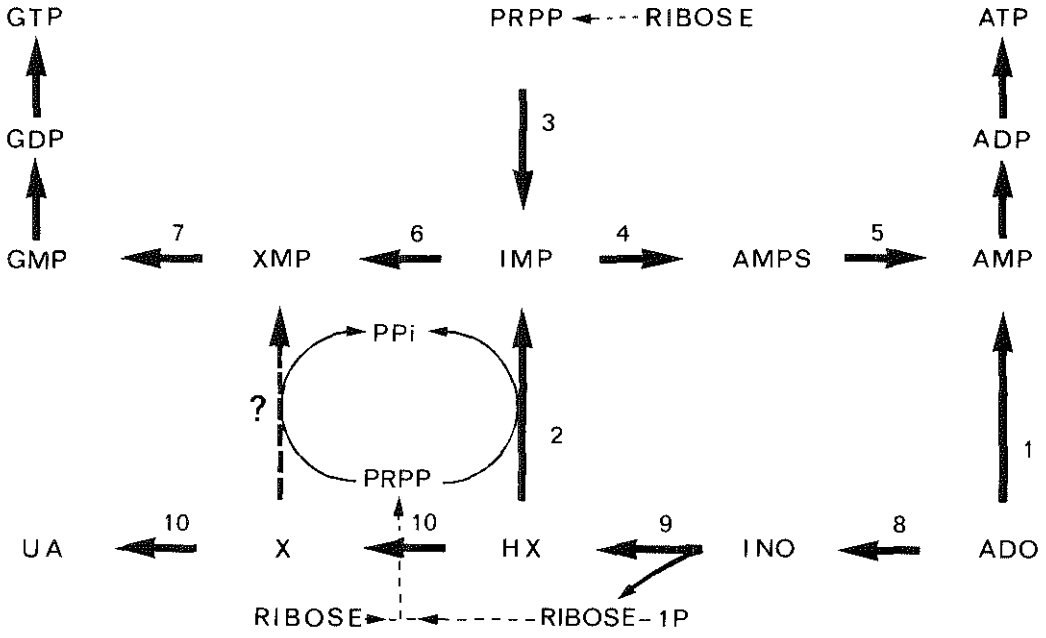

Fig. 6.1. Enzymes involved in the biosynthesis of ATP and GTP.
1 = adenosine kinase, 2 = hypoxanthine phosphoribosyl transferase, 3 = de novo synthesis (a 10-step synthesis), 4 = adenylosuccinate synthetase, 5 = adenylosuccinate lyase, 6 = IMP-dehydrogenase, 7 = GMP-synthetase, 8 = adenosine deaminase, 9 = nucleoside phosphorylase, 10 = xanthine oxidase/dehydrogenase. ADO = adenosine, AMPS = adenylosuccinate, HX = hypoxanthine, INO = inosine, PP_i = pyrophosphate, PRPP = phosphoribosyl pyrophosphate, X = xanthine, UA = uric acid. ? indicates the possibility, that xanthine can be converted to XMP.

6.2.1 Adenosine phosphorylation

Adenosine is directly phosphorylated to AMP by adenosine kinase (AK).

$$\text{Adenosine} + \text{ATP} \longrightarrow \text{AMP} + \text{ADP}$$

In 1957, Goldwaith [16] was the first to show the existence of this process in heart homogenates and since that time adenosine kinase has been demonstrated in hearts of several species [7, 20, 34, 48, 58]. The enzyme has a (low) K_m of about 1 μM for adenosine and a specific activity of about 6-8 μmol/min/g protein [8, 60 and appendix paper 4]. Several isoenzymes are found, all with a molecular weight of about 40,000. In rat heart, it is predominantly located in the cytosol [7]. Together with 5'N, it forms a so-called futile cycle. According to Arch and Newsholm [2] this cycle has a specific task in adenosine regulation (see also chapter 4). At the cost of 1 mol ATP per turn, this cycle provides a direct and fast production of adenosine, which is of utmost importance for flow regulation [3, 38, 42]. In appendix paper 4, the purification of AK from rat heart is described. With a fast, relatively simple two column-step procedure, this enzyme is purified to apparent purity. The enzyme is inhibited by the adenosine deaminase inhibitor dipyridamole [8]. The maximal incorporation rate of adenosine found in isolated rat heart is 50 nmol/min/g dwt [48], which is about 1500 times less than the maximal reported enzyme activity.

6.2.2 Purine salvage

The purine bases adenine, guanine and hypoxanthine are converted to their respective monophosphates, using phosphoribosyl-pyrophosphate (PRPP, a diphosphorylated ribose-5-phosphate).

Adenine	+	PRPP	⟶	AMP + PP_i
Guanine	+	PRPP	⟶	GMP + PP_i
Hypoxanthine	+	PRPP	⟶	IMP + PP_i

Hypoxanthine and guanine are phosphorylated by hypoxanthine phosphoribosyl transferase (HPRT), while adenine is converted by adenine phosphoribosyl transferase [18].

According to Murray [37] and Fox [13] more than 75% of all purine bases in the body (formed by nucleotide- or nucleic acid breakdown or derived from the food) are transformed into intracellular nucleotides by these enzymes. The other 25% is converted to uric acid (or allantoin) and excreted.

The salvage enzymes play an important role in nucleotide metabolism; however, the precise regulatory mechanism remains to be elucidated. The importance of the salvage pathway is stressed by the folowing example: a HPRT deficiency results in the so-called Lesch-Nyhan syndrome. This disease is associated with several severe neurological disturbances and mental retardation, resulting in very aggressive behaviour and a tendency to self mutilation. Also massive deposition of uric acid in the kidneys is found with resulting renal failure [32]. This increased uric acid production is not only derived from "non-salvaged" hypoxanthine but predominantly from the accelerated de novo synthesis.

In heart tissue, HPRT and APRT enzyme activities have been detected [35]. Normally, adenine levels are not detectable in blood, whereas hypoxanthine is present in micromolar amounts. Inosine, the precursor of hypoxanthine, is often used as a cardioprotective agent and cannot be directly incorporated into myocardial nucleotides [34].

Incorporation of hypoxanthine and inosine into myocardial ATP has been reported by several authors [16, 20, 26, 39, 45, 51, 57, 58]. However, till now, no regulatory mechanism for this process has been found in heart.

In appendix paper 8, we described the incorporation of hypoxanthine in isolated rat hearts. The ATP biosynthesis from hypoxanthine was 0.4 nmol/min/g dwt under normoxic conditions.

About 0.2 nmol/min/g dwt hypoxanthine was incorporated in the AMP and ADP fraction, so that the total adenine nucleotide synthesis in rat heart from hypoxanthine is about 0.6 nmol/min/g dwt. The GTP biosynthesis is 0.1 nmol/min/g dwt, which also constitutes about 75% of total guanine nucleotide synthesis. The latter finding was unexpected, because GTP levels in heart are about 5% of the ATP levels [17, 54, 55 and appendix paper 8], while GTP biosynthesis is about 25% of that of the ATP biosynthesis. The total ATP and GTP concentrations are not significant altered after the incorporation period. The biosynthesis rates of ATP and GTP were increased when 0.5 mM ribose is infused together with the hypoxanthine solution [appendix paper 8].

During reperfusion (after a 20 min period of mild ischemia induced by a flow reduction of 70%), the ATP biosynthesis rate from hypoxanthine is doubled. The maximal synthesis rate is 1.5 nmol/min/g dwt, which amounts to 2.0 nmol/min/g dwt synthesis of total adenine nucleotides. GTP biosynthesis in all cases parallels ATP biosynthesis, the maximal total guanine nucleotide synthesis rate being 0.5 nmol/min/g dwt (fig. 6.2). The uptake of the hypoxanthine was comparable in all sets of experiments. We estimate the intracellular hypoxanthine concentration to be between 15 and 20 µM, which is reflected by its concentration in the perfusate. Inosine uptake is about twice the hypoxanthine uptake, while conversion to hypoxanthine maximally amounts to 15%. This indicates a lower cellular hypoxanthine concentration than during perfusion with hypoxanthine, while hypoxanthine and inosine incorporation rates are comparable in normoxic and reperfused hearts. From these observations, we conclude an intracellular factor to be responsible for the regulation of the salvage rate, presumably PRPP.

According to Zimmer et al. [62, 64], PRPP is supplied by the hexose monophosphate shunt (see fig. 6.3). The first step in the oxidative part of the pentose cycle by the enzyme glucose-6-phosphate dehydrogenase (G-6PD) is the rate-limiting step and is regulated by the $NADP^+$/NADPH ratio. After ischemia,

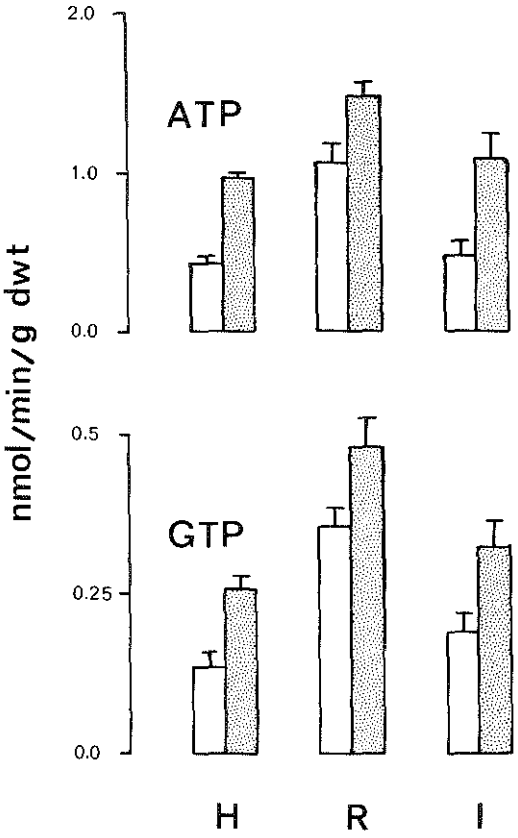

Fig. 6.2. Biosynthesis of ATP and GTP.
Isolated hearts were perfused with a modified Tyrode solution, gassed with 95% O_2 and 5% CO_2. Twenty min after the start of the perfusion, hearts were made ischemic by flow reduction for 20 min (about 25% of control flow). Thereafter, the hearts were reperfused for another 20 min. The experiments were terminated by freeze-clamping the hearts. In the last 15 min of the experiments 20 µM [8-^{14}C]hypoxanthine (H), with 0.5 mM ribose (R) or 20 µM [8-^{14}C]inosine (I) is applied to the hearts. From the freeze-clamped hearts, ATP and GTP were extracted, and ^{14}C was counted. The open bars represent the normoxic values, the shaded bars the reperfused values + SEM. (Data were taken from appendix paper 8.)

this ratio is decreased, the inhibition of G-6PD is abolished, and the PRPP synthesis is increased. It is possible to bypass this cycle, by infusion of ribose, which can be directly converted via ribose-5-P to PRPP [47, 63]. All these conditions

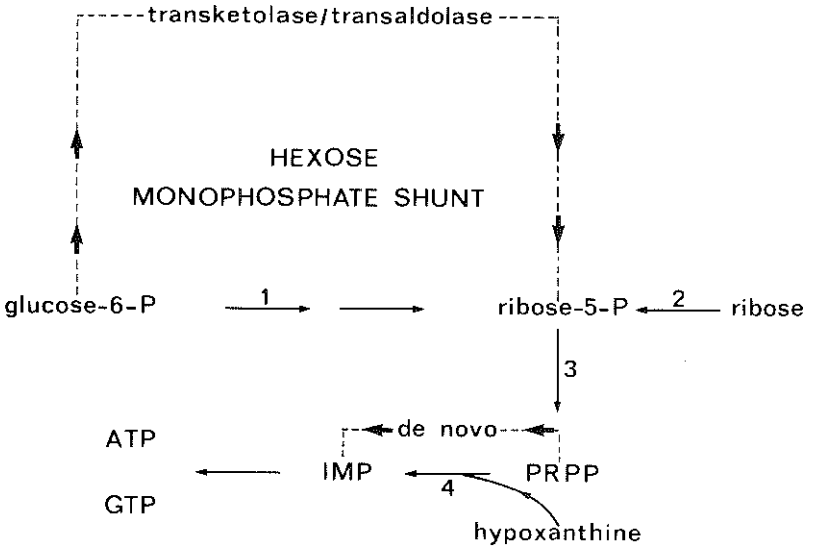

Fig. 6.3. Phosphoribosyl-pyrophosphate (PRPP) synthesis .
1 = Glucose-6-phosphate dehydrogenase, 2 = ribokinase, 3 = PRPP synthetase, 4 = hypoxanthine phosphotransferase.

indeed stimulated hypoxanthine incorporation, confirming the PRPP dependency of the process.

Inosine incorporation into ATP is comparable with hypoxanthine incorporation in normoxic and reperfused hearts (0.7 and 2.0 nmol/min/g dwt, respectively; see appendix paper 8). Inosine cannot directly be incorporated into IMP [34], but is first catabolized to hypoxanthine and ribose-1-phosphate. The last compound can be used as a precursor of PRPP (see fig. 6.1). The increase of total adenine nucleotides after reperfusion with inosine is surprising. After ischemia, total adenine nucleotides fall to about 23 µmol/g dwt. Fifteen minutes of reperfusion with inosine restores total adenine nucleotides levels to about 27 µmol/g dwt, an increase of about 4 µmol/g dwt. Total adenine nucleotide biosynthesis from inosine during that period is no more than 30 nmol/min/g dwt, as measured by the incorporation of ^{14}C-inosine. The reason for this discrepancy between these data is not clear at the moment. It seems that inosine can restore ATP and total adenine nucleotides levels by another pathway than salvage, via a so far unknown mechanism.

ATP and GTP synthesis involves conversion of IMP to AMP and GMP, respectively (see fig. 6.1). The enzymes adenylosuccinate synthetase and -lyase, needed for AMP synthesis, have been detected in heart homogenates, the first enzyme being rate limiting [18, 35]. The enzymes needed for GMP formation, IMP-dehydrogenase and GMP synthethase have not yet been determined in cardiac tissue, but the incorporation of [8-^{14}C]hypoxanthine into myocardial ATP and GTP makes it almost certain that these enzymes exist in rat heart.

IMP concentrations in heart are low (<0.1 μmol/g dwt) [17, 48, 54, 55]. This suggests that conversion of IMP to AMP and GMP is not restricted by the enzymes needed for AMP and GMP synthesis, but that the first step, conversion of hypoxanthine and PRPP to IMP, is rate limiting because of the limited availability of PRPP.

6.2.3. De novo synthesis

IMP is formed from PRPP in a ten-step synthesis. As precursors glutamine, alanine, aspartate, CO_2 and acetic acid (see fig. 6.4), are needed, plus 4 molecules of ATP (4~P) per molecule IMP formed (see fig. 6.4). The first step (PRPP-transferase) is thought to be rate limiting. This enzyme is PRPP dependent and inhibited by ATP [64]. Also high levels of hypoxanthine and inosine are found to inhibit this step.

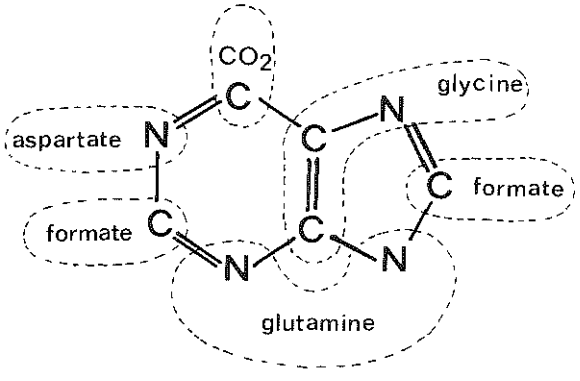

Fig. 6.4. Precursors for de novo synthesis of the purine ring.

Whether this is by direct inhibition [53] or by competition for the available PRPP [61] is unclear at the moment.

In isolated normoxic rat hearts, total adenine nucleotide synthesis rate in the de novo pathway amounts to 0.1 nmol/min/g dwt [64], which can be accelerated after ischemia and during ribose infusion to about 2 nmol/min/g dwt.

6.3 The relative importance of adenosine phosphorylation, hypoxanthine salvage and de novo synthesis

In section 6.2. de novo synthesis, hypoxanthine salvage and adenosine phosphorylation in the myocardium have been described. Adenosine phosphorylation takes place at the highest rate and is energetically the most economical [48]. However, free adenosine concentrations are low in heart and blood [43 and appendix paper 1]. During ischemia, the adenosine concentration increases in heart. This nucleoside is predominantly washed out of the cells and catabolized in the blood as shown in chapter 4. For this reason, adenosine phosphorylation may be restricted.

The de novo synthesis rate in rat heart is 0.1 nmol/min/g dwt. This rate is stimulated after ischemia and during ribose infusion to about 2 nmol/min/g dwt. Also AICA-ribose (5-amino-4-imidazolecarboxamide-ribose), an intermediate of the de novo pathway, has been shown to accelerate this process [50, 55].

The hypoxanthine salvage rate in normoxic heart is about 0.7 nmol/min/g dwt when 20 μM hypoxanthine or inosine is supplied to the heart [39 and appendix paper 5]. This rate is increased after ischemia and ribose infusion to about 3 nmol/min/g dwt.

All the above mentioned processes are slow, compared with ischemic purine release. However, their importance can be indicated by the following example:

When 20% of ATP is depleted during (moderate) ischemia and has to be resynthetized in the following reperfusion phase, it will take two hours to restore normoxic ATP values, provided

adenosine supply is adequate with concentrations above 20 μM. When only de novo synthesis is activated (which is stimulated after ischemia), it takes about 6 days for the heart ATP content to recover. With 20 μM hypoxanthine, ATP restoration will take place within 2 days. Addition of 0.5 mM ribose will accomplish this process within 1 day. Twenty μM inosine probably leads even faster to recovery of proper ATP levels (within 15 min, see appendix paper 8), compared to hypoxanthine, although their incorporation rates are in the same order of magnitude.

It must be stressed that these recovery times are estimated from data derived from isolated rat hearts. Zimmer et al. [64] have found that myocardial de novo synthesis rate in the intact animal is about 5 times faster than in isolated perfused heart. Presumably, in intact animals, glucose metabolism is more active than in isolated hearts [40, 44]. However, the calculation in the above described example shows, that after an ischemic event, restoration of ATP levels can be improved drastically, and will probably decrease the "time at risk", especially when inosine is used as precursor.

6.4 Relevance of ATP biosynthesis in the intact animal and in the clinical setting

Addition of Persantin (dipyridamole, an adenosine transport inhibitor) during ischemia and reperfusion diminishes the adenosine washout from the heart.This adenosine can be used for the resynthesis of ATP [4, 11, 12, 28, 49]. Infarct size has been limited in dogs as a result of this treatment [49]. Furthermore, loss of function caused by 20 min aortic crossclamping and reperfusion was inhibited [11, 12]. Also, addition of erythro-hydroxy-nonyl-adenine (EHNA), an adenosine deaminase inhibitor, plus adenosine increased recovery of function [19]. All factors, which increase adenosine concentration, cause peripheral- and coronary vasodilatation. This also can exert a beneficial effect on the heart.

Allopurinol, a xanthine oxidase inhibitor, decreases the hypoxanthine breakdown to uric acid. In animals, it shows cardioprotective properties [14, 31, 33, 36, 46]. It is thought that at least part of the beneficial effect is mediated by the stimulation of ATP synthesis, caused by an increased hypoxanthine availability.

Inosine has also been used as a cardioprotective agent [15, 25, 52, 59]. It provides hypoxanthine and ribose-1-phosphate and also shows some interesting pharmacological properties, such as vasodilatation, although it is at least 100 times less potent as adenosine [9, 22, 23, 59]. Furthermore, in contrast with adenosine, it acts as a positive inotropic agent [9, 24, 29, 52, 56, 59] and can stimulate the glucose uptake and the glycolysis rate [29, 30, 52]. Part of the positive inotropic effect of inosine is beta-adrenergically mediated, although inhibition of the Na^+-K^+-pump cannot be excluded. Infarct size was limited and scar formation accelerated after acute experimental myocardial infarction by inosine treatment [9, 41]. Data of DeWitt et al. [10] indicate that application of inosine during cardioplegia decreases ATP breakdown and loss of function.

6.5 Conclusions

The three processes which restore diminished ATP levels - adenosine phosphorylation, hypoxanthine salvage and de novo synthesis - all take place in cardiac tissue. Maguire et al. [35] concluded from enzyme activity measurements, that hypoxanthine incorporation would be by far the most important of the three pathways. Literature data and data from our laboratory on direct incorporation studies do not support this view [7, 39 and appendix paper 8]. When high concentrations of purines are supplied to the heart, adenosine incorporation is the most predominant process. Adenosine, however, is quickly catabolized to inosine and hypoxanthine. Treatment with adenosine must be combined therefore with inhibitors of adenosine catabolism.

Hypoxanthine salvage and de novo synthesis are much slower than adenosine phosphorylation. These two processes produce ATP and GTP. Although the significance of the relatively high GTP synthesis rate is not clear at the moment, this relatively high rate could indicate its relevance. Both processes are limited by PRPP availability, i.e., synthesis of PRPP is rate-limiting. PRPP concentration is increased after ischemia and during ribose infusion, resulting in higher rates of de novo synthesis and salvage. When hypoxanthine is present, PRPP is preferentially used by the salvage process, while delaying or inhibiting de novo synthesis.

Inosine is also incorporated into ATP and GTP, via conversion to hypoxanthine. Besides direct incorporation and the delivery of ribose-1-P, inosine seems to have a direct action on ATP concentration. The mechanism of this action is unknown at the moment. Besides this favorable action on ATP levels, it also has desirable pharmacological properties. Inosine acts as a peripheral- and coronary vasodilator and has positive inotropic properties. It can also stimulate the glycolysis.

For clinical purposes, ribose and inosine infusion would seem to be the most important for patients who have suffered from myocardial ischemia (angina pectoris, myocardial infarction, post-operative patients). Especially inosine may have beneficial effects as a cardioprotective agent during and after ischemia, for instance in cardioplegic solutions during open heart surgery.

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CHAPTER 7 FINAL CONCLUSIONS

It is of utmost importance for the myocyte to maintain adequate ATP levels throughout its lifecycle. Most of the ATP needed for contraction is synthetized by the mitochondria via oxidative phosphorylation, an O_2-dependent process. However, O_2 cannot be stored in the cytosol in sufficient amounts and therefore must be supplied continuously by the coronary arterial system. Lack of oxygen caused by inadequate supply of arterial blood, ischemia, leads directly to a decreased ATP production. Ultimately, as a consequence of this low ATP production, the dephosphorylated purine compounds adenosine, inosine, hypoxanthine, xanthine and uric acid leave the heart and enter the coronary venous system. Appearance of adenosine and catabolites in the venous blood therefore directly indicates ATP catabolism as a consequence of impairment of O_2 supply. For this reason, these catabolites are ideal markers for ischemia as discussed in chapter 4.

HPLC has proven to be a good technique to study the above described processes. From an analytical point of view, it is a sensitive technique, and within one run several compounds can be determined. The process can be automated and used quantitatively to separate radiolabeled compounds.

The effectiveness of anti-ischemic drugs can be tested by their ability to reduce ischemic ATP breakdown, and hence to reduce ischemic purine release. Ca^{2+}-antagonists are able to reduce ischemic ATP breakdown, when applied to the heart before or during ischemia. Substantial controversy exists about their mechanism of action. Our results with these drugs can be mainly explained by their negative inotropic properties as discussed

in chapter 5. By their negative inotropic properties, ATP demand will be decreased, which makes the cell more capable to maintain proper ATP levels and ionic homeostasis.

The combination of calcium antagonists and beta-blockers has been shown to have a synergistic effect in prevention of ischemic ATP breakdown. The mechanism of this synergism remains to be elucidated. In contrast to application of either drug alone, this ATP saving effect is not due to their negative inotropic action, and some intracellular action must be assumed. Numerous phosphorylation reactions are regulated by c-AMP and Ca^{2+} and are probably partially inhibited by combined beta-blockade and calcium antagonism.

After ischemia, reduced myocardial ATP levels are found, due to washout of the purine catabolites. These low ATP levels make the heart more vulnerable during a next ischemic attack and must be replenished as quickly as possible. The salvage mechanism, i.e., incorporation of hypoxanthine into ATP, is a promising pathway. We have shown, that this pathway is PRPP dependent and can be stimulated after ischemia and during ribose infusion. This last compound can be used clinically, in combination with hypoxanthine and a xanthine oxidase inhibitor. The precursor of hypoxanthine, inosine, deserves special attention in this context. This compound is able to restore ATP levels at a high rate, which cannot be explained by the incorporation of inosine via the salvage mechanism alone. It also shows some beneficial hemodynamic effects, such as positive inotropism and vasodilatation, which makes it clinically very attractive. Further research in this direction would seem urgently required, if the contribution of modern cardiac biochemistry must have significance for the clinic.

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Hypoxanthine production by ischemic heart demonstrated by high pressure liquid chromatography of blood purine nucleosides and oxypurines

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Summary

An isocratic high pressure liquid chromatographic system was developed for the estimation of purine nucleosides and oxypurines in blood. Use was made of a reversed-phase column. Nucleotides derived from erythrocytes affected the separation; these compounds were removed with Al_2O_3. The recovery of the whole clean-up procedure exceeded 75%, and the lower detection limit of the assay for blood metabolites was 0.1 μmol/l. In 6 healthy volunteers, non-resting, the following blood concentrations (mean values ± S.D. in μmol/l) were observed: adenosine (< 0.1), inosine (0.2 ± 0.1), hypoxanthine (2.2 ± 1.3) and xanthine (0.2 ± 0.1). In plasma and serum the total amount of these compounds was 1.9 and 5.4 times higher, respectively, presumably due to nucleotide breakdown during blood processing. The myocardial arterial-venous differences of blood purine nucleosides, oxypurines and lactate were subsequently measured in blood samples from 13 patients with angiographically documented ischemic heart disease, undergoing an atrial pacing stress test. No significant release of adenosine, inosine and xanthine by the heart was detectable in this study. The myocardial arterial-venous difference of lactate changed from 0.01 ± 0.03 mmol/l (mean ± SEM) at rest, to −0.10 ± 0.04 mmol/l during pacing ($p < 0.002$). Relatively larger changes were observed for hypoxanthine: pacing increased the arterial-venous difference from −0.01 ± 0.05 to −0.51 ± 0.17 μmol/l ($p < 0.02$). We conclude that the high pressure liquid chromatographic assay of blood hypoxanthine is a useful tool in the diagnosis of ischemic heart disease.

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Introduction

During and after hypoxia or ischemia, there is in the heart, as well as in other muscles, excessive ATP breakdown. This degradation of ATP causes an efflux of breakdown products, which are able to pass through the cell membrane, and are released into the blood. The purine derivatives adenosine, inosine and hypoxanthine are thought to be markers for ischemia (for a review, see ref. [1]). Because of high activities of adenosine deaminase (EC 3.5.4.4; ADA) and nucleoside phosphorylase (EC 2.4.2.1; NP), and low or non-detectable amounts of xanthine oxidase (EC 1.2.3.2; XO) in the heart and blood, hypoxanthine seems very promising as marker for myocardial ischemia [2,3]. Enzymatic purine determinations [4,5] are time-consuming and do not differentiate between hypoxanthine and xanthine. Recently high pressure liquid chromatography (HPLC) came into use for the determination of nucleosides and purine bases in urine, plasma and serum [6–8]. We have developed a method to determine these compounds in blood, and have compared the levels of adenosine, inosine, hypoxanthine and xanthine in blood, plasma and serum. With the assay we demonstrated the myocardial release of hypoxanthine in patients with angiographically documented ischemic heart disease, undergoing an atrial pacing stress test.

Materials and methods

Enzymes (XO, 13 U/ml; NP, 10 U/ml; ADA, 140 U/ml) were purchased from Boehringer-Mannheim, F.R.G.; CH_3OH (Uvasol), $NH_4H_2PO_4$, KOH, K_2CO_3, adenosine, hypoxanthine and uridine from Merck (Darmstadt, F.R.G.). Uric acid was supplied by Sigma (Saint Louis, MO, U.S.A.), and the other standards by Koch-Light (Colnbrook, Bucks., U.K.). Al_2O_3 came from Desaga, Heidelberg, F.R.G. [U-^{14}C]nucleosides and oxypurines were obtained from The Radiochemical Centre (Amersham, Bucks., U.K.; spec. act. 50–60 Ci/mol). $HClO_4$ was supplied by Merck, and used as a 1.3 mol/l dilution. Water was purified with the Milli-Ro4/Milli-Q system (Millipore Co., Bedford, MA, U.S.A.).

Blood sample treatment

Blood (2.0 ml) was deproteinized immediately with an equal volume of $HClO_4$ as described by Remme et al. [2]. Plasma was prepared by adding 3 ml blood to a heparinized tube and immediate centrifugation for 15 min at $3000 \times g$ (4°C). Serum was prepared from 3 ml blood which had been allowed to clot at room temperature for 30 min and subsequent centrifugation. Plasma and serum were also deproteinized with equal volumes of $HClO_4$. After centrifugation, the supernatant fluids were stored at −20°C. The acid extracts were brought to pH 5–7 at 0°C with an automatic titration device (Radiometer, Copenhagen) using 150–200 µl 6 mol/l KOH + 1 mol/l K_2CO_3. $KClO_4$ was spun down for 10 min at $12000 \times g$ at 4°C. For plasma and serum 200 µl of these extracts were directly injected onto the HPLC column. For whole blood extracts, removal of nucleotides appeared to be necessary. We used the method of Chatterjee et al. [9], with some minor differences. We applied

1.5 ml of the deproteinized, neutralized blood sample onto a pre-washed column of Al_2O_3 (0.6 g) in a Pasteur pipette, and eluted it with 5.0 ml 10 mmol/l Tris/HCl, pH 7.4. For faster elution, a vacuum was applied; with a 1225 sampling manifold (Millipore Co.) 12 samples were treated at the same time. With standards (about 10 μmol/l oxypurines and nucleosides, and about 5 mmol/l nucleotides), we found recoveries exceeding 90%, whereas the nucleotides were fully retained. These values are in close agreement with values in the literature [9].

Recovery studies for blood treatment

To carry out recovery studies, it is necessary to inactivate first the purine metabolizing enzymes in the blood. We achieved this by rapid mixing of blood with cold $HClO_4$. This procedure is comparable to the determination of recoveries after inactivation of enzymes by freeze-clamping heart tissue, or removal of cells from blood, and subsequent addition of standards [4,5,7,8,10–12]. If radioactive standards were used 10 μl ^{14}C-labelled hypoxanthine, xanthine, inosine or adenosine were mixed with 10 ml $HClO_4$. For the determination of radioactivity 50 μl aliquots were mixed with 10 ml Insta-gel (Packard Instr. Co., Downers Grove, IL, U.S.A.) and counted with a Packard Tricarb 2650 liquid scintillation counter.

High pressure liquid chromatography

A Varian 8520 high pressure liquid chromatograph (Varian, Palo Alto, CA, U.S.A.) was used with a pneumatic sampling device (Valco Instr. Co., Houston, TX, U.S.A.), an autosampler (Varian), a Model 440 fixed wavelength UV-detector (Waters Assoc., Milford, MA, U.S.A.) and a chromatographic data system C-111 (Varian). A 4 mm I.D. $\times$ 30 cm prepacked μBondapak/C_{18} column (Waters Assoc.) was used in these studies. The packing material has an average particle size of 10 μm and consists of porous silica to which a monolayer of octadecyltrichlorosilane is chemically bound. Chromatographic conditions were adapted from earlier work [13]: 200 μl samples were eluted from this column with 10 mmol/l $NH_4H_2PO_4/CH_3OH$ (10 : 1, v/v), pH 5.50. The flow rate was 60 ml/h ($p = 80$ bar).

Lactate assay

Lactate was determined in acid supernatants in duplicate with an AutoAnalyzer II (Technicon, Tarrytown, NY, U.S.A.) as described by Apstein et al. [14].

Statistical analysis

Statistical analysis in Table II was performed with a two-group comparison test based on Student's t distribution, and those in Table III and Fig. 5 by Student's paired t test. p-values < 0.05 (two-sided) were considered significant.

Results

HPLC

Fig. 1 represents the separation of several nucleosides and purine bases. The lower detection limit with a 200 μl sample loop is 0.01 μmol/l; the method is linear up to

200 μmol/l (Fig. 2). The standard deviation at the 10, 1 and 0.1 μmol/l level is $< 0.12\%$, $< 5\%$ and $< 9\%$, respectively ($n = 9$). Identification of the compounds in pre-purified blood samples was achieved by retention times (Fig. 3a), co-chromatography of standards (Fig. 3b) and enzyme shifts (Fig. 3c), as described by Hartwick et al. [7]. The clean-up procedure of the blood samples causes a 9-fold dilution, which increased the lower detection limit in blood to 0.1 μmol/l.

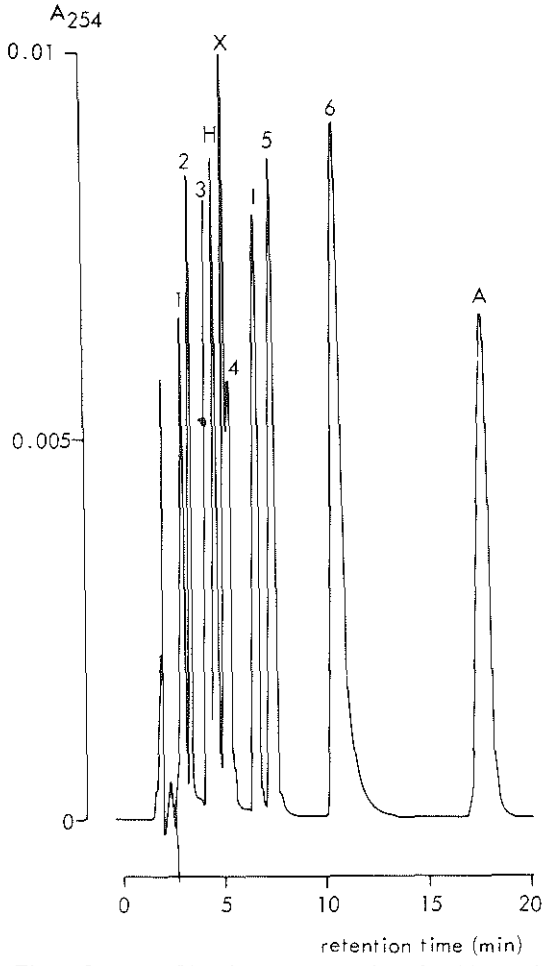

Fig. 1. Isocratic HPLC separation of nucleosides and purine bases (2–4 μmol/l). Column: μBondapak C_{18} (4×300 mm). Buffer: 10 mmol/l $NH_4H_2PO_4/CH_3OH$ (10:1, v/v), pH 5.50. Flow rate: 60 ml/h. Injected sample volume: 200 μl. 1, uric acid; 2, uracil; 3, uridine; H, hypoxanthine; X, xanthine; 4, xanthosine; I, inosine; 5, guanosine; 6, adenine; A, adenosine.

Recoveries

With radioactive standards the recoveries for the whole clean-up procedure exceeded 75% (Table I). With non-radioactive standards, assayed by HPLC, we found comparable recoveries in the concentration range of our interest (Fig. 4).

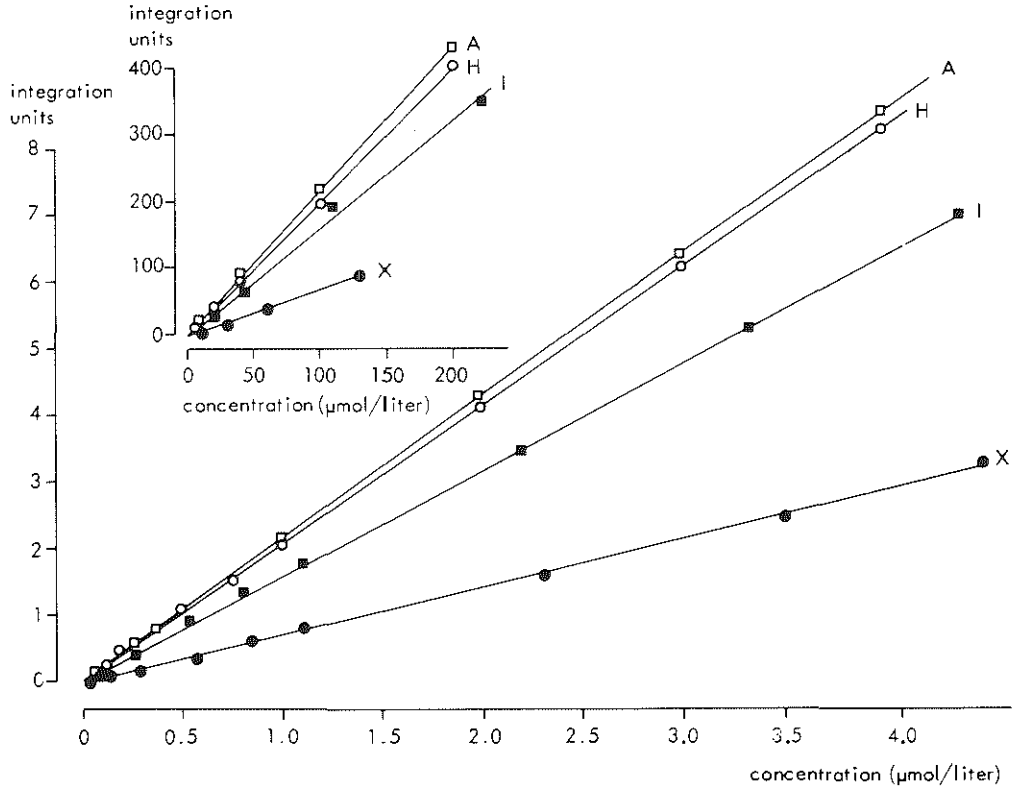

Fig. 2. Calibration curves for HPLC of hypoxanthine (○ ——— ○) $y=2.16x-0.09$, $r=1.000$; xanthine (● ——— ●) $y=0.73x+0.01$, $r=0.999$; inosine (■ ——— ■) $y=1.59x+0.02$, $r=1.000$; adenosine (□ ——— □) $y=2.23x-0.05$, $r=0.999$.

Purine derivatives in blood, plasma and serum

Six healthy non-fasting, non-resting volunteers donated venous blood. In their blood, plasma and serum, hypoxanthine, xanthine, inosine and adenosine were

TABLE I

RECOVERY OF PURINE NUCLEOSIDES AND OXYPURINES DURING SAMPLE PREPARATION

(Data represent mean values of 6 experiments ± S.E.M.)

Clean-up stage	Adenosine	Inosine	Hypoxanthine	Xanthine
	dpm added			
Acidified human blood (= 100%)	50000	330000	230000	44000
	recovery (%)			
Acid supernatant	96 ± 3	106 ± 1	101 ± 1	93 ± 4
Neutralized extract	88 ± 2	101 ± 2	92 ± 2	99 ± 4
Alumina eluate	79 ± 2	91 ± 4	86 ± 1	76 ± 6

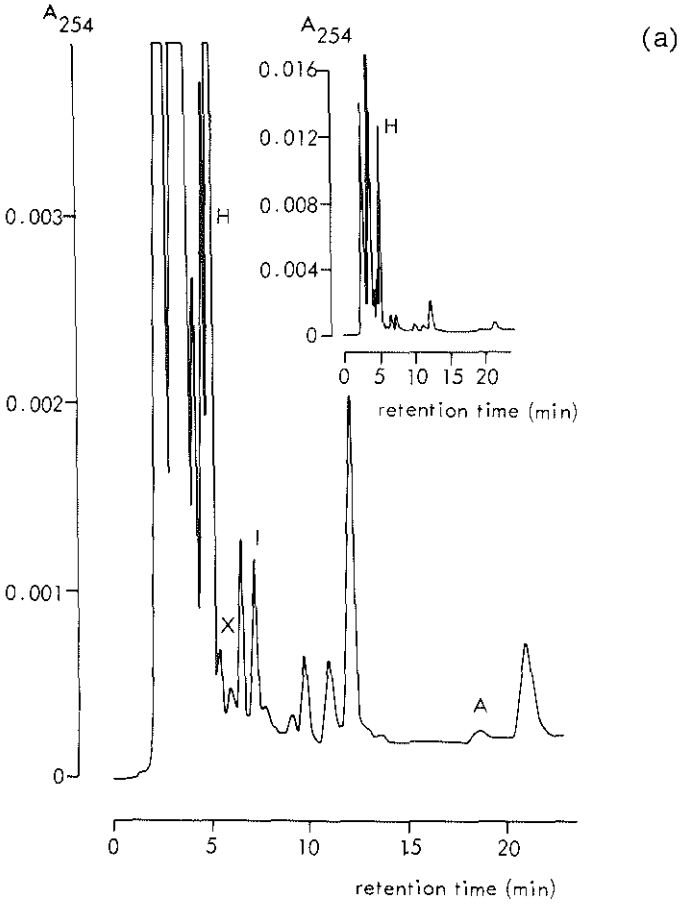

Fig. 3 (a, b and c). Chromatogram of blood from a normal donor. (a) Blood was deproteinized and purified as described under "Materials and methods". Conditions, see Fig. 1. (The upper panel represents the same chromatogram with a 4 times lower detector sensitivity.) H, hypoxanthine; X, xanthine; I, inosine; A, adenosine.

TABLE II

CONCENTRATION OF ADENOSINE, INOSINE AND (HYPO)XANTHINE IN HUMAN BLOOD, PLASMA AND SERUM

(Data represent mean values in μmol/l of 5–6 volunteers ± S.D.)

Preparation	Adenosine (μmol/l)	Inosine (μmol/l)	Hypoxanthine (μmol/l)	Xanthine (μmol/l)
Blood	<0.1	0.2 ± 0.1	2.2 ± 1.3	0.2 ± 0.1
Plasma	<0.1	0.2 ± 0.2	3.6 ± 0.8	1.1 ± 0.7 *
Serum	0.9 ± 0.2 *▲	1.0 ± 0.4 *▲	5.6 ± 1.9 *	6.6 ± 2.1 *▲

* $p<0.05$ vs. blood, ▲ $p<0.05$ vs. plasma.

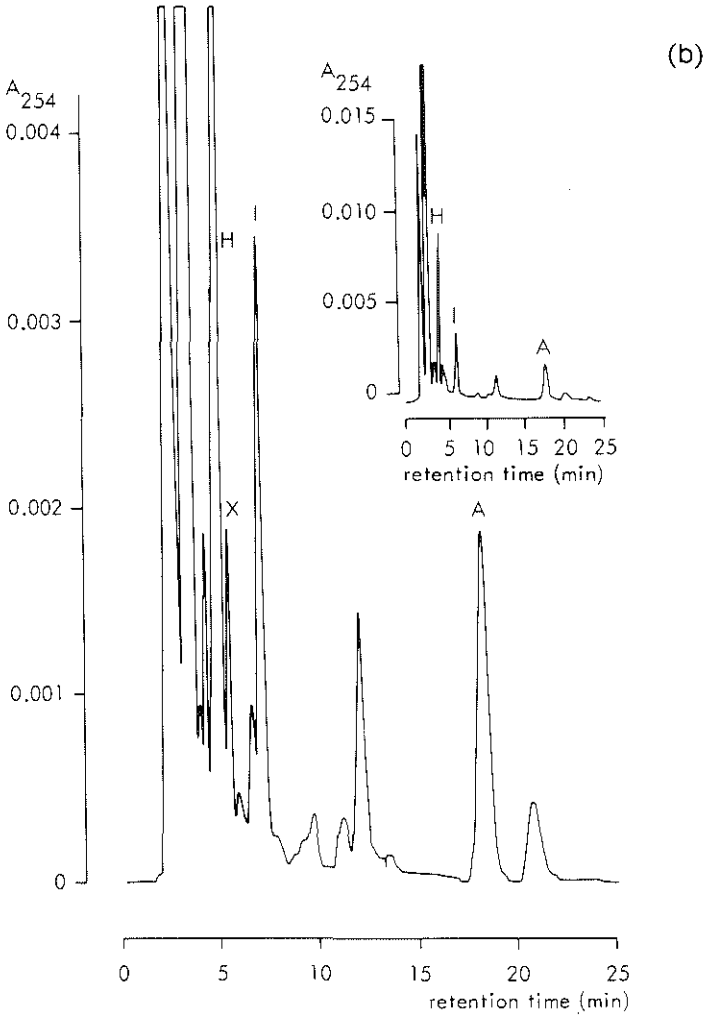

Fig. 3(b). Chromatogram as in (a), after co-injection of a solution containing hypoxanthine, xanthine, inosine and adenosine.

determined. The results are listed in Table II. As can be seen plasma shows 1.6 times higher levels of hypoxanthine and 5.5 times higher levels of xanthine than blood. Serum has significantly higher values of the AMP-catabolites. Here the values are 2.5 times (for hypoxanthine) to 33 times (for xanthine) higher, compared to blood.

Determination of blood purine nucleosides, oxypurines and lactate of patients with ischemic heart disease

Thirteen patients with angina pectoris were catheterized as described by Remme et al. [2]. The patients were fasted overnight and 36 h before catheterization all medication was stopped. Before catheterization 50000 IE of heparin were infused. The diagnosis, ischemic heart disease, was established by angiography (obstruction of at least one coronary artery $>50\%$). Before angiography an atrial pacing stress

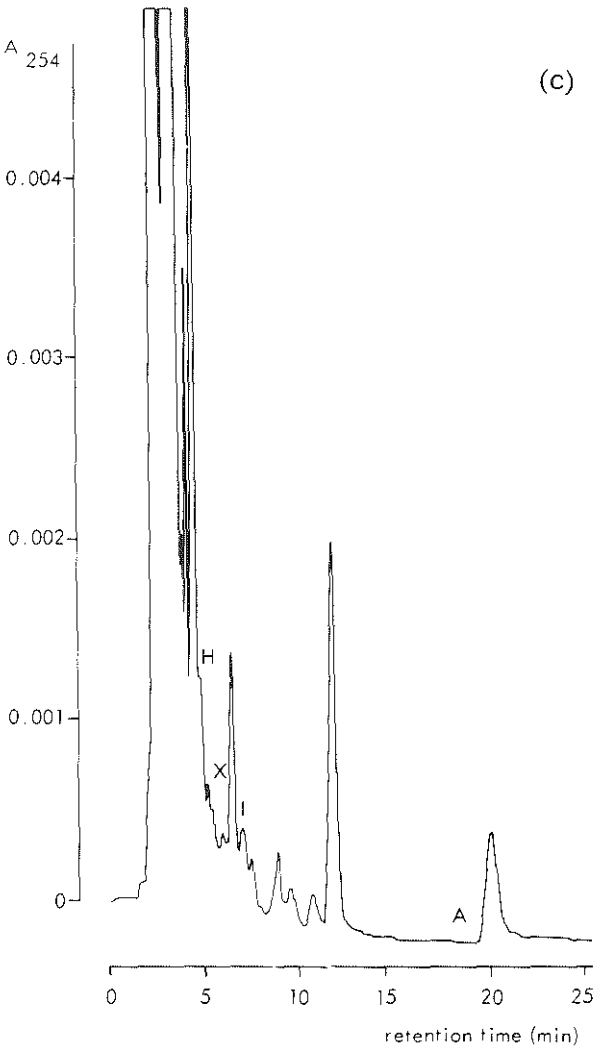

Fig. 3(c). Chromatogram as in (a), after 30 min incubation with XO, NP and ADA, 5 μl each.

test was performed. During a control period, maximal pacing, and 5 and 20 min after pacing, arterial and coronary sinus blood samples were taken. At rest no significant myocardial arterial-venous hypoxanthine or lactate difference was seen (Fig. 5). During maximal pacing, a rise in coronary sinus hypoxanthine (from 0.83 to 1.43 μmol/l, $p < 0.01$) and lactate (from 0.87 to 0.98 mmol/l, $p < 0.005$) was observed, which fell off after pacing. The arterial levels of these compounds remained constant. The myocardial arterial-venous difference of hypoxanthine changed from -0.01 μmol/l at rest to -0.51 μmol/l ($p < 0.02$) during pacing (Table III). We showed in earlier work [2,15] that patients with anginal pain, but with patent coronary arteries ($< 50\%$ obstruction), did not produce myocardial hypoxanthine. Relatively smaller changes were observed for lactate: pacing in-

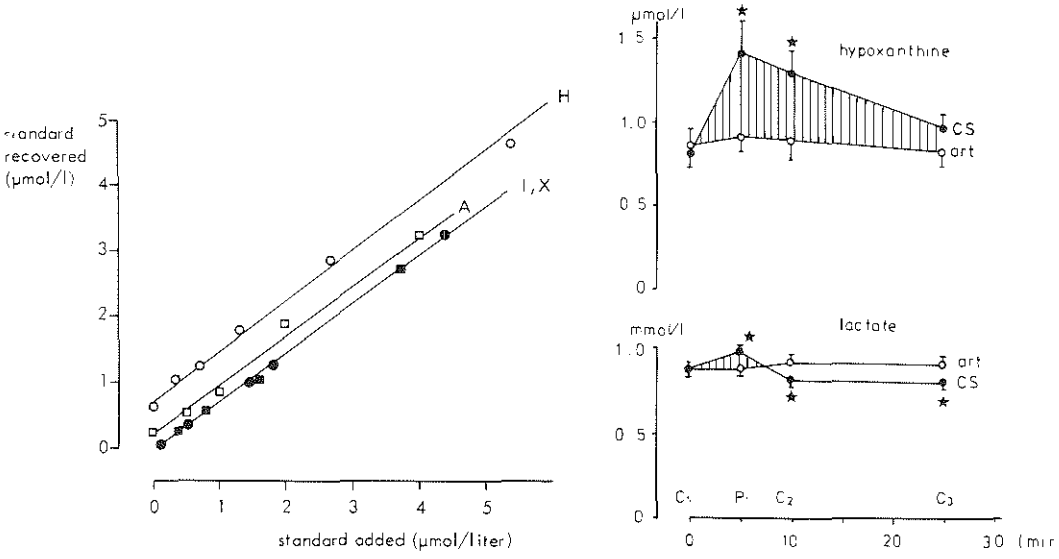

Fig. 4. Recoveries determined by HPLC. Standards were dissolved in $HClO_4$, before blood was added. Hypoxanthine: (○——○) $y=0.72x+0.77$, $r=0.997$; xanthine: (●——●) $y=0.68x+0.01$, $r=0.999$; inosine: (■——■) $y=0.72x-0.03$, $r=0.993$; adenosine: (□——□) $y=0.77x+0.20$, $r=0.995$. For conditions, see legends to Figs. 1 and 3.

Fig. 5. Arterial (○——○) and coronary sinus (●——●) hypoxanthine and lactate levels during an atrial pacing stress test of 13 patients with ischemic heart disease. C_1, control period; P_1, maximal pacing; C_2 and C_3, 5 and 20 min after maximal pacing, respectively. Mean values are given with 1 S.E.M. (★ $p<0.05$ vs. arterial levels).

TABLE III

MYOCARDIAL ARTERIAL-VENOUS DIFFERENCES AND EXTRACTION VALUES OF HYPOXANTHINE AND LACTATE DURING AN ATRIAL PACING STRESS TEST OF PATIENTS WITH ISCHEMIC HEART DISEASE

The extraction value is defined as the arterial concentration minus coronary sinus concentration divided by the arterial concentration, times 100%. Mean values are given ± S.E.M. ($n=13$).

	Heart rate (beats/min)	Hypoxanthine		Lactate	
		A−CS (μmol/l)	Extraction (%)	A−CS (mmol/l)	Extraction (%)
C_1	79±4	−0.01±0.05	−6± 6	0.01±0.03	4±4
P_1	144±4 *	−0.51±0.17 *	−68±18 *	−0.10±0.04 *	−9±6 *
C_2	78±4	−0.33±0.14 *	−49±14 *	0.08±0.03	13±6
C_3	80±4	−0.15±0.08	−35±14	0.12±0.04 *	19±6 *

* $p<0.05$ vs. C_1.

A−CS, arterial-venous difference; C_1, control period; P_1, maximum pacing; C_2, 5 min after pacing; C_3, 20 min after pacing.

creased the arterial-venous difference from 0.01 to -0.10 mmol/l ($p < 0.002$, see Table III). Significant production of lactate, calculated either as arterial-venous difference or extraction, only took place during maximal pacing, but significant hypoxanthine release was also present 5 min after pacing was stopped (Table III). The coronary sinus levels of adenosine, inosine and xanthine during the control period were 0.17 ± 0.04, 0.53 ± 0.11 and 0.20 ± 0.14 μmol/l, respectively. No significant arterio-venous differences were observed throughout the test.

Discussion

Hypoxanthine, xanthine, inosine and adenosine levels in human blood (or plasma or serum) have been measured in normal adults [7–11,16], patients with gout [17], immunological disorders [18], ischemic heart disease [2,3,19,20], pregnant women [21], and children [21]. The values reported show considerable variation, which could be caused by:

(1) Differences in assay methods. The enzymatic determination of hypoxanthine, for instance, does not differentiate between hypoxanthine and xanthine.

(2) As we have shown in Table II, large differences occur in the levels of these compounds when these are measured in blood, plasma or serum from the same person. These differences can be explained by the enzymatic degradation of ATP, derived from blood cells, and ADP, released from platelets during clotting [7,8]. Furthermore xanthine, as the end-product of guanine nucleotide catabolism, can be expected to occur in higher concentrations in plasma and serum than in blood. For instance, blood xanthosine, guanosine and guanine are rapidly converted to xanthine [22]. For this reason instant inactivation by acid seems indicated, when one wants to determine the nucleoside and oxypurine concentration in the blood.

(3) Physical activity also influences the levels of these AMP catabolites in blood. Sutton et al. [23] showed that the plasma levels of oxypurines are twice as high during and after physical training compared with control levels. Although they did not measure inosine levels in the plasma, urinary inosine during excercise is elevated tenfold, possibly indicating increased inosine levels in the blood. Therefore, if one wants to measure normal values, it is also important to define the physical state of the group under study.

We feel that measuring myocardial arterial-venous differences of blood hypoxanthine levels could give insight into the metabolic state of the heart; the method described here makes it possible to measure a number of purine metabolites in blood. The observations on the patients undergoing an atrial pacing stress test indicate that hypoxanthine is a more sensitive parameter for myocardial ischemia than adenosine, inosine, xanthine or lactate, because hypoxanthine release is more pronounced and of a longer duration than that of the other compounds.

Acknowledgements

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APPENDIX PAPER 2

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Simultaneous determination of myocardial adenine nucleotides and creatine phosphate by high-performance liquid chromatography

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ATP is one of the most important substances for the proper functioning of the heart [1]. Creatine phosphate (CrP) serves as an "energy buffer", and could also play a role in the rapid transport of energy between cell compartments [2]. Separations by high-performance liquid chromatography (HPLC) of nucleotides on an ion-exchange column with detection at 254 nm have been carried out [3, 4]. Recently, determinations of nucleotides on reversed-phase columns [5], with or without ion-pairing [6, 7], have also been made. CrP, however, cannot be detected at 254 nm. Juengling and Kammermeier [6] detected CrP at 210 nm, but their HPLC system could not separate adenine nucleotides. Heldt et al. [8] used a phosphate analyzer as a detector and were able to detect CrP in one run with nucleotide quantitation. We modified the separation and detection conditions of Edelson et al. [9]. Analysis time was decreased by a factor of two and detection at 210 nm was introduced. We obtained sufficient resolution to quantitate CrP and adenine nucleotides within 30 min.

EXPERIMENTAL

Reagents

Hexokinase (EC 2.7.1.1, 140 U/ml), creatine kinase (EC 2.7.3.2, 25 U/ml), and adenylate kinase (EC 2.7.4.3, 360 U/ml) were purchased from Boehringer, Mannheim, G.F.R.; AMP-deaminase (EC 3.5.4.6, 30—60 U/mg) was obtained from Sigma (St. Louis, MO, U.S.A.). KH_2PO_4, H_3PO_4, KOH and $HClO_4$ were from Merck (Darmstadt, G.F.R.), the nucleotides and CrP were from Boehringer and creatine was from Technicon (Tarrytown, NY, U.S.A.). Water

was purified with the Milli-Ro4/Milli-Q system (Millipore, Bedford, MA, U.S.A.).

High-performance liquid chromatography

A Varian 8520 HPLC system (Varian, Palo Alto, CA, U.S.A.) was used, which consisted of two positive displacement pumps, a variable-wavelength UV detector (Varichrom) set at 210 nm, a pneumatic sampling device, and a chromatographic data system (CDS-111) connected with a chart recorder. Buffers were prepared on the day of use and filtered through a 0.45-μm filter (Millipore). Buffer A consisted of 0.01 *M* H_3PO_4, adjusted to pH 2.85 with KOH; buffer B consisted of 0.75 *M* KH_2PO_4, pH 4.40. The column (Partisil-10-SAX, 0.4 × 25 cm, particle size 10 μm; Whatman, Maidstone, Great Britain) was eluted with buffer A at a flow-rate of 2.0 ml/min. Five minutes after injection, a gradient started with an increase of 4% B per minute until 100% B.

Rat heart perfusions and sample treatment

Isolated hearts from male rats (Wistar strain, 300—400 g) were perfused retrogradely for 30 min and quickly frozen as described before [10]. After weighing, cardiac tissue was ground in a mortar, precooled with liquid nitrogen. One half of the sample (about 0.5 g) was freeze-dried for determination of percentage dry weight; the other half was extracted with 3.0 ml of 0.8 *M* $HClO_4$. After thawing and centrifugation, 2.0 ml of the supernatant fluid were neutralized at 0°C with about 200 μl of 6 *N* KOH and the $KClO_4$ was spun down at 4°C. A 20-μl volume of this supernatant was applied to the HPLC column.

RESULTS AND DISCUSSION

Chromatograms

Fig. 1 shows the separation of a standard mixture of thirteen nucleotides and creatine compounds. Fig. 2A gives the separation of the adenine nucleotides and CrP in an extract of an oxygenated rat heart.

Linearity, recovery and sensitivity

The lower detection limit with a 20-μl sample loop varies between 20 pmol for AMP and 100 pmol for CrP, and the determination is linear up to 400 nmol for all compounds indicated. Recoveries, determined in a model system (high-energy phosphates added to a 1 g/ml albumin solution), after deproteinization and neutralization, exceeded 95%, with a standard deviation of $<3\%$ for the adenine nucleotides and $<6\%$ for CrP ($n = 5$).

Peak identification

Fig. 2A gives the chromatogram of a rat heart extract. The main peaks in the extract are creatine, NAD, CrP, ADP and ATP. Peak identification was made by comparing retention times with standards and by enzymic peak shifts [3]. Fig.

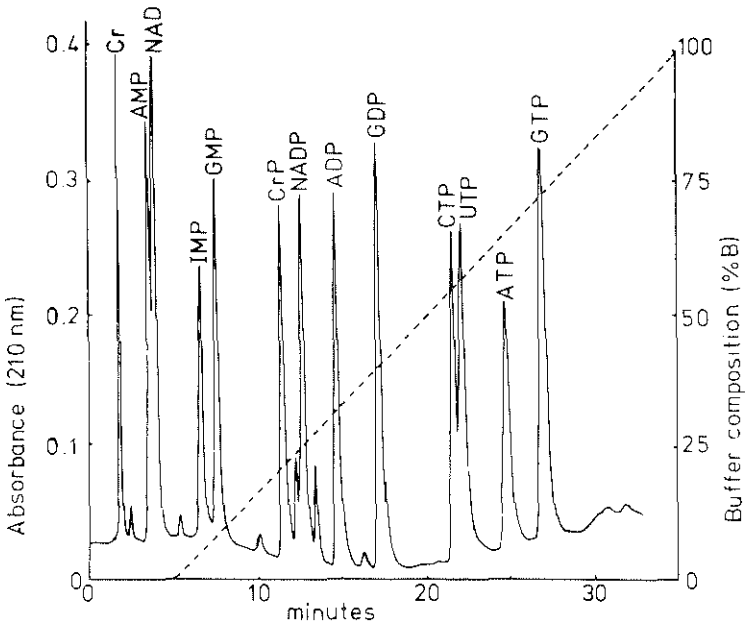

Fig. 1. Separation of standard nucleotides and creatine (Cr) compounds (0.2—0.5 mmol/l). Injection volume, 20 µl; flow-rate, 2.0 ml/min; eluents, A = 0.01 *M* H_3PO_4, adjusted to pH 2.85 with KOH, B = 0.75 *M* KH_2PO_4 (pH 4.40); column, Partisil-10-SAX.

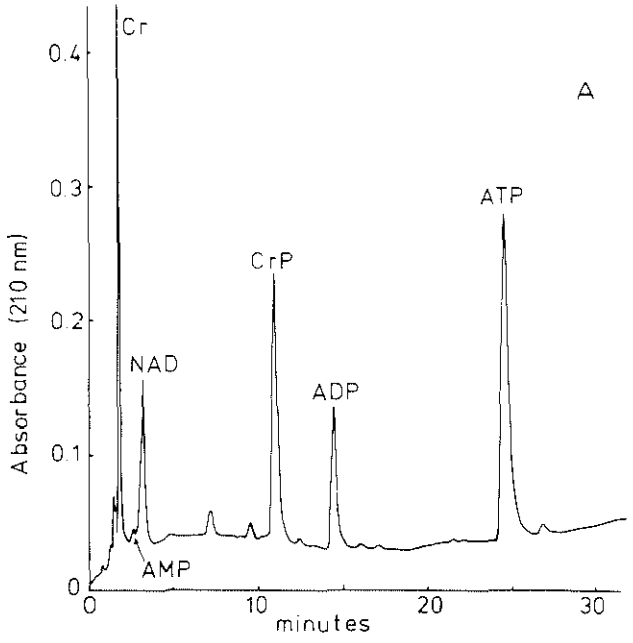

Fig. 2. Chromatogram of a rat heart extract and peak identification by enzymic conversions. HPLC was carried out with a 20-µl extract, prepared as described in the text. For HPLC conditions, see Fig. 1. Panel A gives the chromatogram of untreated extract. Enzymic peak shifts were carried out according to the method of Brown [3]. An aliquot of the extract was

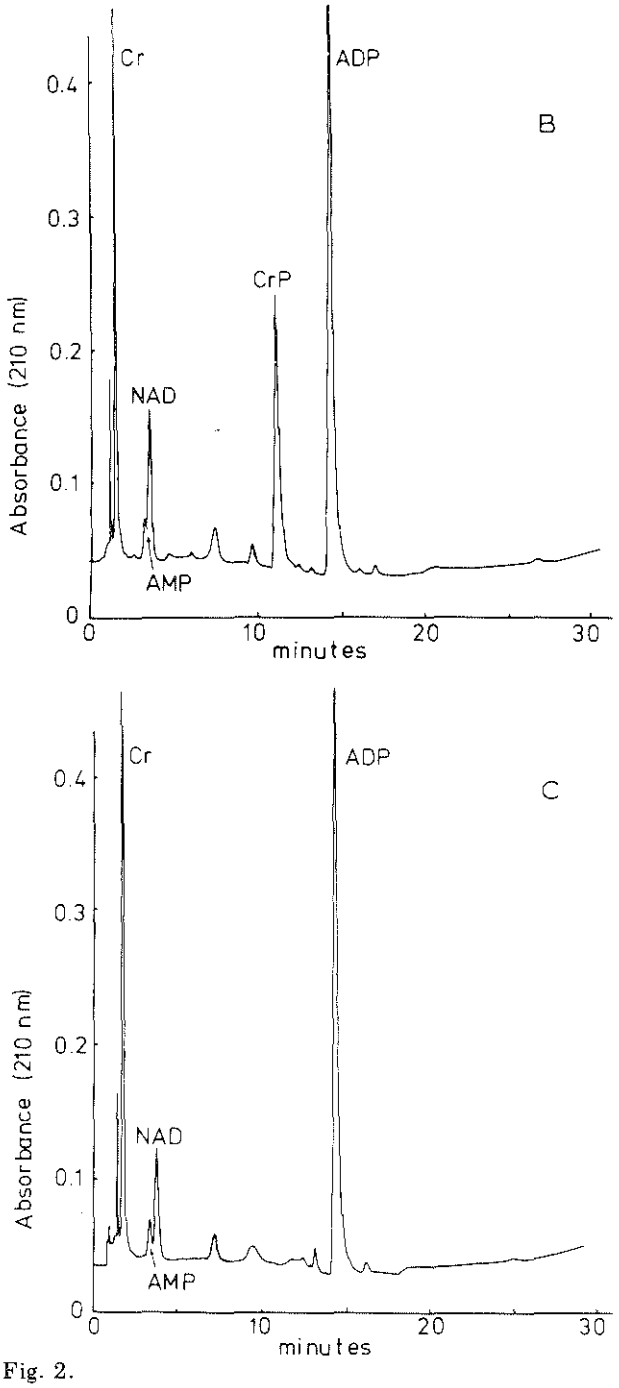

Fig. 2.

incubated for 30 min at 37°C with 0.7 mU hexokinase and D-glucose to convert ATP: ATP + glucose → ADP + glucose-6-phosphate (panel B). Subsequently CrP was broken down by addition to the extract of 0.1 mU of creatine kinase and $MgCl_2$. Under the influence of creatine kinase and hexokinase, the overall reaction is: CrP + glucose → creatine + glucose-6-

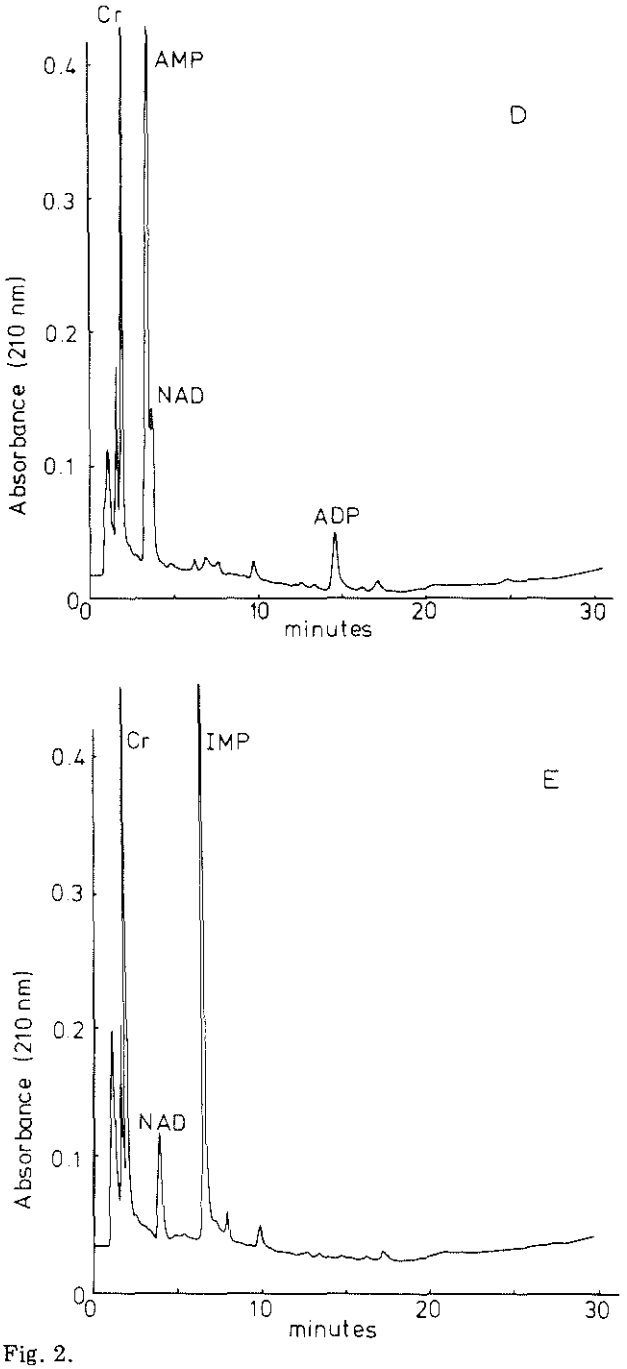

Fig. 2.

phosphate (panel C). In a similar way ADP was converted with 1.8 mU of adenylate kinase: 2ADP + glucose → 2AMP + glucose-6-phosphate (panel D). Finally, the AMP peak was shifted with 0.4 mU of AMP-deaminase: AMP → IMP + NH_3 (panel E).

2B—E shows chromatograms after subsequent incubation with hexokinase (B), creatine kinase (C), adenylate kinase (D) and AMP-deaminase (E). Complete removal of ATP, CrP and AMP was observed, with a concomitant increase of the conversion products ADP, creatine and IMP. ADP in the chromatogram shown in Fig. 2D is not completely removed, presumably because of an unfavorable equilibrium of the adenylate kinase reaction. After addition of AMP-deaminase, which removes AMP, all ADP is converted (Fig. 2E).

Rat heart concentrations

In Table I concentrations are given for adenine nucleotides and CrP, measured in normal and anoxic rat hearts. These values are in the same range as found in the literature [1, 11, 12].

TABLE I

CONCENTRATION OF ADENINE NUCLEOTIDES AND CREATINE PHOSPHATE IN RAT HEART

Hearts were perfused retrogradely (Langendorff perfusion) for 30 min with a modified Tyrode solution, equilibrated with 95% O_2—5% CO_2 (normoxia), or for 15 min perfused with 95% O_2—5% CO_2 and 15 min with 95% N_2—5% CO_2 (anoxia). Heart extracts were prepared as described in the text. For HPLC conditions and peak identification, see Figs. 1 and 2.

Condition	μmol/g dry weight (± S.E.M.)			
	ATP	ADP	AMP	CrP
Normoxia	20.9 ± 0.5	7.3 ± 0.4	0.2 ± 0.1	29.3 ± 2.8
Anoxia	9.9 ± 1.9*	9.5 ± 2.2	5.1 ± 1.6*	7.4 ± 1.8*

*$p < 0.005$ vs. normoxia, n = 5—7.

Conclusion

We conclude that the method presented here is a quick and accurate way to determine myocardial high-energy phosphates. Within 30 min the concentration, energy charge [13] and ATP/CrP ratio can be read from the chromatogram.

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REGULATION OF PORCINE HEART AND SKELETAL MUSCLE AMP-DEAMINASE BY ADENYLATE ENERGY CHARGE

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Abstract—1. Cytosol from pig skeletal muscle, but not heart, contains an inhibitor of AMP-deaminase (AMP-D, EC 3.5.4.6) which reduces AMP-D activity 8-fold.

2. Heart and skeletal muscle AMP-D have been purified to apparent homogeneity by cellulose phosphate and DEAE–Sephacel chromatography.

3. AMP-D from skeletal muscle is inhibited more severely than the heart enzyme by an increase in adenylate energy charge to levels exceeding 0.4. Nevertheless both enzymes seem to be regulated by the energy charge, which contrasts with reports for rabbit heart AMP-D.

INTRODUCTION

AMP-deaminase (AMP-D, EC 3.5.4.6) catalyzes the hydrolytic deamination of AMP to IMP and NH_3. The enzyme seems important in the regulation of the adenylate energy charge (ATP + 0.5 ADP)/(ATP + ADP + AMP) and the adenine nucleotide pool, together with 5'-nucleotidase (EC 3.1.3.5) and adenylate kinase (EC 2.7.4.3) (see review: De Jong, 1979). Relatively little is known about AMP-D in heart. In view of the presence of tissue specific isozymes of AMP-D (Ogasawara *et al.*, 1978; 1982), it seems likely that the physiological role of this enzyme may vary from tissue to tissue.

AMP-D has been purified to homogeneity from skeletal muscle of rabbit (Smiley *et al.*, 1967; Stankiewicz *et al.*, 1979), rat (Coffee & Kofke, 1975; Ranieri-Raggi and Raggi, 1976; Ogasawara *et al.*, 1978; Shiraki *et al.*, 1979; Stankiewicz *et al.*, 1979) and man (Stankiewicz, 1981; Ogasawara *et al.*, 1982). The heart enzyme has been purified only partially from a number of mammalian sources: rabbit (Chung & Bridger, 1976; Solano & Coffee, 1978; Barsacchi *et al.*, 1979), rat (Ogasawara *et al.*, 1975; Kaletha & Skladanowski, 1979), pig (Purzycka-Preis *et al.*, 1978), beef (Skladanowski *et al.*, 1981) and man (Kaletha *et al.*, 1979; Ogasawara *et al.*, 1982). Because of our interest in pig purine metabolism (De Jong & Goldstein, 1974; De Jong *et al.*, 1977) we decided to purify porcine heart and skeletal muscle AMP-D to homogeneity and studied the response of the enzymes to various adenylate energy charges*.

MATERIALS AND METHODS

Nucleotides

These were purchased from Boehringer, Mannheim, GFR.

AMP-D assay (see Smiley *et al.*, 1967)

The enzyme was incubated at 30°C with 50 mM imidazole.HCl, 20 mM sodium cacodylate, 0.5 M KCl, 1 mM 2-mercaptoethanol and 5 mM 5'-AMP (except for the experiments shown in Fig. 1). The pH was 6.5 and the final volume 1.0 ml. IMP formation was detected at 285 nm with a Gilford 2600 spectrophotometer (Gilford, Oberlin, OH).

Protein determination

The Coomassie Brilliant Blue (Biorad Laboratories, Munich, GFR) assay was used with bovine serum albumin (Sigma, St. Louis, MO) as the standard.

Purification of AMP-D (see Coffee & Kofke, 1975; Purzycka-Preis *et al.*, 1978)

Each buffer contained 1 mM 2-mercaptoethanol. All steps were performed at 0–5°C. Heart or white skeletal muscle (100 g) from piglets (*Sus scrofa*, about 25 kg) was homogenized with 3 vol (v/w) of 0.18 M KCl–0.054 M KH_2PO_4–0.035 M K_2HPO_4 (pH 6.5). The homogenate was centrifuged for 30 min at 25,000 *g*, and the supernatant fluid passed through Nuova filter material (Hoffmann, Overath-Eulenthal, GFR). The eluate was stirred with 30 ml washed cellulose phosphate P11 (Whatman, Maidstone, UK; 5 g) for 1 hr. The slurry was then transferred to a glass column (1.5 × 12 cm) and washed with the extraction buffer until the effluent had an absorbance at 280 nm below 0.01. The enzyme was eluted with 1 M KCl (pH 6.5). Fractions with the highest specific activity were pooled and dialyzed against 0.045 M potassium phosphate (pH 7.2). The dialyzate was applied to a DEAE–Sephacel column (Pharmacia, Uppsala, Sweden; 1.5 × 15 cm), which had been equilibrated with this phosphate buffer, and washed with 100 ml buffer. The enzyme was eluted with a linear gradient consisting of 100 ml each of 0.045 and 0.45 M potassium phosphate (pH 7.2).

Generation of energy charge ratios

AMP and ATP were incubated with adenylate kinase as described by Coffee & Solano (1977). The concentrations of AMP, ADP and ATP were determined by high performance liquid chromatography (Harmsen *et al.*, 1982). The individual nucleotide concentrations were found to be within 10% of the calculated ones.

* Part of this study has been presented at the 4th International Symposium on Human Purine and Pyrimidine Metabolism, Maastricht, The Netherlands (Verwoerd *et al.*, 1982).

Table 1. AMP-deaminase activity (U/mg protein) of pig heart and skeletal muscle cytosol

Treatment	Heart	Skeletal muscle
None	0.099 ± 0.006	0.340 ± 0.018*†
Gel filtration	0.104 ± 0.010	2.74 ± 0.038*

Mean values of 3 to 4 experiments ± SEM. Student's *t*-test: *P < 0.001 vs heart, †P < 0.001 vs treatment with Sephadex G-25 gel.

RESULTS

Activity

Table 1 shows that the activity of AMP-D in the cytosol of skeletal muscle could be increased 8-fold (to 2.7 U/mg protein) by removal of endogenous inhibitors. This treatment did not influence the heart enzyme, which had an activity of 0.10 U/mg protein.

Purification

In Table 2 data of the purification of AMP-D from heart and skeletal muscle are presented. With cellulose phosphate and DEAE–Sephacel chromatography they were purified 426 and 526 times, respectively. Disc electrophoresis on polyacrylamide (10% acrylamide, plus sodium dodecylsulfate) gave only one band. The cardiac enzyme did not show 5'-nucleotidase or adenosine deaminase (EC 3.5.4.4) activity. It was quite labile: 10% of enzymic activity disappeared when the preparation was kept overnight at −20°C. Repeated freeze-thawing caused the activity to disappear completely. Purified skeletal muscle AMP, on the other hand, remained active, when treated like this.

Properties of the purified enzymes

The apparent K_m of purified heart and skeletal muscle AMP-D was 3.8 and 1.2 mM, respectively. The Hill constant, n_H, was comparable for the two enzymes: 1.0 (heart AMP-D) and 1.1 (skeletal muscle AMP-D).

Effect of energy charge

Skeletal muscle AMP-D was inhibited by increasing adenylate energy charge (Fig. 1). Heart AMP-D showed a biphasic response. At energy charges which are probably physiological (>0.5), skeletal muscle AMP-D is inhibited 1.5–2.7 times more than heart AMP-D. The effects were most pronounced at lower (5 mM) total adenine nucleotide pool sizes.

DISCUSSION

The activity of AMP-D in pig skeletal muscle cytosol increased 8-fold, when low molecular weight components were removed (Table 1). It is of interest that Fishbein *et al.* (1981) found a specific inhibition of muscle AMP-D in serum and plasma of humans and animals. Apparently pig heart cytosol did not contain inhibitors which could be removed by gel filtration (Table 1). The V_{max} is 25 times lower than that in skeletal muscle cytosol (after gel filtration). Ogasawara *et al.* (1982) did a similar observation in human tissues.

Skeletal muscle AMP-D has been purified to homogeneity from a variety of species. However, the heart enzyme has only been completely purified from duck (Pekkel' and Kirkel', 1979), but not from the mammalian heart. Purzycka-Preis *et al.* (1978) obtained AMP-D from porcine heart by cellulose phosphate chromatography, but their preparation was presumably impure. We found it necessary to use DEAE–Sephacel chromatography after the cellulose phosphate step to obtain an apparently homogeneous preparation. Our procedure is considerably simpler than the one used by Pekkel' & Kirkel' (1979).

Purzycka-Preis *et al.* (1978) found a half-saturating substrate concentration for porcine heart AMP-D of 5 mM. This is comparable to our value (3.8 mM). For pig skeletal muscle AMP-D we found the K_m to be 3-fold lower. This contrasts findings by Ogasawara *et al.* (1975) who reported that rat skeletal muscle AMP-D has a K_m which is 3–4 times higher than that of heart.

Purzycka-Preis *et al.* (1978) reported cooperative binding of AMP for pig heart AMP-D ($n_H = 2.35$). This value decreased to about 1 on addition of ATP and liposomes. In our hands $n_H = 0.96$ was found for the purified enzyme without any addition, which is similar to the value reported for rat heart AMP-D (Kaletha & Skladanowski, 1979). We conclude from our studies that the number of binding-sites for AMP on AMP-D from pig heart and skeletal muscle is the same.

Solano & Coffee (1978) reported that rabbit heart AMP-D was unaffected by energy charges below 0.9, in contrast to the skeletal muscle enzyme which increased gradually in activity when the energy charge was decreased from 1.0 to 0. We observed that pig skeletal muscle enzyme AMP-D behaved comparably to that from rabbit (Fig. 1). Although the inhibition observed with physiological energy charges was less for the heart enzyme (Fig. 1), AMP-D from porcine heart seems to be regulated by the energy charge, whereas rabbit heart AMP-D always seems to be in

Table 2. Purification of pig heart and skeletal muscle AMP-deaminase

Purification step	Specific activity (U/mg protein)		Purification (×)		Yield (%)	
	Heart	SM	Heart	SM	Heart	SM
Supernatant fluid	0.10	0.30	(1)	(1)	(100)	(100)
Cellulose phosphate	20	75	208	252	20	62
DEAE–Sephacel	30	156	426	526	11	42

SM, skeletal muscle.

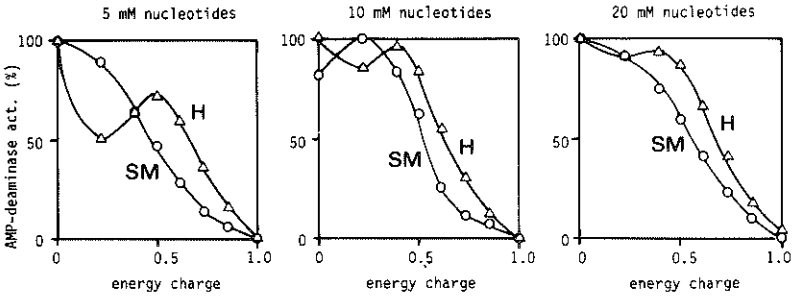

Fig. 1. Response of AMP-D to variation in the adenylate energy charge at different total adenine nucleotide pool sizes. Purified enzyme of pig skeletal muscle (SM, 3 μg) and heart (H, 10 μg) were tested in triplicate. Maximal activities (μmol/min) observed at 5 mM nucleotides: 0.39 (SM) and 0.12 (H); at 10 mM nucleotides: 0.53 (SM) and 0.30 (H); at 20 mM nucleotides: 0.49 (SM) and 0.38 (H).

the activated state (Solano & Coffee, 1978; Barsacchi *et al.*, 1979).

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Further Purification of Adenosine Kinase from Rat Heart Using Affinity and Ion-Exchange Chromatography

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Adenosine kinase (EC 2.7.1.20) in a cytoplasmic fraction of rat heart was subjected to 5′-AMP–Sepharose 4B chromatography. The enzyme showed affinity for the column in contrast to adenosine deaminase, and was eluted with adenosine *plus* MgATP. Fractions containing adenosine kinase were put on a column of DEAE–Sephacel and eluted with a gradient. The enzyme was purified up to 3000-fold (yield 10%). The specific activity exceeded 8000 units per gram of protein and sodium dodecyl sulfate–polyacrylamide gel electrophoresis showed only one band. We conclude that the method presented is a simple, quick, and elegant way of purifying myocardial adenosine kinase to virtual homogeneity.

Relatively little work has been published on myocardial adenosine kinase (ATP: adenosine 5′-phosphotransferase, EC 2.7.1.20). Nevertheless this enzyme may play an important role in the maintenance of adequate adenine nucleotide levels in the heart (1–3). Purification of myocardial adenosine kinase is useful for kinetic measurements (4–6), assays of adenosine (7), and the study of the effect of drugs, for instance, dipyridamole[1] (6,8,9). In an earlier publication we showed that rat heart adenosine kinase could be purified up to 80-fold with Sephadex G-100 and DEAE–Sephadex A-50 chromatography (4). Subsequently we reported briefly (8) on the use of 5′-AMP–Sepharose 4B affinity chromatography to separate adenosine kinase and adenosine deaminase (EC 3.5.4.4). In this paper we describe a combination of affinity and ion-exchange chromatography for the purification of adenosine kinase. A purification factor of 3000-fold can be reached with a yield of about 10%. A preliminary report has been published as an abstract.[2]

MATERIALS AND METHODS

Adenosine kinase assay. See Ref. (1). Incubations were carried out at 30°C in a volume of 100 μl in closed micro-test tubes (Eppendorf, Hamburg). Incubation conditions were: 40 mM potassium phosphate, pH 7.0, 1.0 mM potassium guanosine 5′-triphosphate, 1.0 mM $MgCl_2$, 10 μM [U-^{14}C]adenosine (Radiochemical Centre, Amersham; specific activity about 50 Ci/mol), and bovine serum albumin (0.1 mg/ml). Reactions were initiated by the addition of adenosine kinase (up to 10 μg protein). After 10–20 min 25-μl aliquots of the reaction mixture were distributed on squares of DEAE-cellulose (1.5 × 1.5 cm Whatman DE81) which binds nucleotides. The squares were washed with 1 mM ammonium formiate, water, and ethanol. A detailed description of the washing and

[1] Abbreviations used: dipyridamole, 2,6-bis(diethanolamino-4,8-dipiperidinopyrimido-[5,4-(+)]pyrimidine; EHNA, *erythro*-9-(2-hydroxy-3-nonyl)adenine; Tricine, *N*-tris(hydroxymethyl)methylglycine.

[2] Uitendaal, M. P., De Jong, J. W., Harmsen, E., and Keijzer, E. (1979) *J. Clin. Chem. Clin. Biochem.* 17, 446.

counting procedure can be found elsewhere (1).

Adenosine assay. Proteins were denatured by heating the assay mixture to 100°C for 3 min. After cooling on ice the samples were filtered (Millipore filters, 1.2 μm). Analysis of adenosine (and inosine) was performed by automatic high-pressure liquid chromatography (LC 8500, Varian, Palo Alto, Calif.) on a μBondapak C_{18} column (Waters, Milford, Mass.; 0.39 × 30 cm). The column was eluted with 10 mM ammonium phosphate at pH 5.5/methanol (87/13) at a rate of 1.0 ml/min and a pressure of 60 bar. Peaks were detected at 260 nm (VariChrom, Varian) and peak areas calculated automatically with a Chromatography Data System 111C (Varian). The concentration of standard solutions was determined according to Olsson (10).

Adenosine deaminase activity, chloride concentrations, and protein content. Adenosine deaminase activity was measured (4) at room temperature (22°C). *Erythro*-9-(2-Hydroxy-3-nonyl)adenine (EHNA; 2 μM) inhibited the enzyme completely. Chloride concentrations were determined titrimetrically (11) with NaCl as standard. Protein content was measured according to Bradford (12) using the Bio-Rad (Richmond, Calif.) protein assay with bovine serum albumin as standard. No interference was noted between the Coomassie blue reagent and P_i, Tris, or GSH. We found that the presence of these compounds in our samples made it impossible to use the Folin–Ciocalteu reagent (13).

Gel electrophoresis. Gel electrophoresis was carried out as described by Weber and Osborn (14) with P_i buffer containing sodium dodecyl sulfate and bromphenol blue as the tracking dye. The separation took 2 h (constant current 16 mA). Gels were stained for 1.5 h with Coomassie brillant blue in methanol/acetic acid (91/9) and destained overnight with methanol/acetic acid/water (50/75/875).

Concentration of samples. Concentration of samples after ion-exchange chromatography was necessary for the measurement of protein content and for gel electrophoresis. We used the single hollow fiber method for fast concentration described by Rommerts *et al.* (15).

Materials. Coenzymes were purchased from Boehringer–Mannheim, agarose derivatives from P-L Biochemicals (Milwaukee, Wis.), and methanol (Uvasol) from Merck (Darmstadt). Adenosine, hypoxanthine, and uridine were also supplied by Merck, uric acid by Sigma (St. Louis, Mo.), and the other standards by Koch–Light (Colnbrook, Buckinghamshire, U. K.).

Purification. Male rats of the Wistar strain (200–250 g) were killed by cervical fracture and subsequent bleeding. Hearts were removed and kept at −25°C until used. A 5% myocardial homogenate was prepared with a Virtis homogenizer in 0.25 M sucrose containing 10 mM Tricine–KOH and 1.0 mM Na_2EDTA (pH 7.4). Homogenization and further purification steps were performed at 0–8°C. The homogenate was centrifuged at 7500*g* for 10 min. The supernatant fraction was centrifuged at 100,000*g* for 1 h. The cytoplasmic fraction, 10–80 ml (which could be stored at −25°C, if necessary), was applied at a rate of 1 ml/min to a column of 2 g N^6(6-aminohexyl-)-5′-AMP–Sepharose 4B (Pharmacia, Uppsala; 1.6 × 5 cm) and washed with 20 mM potassium phosphate containing 1 mM GSH at pH 7.0. The absorbance of the eluate was monitored with a Uvicord III (LKB, Stockholm). When this absorbance was minimal, the buffer was changed to 20 mM Tris–HCl/1 mM GSH, pH 7.0. Adenosine kinase was eluted from the column with 10 ml 1 mM ATP/1 mM $MgCl_2$/0.1 mM adenosine in Tris/GSH buffer. The combined fractions containing adenosine kinase were applied to a column of DEAE–Sephacel (Pharmacia; 1.6 × 15 cm) equilibrated with 20 mM Tris–HCl/1 mM GSH, pH 7.0 (1

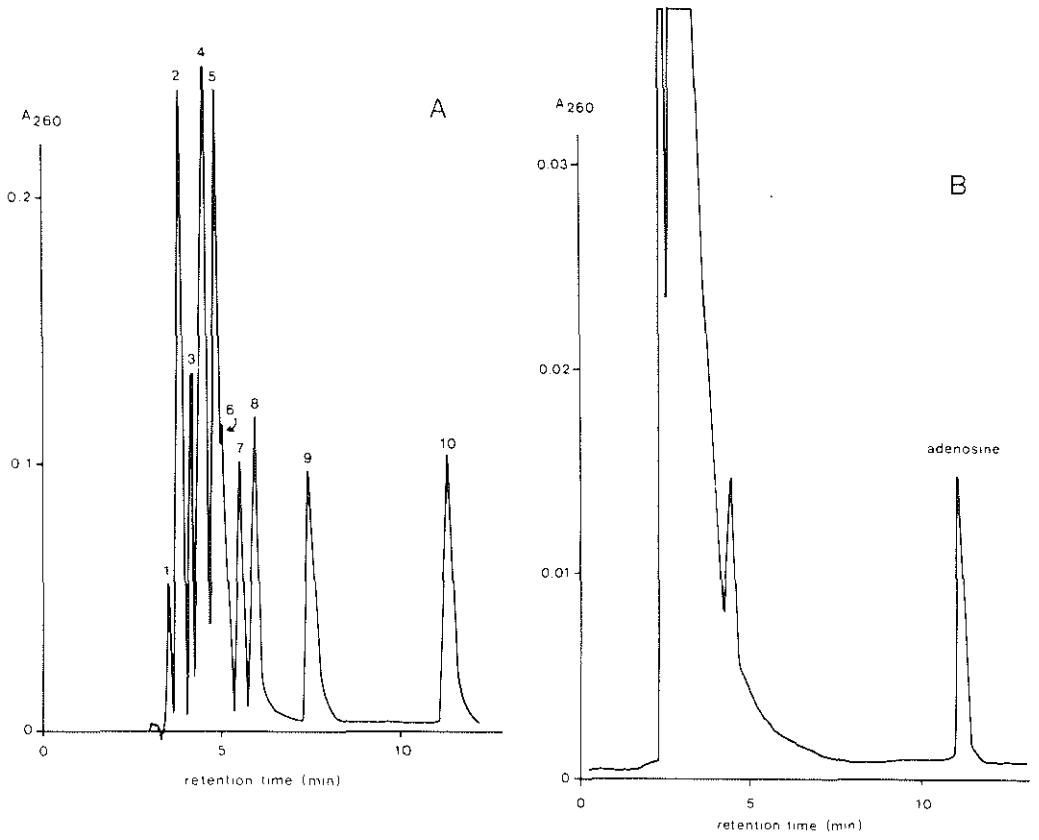

FIG. 1. High-pressure liquid chromatography of nucleosides and derivatives. (A) Chromatogram obtained with the following standards (2–5 nmol): **1**, uric acid; **2**, uracil; **3**, uridine; **4**, hypoxanthine; **5**, xanthine; **6**, xanthosine; **7**, inosine; **8**, guanosine; **9**, adenine; **10**, adenosine. (B) Determination of adenosine concentration in sample from 5'-AMP–Sepharose 4B column eluted with MgATP/adenosine. A_{260} = Absorbance at 260 nm.

ml/min). After elution of adenosine, a linear gradient of 75 ml 20 mM Tris–HCl/1 mM GSH, pH 7.0, and 75 ml 100 mM Tris–HCl/1 mM GSH, pH 7.0, was started.

RESULTS AND DISCUSSION

Assay of Adenosine Kinase

The assay of adenosine kinase is based on a sensitive radiometric determination in which the formation of [^{14}C]AMP is measured (1.4). We changed the incubation buffer from Tris–maleate, pH 5.6, to P_i, pH 7.0, because the pH of the chromatographic eluate was 7.0. This change made certain that adenosine kinase was assayed at a well-defined pH, even when a relatively large amount (50 μl) of eluate was assayed. Second, GTP replaced ATP as phosphate donor which allowed us to work without an ATP-regenerating system and oligomycin. GTP, which is superior to ATP as a phosphate donor (4), gave an optimal activity at a 1 to 5 mM concentration. Third, we omitted dipyridamole (used as an inhibitor of adenosine deaminase), because it has an effect on adenosine kinase per se (4). EHNA, 2 μM, which completely inhibited

rat heart adenosine deaminase (cf. Ref. (16)), influenced adenosine kinase activity, and was not used in the assay. However, only if the adenosine deaminase activity in the sample exceeds that of adenosine kinase many times does care have to be taken for depletion of adenosine (unpublished observations). This is presumably due to the fact that we used 10 μM adenosine which saturates adenosine kinase (K_m 0.5 μM; Ref. (4)), but gives suboptimal activity of adenosine deaminase (K_m 72 μM, Ref. (17)). We found the activity of adenosine kinase measured with the modified system described above comparable to the value obtained with the more complex system (1).

Assay of Adenosine

If an adenosine kinase sample contains unknown amounts of adenosine, the method described above is inaccurate because the radioactive substrate is diluted. Therefore we developed a high-pressure liquid chromatographic procedure to assay adenosine. Figure 1A shows the separation of 10 nucleosides and derivatives. The retention times of inosine and adenosine were 5.5 and 11–11.5 min, respectively. We used the system to measure the concentration of adenosine in the eluate of the AMP–Sepharose column (see Fig. 1B). Nucleotides and GSH showed retention times of less than 5 min. When a 2-μl sample was used in the assay mixture for adenosine kinase, the specific activity of [^{14}C]adenosine decreased up to 20%. We found the high-pressure liquid chromatographic system also useful to check our adenosine kinase and adenosine deaminase measurements. For this purpose incubations and subsequent denaturations were performed as described under Materials and Methods.

Preparation of Cytoplasmic Fraction

Myocardial adenosine kinase is a cytoplasmic enzyme (1). We therefore started the purification with the preparation of a high-speed supernatant fraction. The yield of adenosine kinase activity usually exceeded 100%, which could indicate that an inhibitor is removed by this step. The specific activity increased six-fold. The cytoplasmic fraction also contains the adenosine deaminase activity (17).

Affinity Chromatography

Adenosine kinase and adenosine deaminase can be separated by affinity chromatography. Adenosine deaminase does not bind to the column and virtually all of the activity is eluted with P_i/GSH buffer. Most of the adenosine kinase, on the other hand, shows affinity for AMP–Sepharose, at least when the sample applied to the column is prepared in sucrose/Tricine/EDTA (see Materials and Methods). If P_i/GSH buffer is used for the preparation of the homogenate, binding of adenosine kinase is poor. Adenosine kinase activity is eluted from

TABLE I

PURIFICATION OF RAT HEART ADENOSINE KINASE[a]

Purification step	Volume (ml)	Protein (mg)	Activity (mU)	Specific activity (U/g)
Cell-free extract	79	230	631	2.74
5'-AMP–Sepharose 4B	30	3.51	131	37.3
DEAE–Sephacel (fractions 39 to 41)	15.2	0.0072	60	8280

[a] The activity of the enzyme is given in nmol/min (mU); the specific activity is expressed per gram of protein. "Cell-free extract" refers to the 100,000g supernatant fraction.

Sepharose–AMP, when MgATP and adenosine are present in the eluting buffer, either P_i/GSH or Tris/GSH. ATP can be replaced by GTP, but omission of magnesium, ATP, or adenosine results in minimal recovery.

Larger amounts of cytoplasmic fraction lead to overloading of the column and consequently a lower yield in the MgATP/adenosine eluate. However, in the last step of the purification, ion-exchange chromatography, larger amounts result in a higher yield and specific activity, possibly because less denaturation takes place.

Results, similar to those given in Table 1, were obtained when N^6-(6-aminohexyl)-5'-AMP–agarose was used, although the number of AMP molecules per gram matrix was twice as high as in the case of 5'-AMP–Sepharose 4B. C^8-(Hexyl)-adenosine–agarose and C^8-(hexyl)-5'-AMP–Sepharose could not replace the N^6-(6-aminohexyl)-derivatives of 5'-AMP–Sepharose or 5'-AMP–agarose for the purification of adenosine kinase.

The preparation is relatively stable at this stage, and can be stored at −25°C.

Ion-Exchange Chromatography

The enzyme could be further purified by ion-exchange chromatography. Figure 2 shows the elution pattern of adenosine kinase on DEAE–Sephacel. Gel electrophoresis of the pooled fractions 39 to 41 shows one band. The yield of the purified enzyme is 10% and the specific activity exceeds 8000 units per gram of protein (Table 1). The preparation does not contain detectable activity of adenosine deaminase, adenylate kinase (EC 2.7.4.3), nucleoside phosphorylase (EC 2.4.2.1), or AMP deaminase (EC 3.5.4.6). The enzyme loses its activity rapidly at −25°C. A similar finding has been reported for (partially) purified yeast and liver adenosine kinase (18,19).

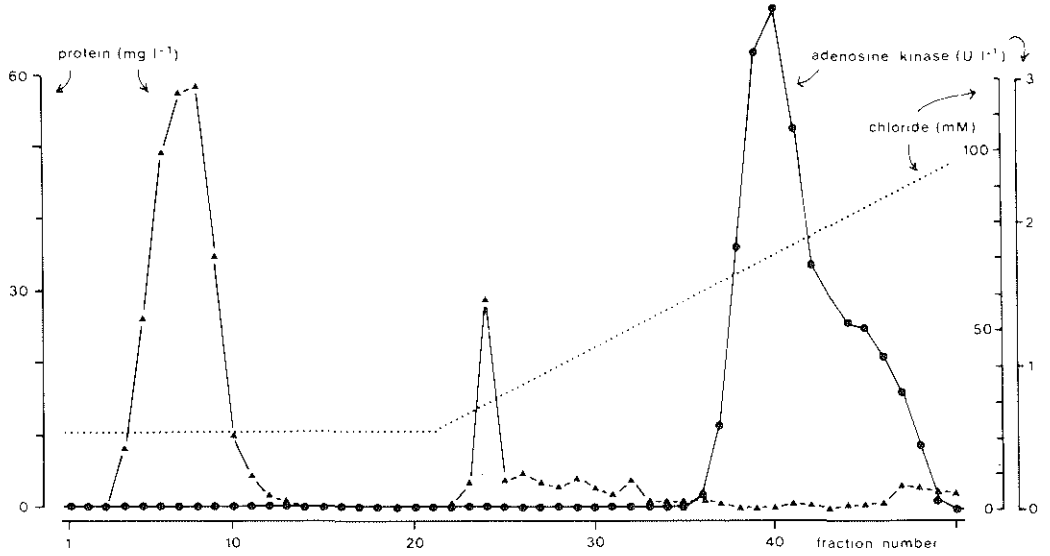

FIG. 2. Ion-exchange chromatography of adenosine kinase on a DEAE–Sephacel column. Fractions containing the partly purified enzyme after affinity chromatography (see Materials and Methods) were pooled and put on the column. The column was eluted with 20 mM Tris–HCl/1 mM GSH, pH 7.0, until the absorbance at 260 nm was minimal. Fractions of 5 ml were collected. The first peak contained protein and adenosine, but no adenosine kinase. With a Tris–HCl gradient the enzyme could be recovered from the column.

Concluding Remarks

The purification described here is simple and the purification factor (3000-fold) much higher than obtained by other methods (4–7). Only a few rat hearts are needed and within a few hours the enzyme is obtained free of adenosine deaminase. The preparation and assay of adenosine kinase described in this paper should prove useful.

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APPENDIX PAPER 5

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NIFEDIPINE REDUCES ADENINE NUCLEOTIDE BREAKDOWN IN ISCHEMIC RAT HEART

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An ATP-sparing effect has been demonstrated for a number of calcium antagonists. Nifedipine probably has a similar action, but data supporting this view are limited. Therefore we decided to study the effect of nifedipine on high-energy phosphate (and carbohydrate) metabolism in the ischemic rat heart. Langendorff preparations were made ischemic for less than 15 min. The reduction in coronary flow was 60 or 70%. Apex displacement during ischemia, a measure of contractility, was comparable for nifedipine-treated and untreated hearts. Ischemia caused a considerable release of the AMP catabolites adenosine, inosine and (hypo)xanthine, and of lactate. Nifedipine (10–100 μg/l) prevented this in a dose-dependent way. The highest dose reduced the release of purines and lactate by 90% ($P<0.01$) and 60% ($P<0.001$), respectively. The drug acted in a similar way during reperfusion. Due to ischemia, the adenylate energy charge (ATP+0.5 ADP)/(ATP+ADP+AMP), decreased 15% ($P<0.001$); nifedipine at a concentration of 100 μg/l prevented this decrease ($P<0.05$). We conclude that nifedipine exerts a benificial effect on myocardial adenine nucleotide metabolism during ischemia and reperfusion.

Calcium antagonist Purine nucleoside Oxypurine Lactate Energy charge Reperfusion

1. Introduction

Nifedipine [1,4-dihydro-2,6-dimethyl-4-(o-nitrophenyl)-pyridine-3,5-dicarboxylic acid dimethyl ester, Adalat®] is a blocker of slow calcium channels (Fleckenstein et al., 1972; Nayler and Poole-Wilson, 1981). The drug has negative inotropic, dromotropic and chronotropic properties and is a powerful vasodilator (cf. review by Henry, 1980). It is presently used in patients for the treatment of angina pectoris and hypertension (see, for instance, Antman et al., 1980; Serruys et al., 1981, Hugenholtz et al., 1981).

Calcium antagonists are thought to block transmembrane Ca^{2+} transport through slow channels or deplete Ca^{2+} stores in the sarcolemma (Fleckenstein, 1980). They prevent the mitochondrial accumulation of calcium ions during ischemia and reperfusion (Nayler et al., 1980). Nifedipine, like other calcium antagonists, is thought to have an ATP-sparing effect (Fleckenstein, 1980; Hugenholtz, 1980; Magovern et al., 1981) but data supporting this view are limited. Nayler et al. (1980) showed a beneficial effect of nifedipine on high-energy phosphate metabolism in rabbit heart which had been totally ischemic for 90 min. Furthermore, Higgins et al. (1980) demonstrated this drug to increase the ATP content of anoxic cultered neonatal rat heart cells. On the other hand, Ichihara and coworkers (1979) were unable to measure an effect of nifedipine on the ATP concentration in ischemic dog heart.

We decided to study the effect of nifedipine on nucleotide metabolism in the isolated perfused rat heart. The preparation was made ischemic for less than 15 min. We used release of adenosine and its catabolites as a sensitive indicator for nucleotide breakdown. This had proved useful in the isolated rat heart (Stam and De Jong, 1977), the open-chested pig heart (De Jong et al., 1977) and

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patients undergoing an atrial pacing stress test (Fox et al., 1974; Remme et al., 1977). The present report shows that nifedipine decreased the release of purine nucleosides, oxypurines and lactate during ischemia and reperfusion.

2. Materials and methods

2.1. Preparation of the ischemic heart

Male Wistar rats [291 ± 51 (S.D.) g], fed ad libitum, were anesthetized with an intraperitoneal (i.p.) injection of 0.5 ml sodium pentobarbital (60 g/l, Abbott, Saint-Remy sur Avre, France). Hearts were rapidly removed and cooled in ice-cold 0.9% NaCl. Retrograde perfusion of the aorta according to Langendorff was immediately started with a modified Tyrode solution (Meyler et al., 1958; De Jong, 1972), containing 10 mM glucose and equilibrated with 95% O_2 + 5% CO_2. The perfusion pressure was 72 mmHg (Statham pressure transducer, model P23D6, Gould Inc., Oxnard, CA). The temperature was measured in the canula tip with a telethermometer (temperature module: Gould Inc., Cleveland, OH; with model 511 probe: Yellow Springs Instr. Inc., Yellow Springs, OH) and maintained at 37.0 ± 0.5°C. Ventricles were paced at 4 V with 2 ms duration. In Experiment 1, hearts were paced at 300 beats/min and ischemia was induced by lowering the perfusion pressure to 17 mmHg. This resulted in an ischemic flow of about 4 ml/min. In Experiment 2, the heart rate was 360 beats/min and during ischemia, flow was restricted with a model 2132 peristaltic pump (LKB, Stockholm, Sweden) to about 2.5 ml/min.

2.2. Chemicals

All chemicals were analytical grade. Water was purified with the Milli-Ro4/Milli-Q system (Millipore Co., Bedford, MA). Bayer AG (Leverkusen, FRG) supplied ampoules with a 0.1 g/l solution of nifedipine or solvent alone ('placebo', ethanol/polyethylene glycol/water, 15:15:70). Care was taken to keep the nifedipine-containing fluid in the dark (Clark et al., 1981).

2.3. Apex displacement

Apex displacement was used as a measure of contractility (Stam and De Jong, 1977). It was measured with a displacement transducer (model 7-DCDT-1010, Hewlett-Packard, Palo Alto, CA), fitted with a steel tampon hooked to the apex of the ventricle, and monitored with a Brush 2800 recorder (Gould, Cleveland, OH). The displacement, observed 5 min after the start of the perfusion, was taken as 100%.

2.4. Protocol

Hearts were allowed to equilibrate with the perfusion fluid for 5 min plus an additional 10 min with medium containing nifedipine or solvent (preperfusion). Collection of perfusate was then started. Two series of experiments were carried out. In Expt. 1, hearts (300 beats/min) were perfused for a control period of 10 min, followed by 14 min ischemic period. Reperfusion for another 14 min only took place in this experiment. In Expt. 2, with hearts at 360 beats/min, control perfusion was also 10 min with severe ischemia for an additional 10 min. The hearts were freeze-clamped at the end of the control or the ischemic period in this second series of experiments for analysis of adenine nucleotides.

2.5. Coronary flow

Coronary flow was measured by timed collection of perfusate. Two-min fractions were collected in Expt. 1. Only two 10 min fractions were collected in Expt. 2: control and ischemic.

2.6. Assay of purines

Purines were assayed in a slightly modified version of the high-performance liquid chromatography (HPLC) described by Harmsen et al. (1981). The μBondapak C_{18} column was eluted at a rate of 3 ml/min with 0.07 M K-PO_4 buffer, pH 4.5, to which methanol (30 ml/l) had been added. Samples of perfusate were collected on ice. NaN_3 (0.02% final concentration) was added to prevent

bacterial purine breakdown. Analysis took place within 24 h.

2.7. *Analysis of myocardial adenine nucleotides*

Analysis of myocardial adenine nucleotides was also done by HPLC (see Harmsen et al., 1982). The elution profile of ATP, ADP and AMP from the ion exchange column was used to calculate adenylate energy charge (ATP + 0.5 ADP)/(ATP + ADP + AMP). Freeze-clamped hearts were stored in liquid N_2 until analysis.

2.8 *Assay of lactate*

Lactate was assayed enzymatically in the perfusates according to Apstein et al. (1970) with the AutoAnalyzer II (Technicon, Tarrytown, NY).

2.9. *Statistical analysis*

Statistical analysis was performed with Student's t-test: $P > 0.05$ (two-sided) was considered not significant (NS). Results are given as mean values ±S.E.M.

3. Results

3.1. *Coronary flow*

Fig. 1 shows the coronary flow in the first experiment. Nifedipine at 10 μg/l concentration increased the control flow from 40 to 100 ml/min per g dwt ($P < 0.001$). When the perfusion pressure was lowered from 72 to 17 mmHg, coronary

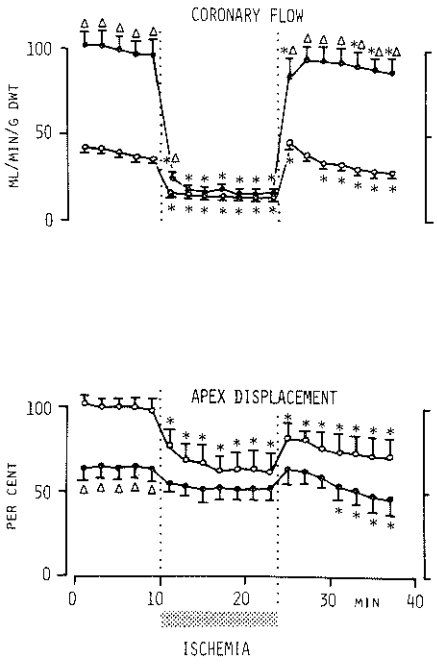

Fig. 1. Coronary flow (ml/min per gram dry weight) and apex displacement (%) during control, ischemia and reperfusion in Expt. 1. The displacement measured before the addition of 10 μg/l nifedipine (●) or solvent (○) was taken as 100%. The heart rate was 300 beats/min. Fourteen minutes of ischemia were induced by lowering the perfusion pressure from 72 to 17 mmHg. Means ± S.E.M. (n = 5), △ $P < 0.05$ vs. solvent (unpaired t-test), * $P < 0.02$ vs. control (paired t-test with values at t = 9 min).

TABLE I

Dose-dependent effect of nifedipine on coronary flow and apex displacement in Expt. 2. Addition of solvent alone to the perfusion fluid had no significant influence on flow and contractility. Apex displacement before the addition of drug was taken as 100%. Heart rate was 360 beats/min. After a control period of 10 min, flow was reduced with a pump for the same period of time.

Nifedipine (μg/l)	Coronary flow (ml/10 min)		Apex displacement (%)	
	Control	Ischemia	Control	Ischemia
0	103 ± 10 (15)	26 ± 2.2 (9) [b]	86 ± 4 (14)	27 ± 5 (8) [b]
3	125 ± 5 (9)	23 ± 0.3 (6) [b]	101 ± 6 (9) [a]	19 ± 6 (6) [b]
10	174 ± 8 (11) [a]	25 ± 2.4 (8) [b]	71 ± 3 (10) [a]	28 ± 6 (7) [b]
30	182 ± 11 (9) [a]	25 ± 1.0 (6) [b]	41 ± 4 (9) [a]	26 ± 9 (6)
100	146 ± 10 (9) [a]	24 ± 0.3 (5) [b]	7 ± 2 (9) [a]	23 ± 6 (5) [b]

Means ± S.E.M. (n); [a] $P < 0.05$ vs. 0 μg/l (unpaired t-test); [b] $P < 0.05$ vs. control (paired t-test).

flow in the untreated hearts decreased by 60% ($P < 0.001$). During ischemia, flow in the presence and absence of nifedipine was comparable, about 15 ml/min per g dwt. During reperfusion, flow again increased to control levels (fig. 1).

Experiment 2 shows that even 3 μg/l of drug acted as a vasodilator during the control period (table 1). In this second experiment ischemia was induced with a pump. Coronary flow in untreated hearts diminished by 75% ($P < 0.001$). Also in Expt. 2 ischemic flow was comparable for treated and untreated hearts.

3.2. Apex displacement

In Expt. 1 nifedipine at a concentration of 10 μg/l decreased apex displacement by 35% ($P < 0.01$, fig. 1). Experiment 2 shows that the negative inotropic effect was dose-dependent (table 1). At 100 μg/l, nifedipine diminished contractility during the control perfusion to 10% of the value observed with solvent ($P < 0.001$). During ischemia, apex displacement was about 25% of preperfusion values ($P < 0.001$), both in treated and untreated hearts. This means that ischemia induced a three-fold increase ($P < 0.02$) in apex displacement, when a high dose of nifedipine (100 μg/l) was present (table 1).

3.3. Biochemical changes

Experiment 1 shows the release of adenosine, inosine and (hypo)xanthine during control perfusion, ischemia and reperfusion (fig. 2). Purine release during the control period was 10 ± 5 nmol/min per g dwt. At the end of the ischemic

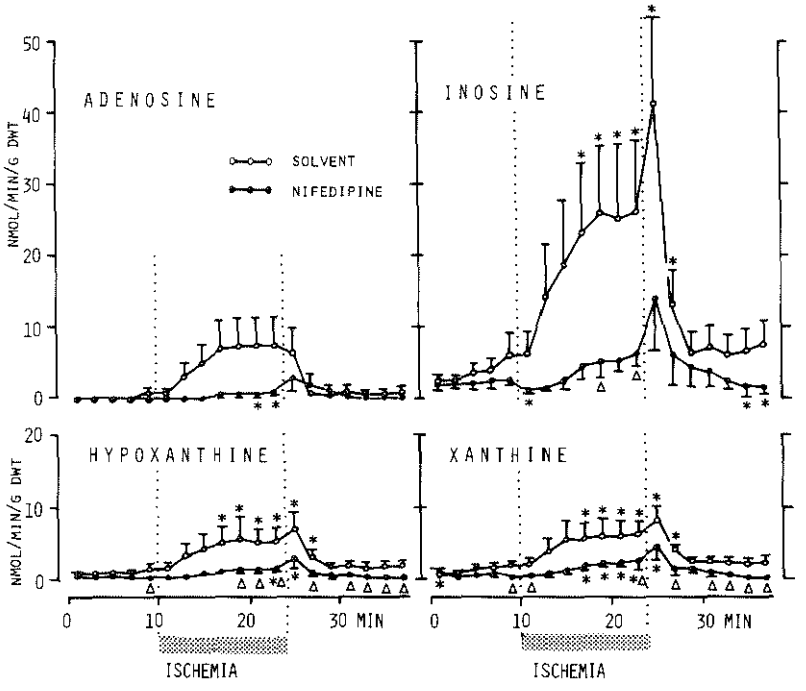

Fig. 2. Nifedipine (10 μg/l) reduced the myocardial release of purine nucleosides (upper panel) and oxypurines (lower panel). Conditions as in Expt. 1, see fig. 1. Means ± S.E.M. ($n = 5$). △ $P < 0.05$ vs. solvent (unpaired t-test). * $P \leq 0.05$ vs. control (paired t-test with values at $t = 9$ min).

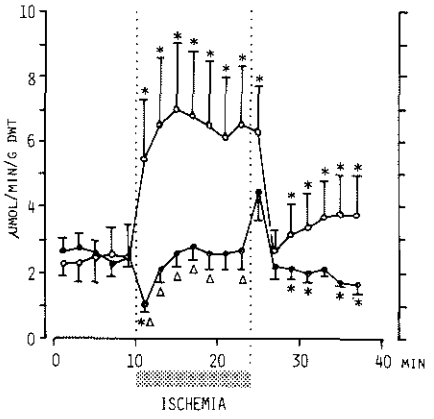

Fig. 3. Nifedipine (●) reduced the myocardial release of lactate. Conditions as in Expt. 1, see fig. 1. Means ± S.E.M. (n = 5), △ $P < 0.05$ vs. solvent (○), (unpaired t-test), * $P < 0.05$ vs. control (paired t-test with values at t = 9 min).

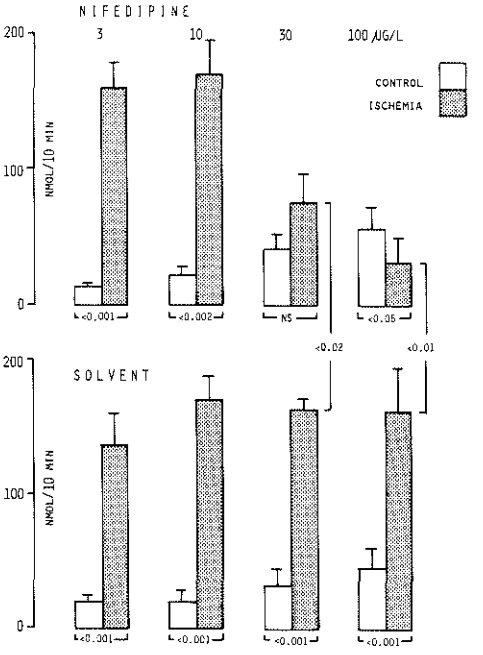

Fig. 4. Nifedipine reduced purine release dose-dependently during ischemia. The sum of the purines adenosine, inosine, hypoxanthine and xanthine is shown. Conditions as in Expt. 2, see table 1. Means ± S.E.M. (n = 4–9). P values vs. control (paired t-test) and vs. solvent (unpaired t-test) are indicated.

period, purine release was 46 ± 17 nmol/min per g dwt ($P < 0.05$ vs. control). Nifedipine (10 µg/l) reduced this release by 75% ($P < 0.05$).

These relatively mild ischemic conditions resulted in a lactate release which was 2.6 times higher than the control value ($P < 0.02$). This was completely abolished by 10 µg/l nifedipine ($P < 0.05$, fig. 3). During reperfusion, purine and lactate production went down again but the hearts released more of these compounds in the untreated group (figs. 2 and 3).

Experiment 2 shows that nifedipine reduced purine release during ischemia in a dose-dependent way (fig. 4). Ischemia was more severe than in Expt. 1, because flow was reduced to a larger extent (and heart rate was higher, see Baller et al., 1981). The highest dose of nifedipine, 100 µg/l, prevented adenosine release during 10 min of ischemia by more than 90% ($P < 0.02$, fig. 5). This dose decreased the release of inosine, hypoxanthine and xanthine during ischemia by 85% ($P < 0.002$), 75% ($P < 0.05$), and 64% ($P < 0.05$), respectively (data not shown). Figs. 4 and 5 also show that the solvent itself had an effect on purine production during the control period. Increasing the dose of nifedipine (and solvent) from 3 to 100 µg/l doubled the release of purines (NS). The release of adenosine was even more dramatic: solvent increased it dose-dependently from levels < 1 nmol/10 min to 7–11 nmol/10 min ($0.05 < P < 0.10$).

In Expt. 2, differences in lactate release during the control period were only observed with 3 µg/l nifedipine: this induced a 42% decrease ($P < 0.05$, fig. 6). The nifedipine dose had to be increased to levels higher than in Expt. 1 to show an effect during ischemia: ten times more drug was needed to prevent lactate release in Expt. 2 ($P < 0.001$, please compare figs. 3 and 6).

We were unable to measure a significant decrease in myocardial ATP content under the ischemic conditions of Expt. 2. However, adenylate energy charge decreased by about 15% due to ischemia ($P < 0.02$, table 2). This decrease was prevented by 100 µg/l nifedipine ($P < 0.05$ vs. solvent).

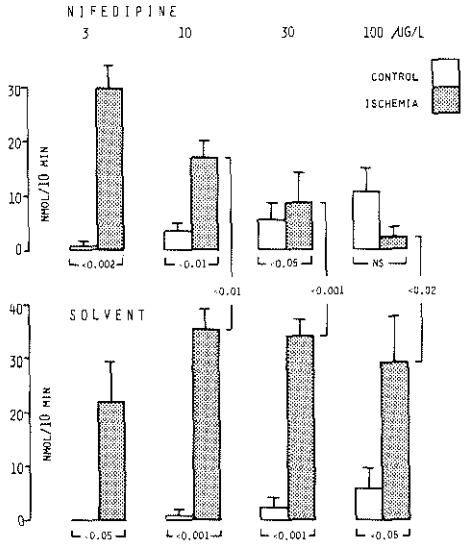

Fig. 5. Nifedipine reduced adenosine release from the ischemic heart dose-dependently. Conditions as in Expt. 2, see table 1. Means ± S.E.M. (n = 4–11), P values vs. control (paired t-test) and vs. solvent (unpaired t-test) are shown.

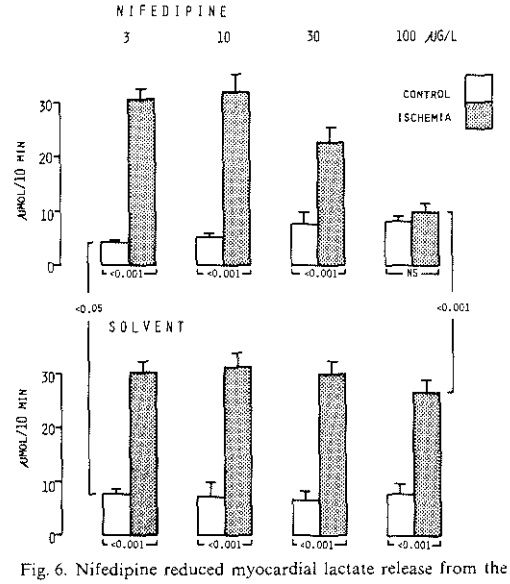

Fig. 6. Nifedipine reduced myocardial lactate release from the ischemic heart dose-dependently. Conditions as in Expt. 2, see table 1. Means ± S.E.M. (n = 4–11). P values vs. control (paired t-test) and vs. solvent (unpaired t-test) are indicated.

TABLE 2

Ischemia-induced decrease of adenylate energy charge, and its prevention by nifedipine. 'Solvent' refers to a vehicle volume corresponding to a nifedipine concentration of 100 μg/l. Perfusion conditions as in Expt. 2, see table 1.

Addition	Adenylate energy charge	
	Control	Ischemia
None	0.890 ± 0.007 (5) [a]	0.793 ± 0.012 (7) [b]
Solvent	0.896 ± 0.004 (4) [c]	0.761 ± 0.029 (4) [d]
Nifedipine 10 μg/l	0.889 ± 0.004 (3) [e]	0.773 ± 0.023 (5) [f]
100 μg/l	0.897 ± 0.007 (4)	0.864 ± 0.024 (4) [g]

Means ± S.E.M. (n); P-values, calculated with an unpaired t-test: [a–b] $P<0.001$; [c–d] $P<0.02$; [e–f] $P<0.01$; [b–g] $P<0.02$; [d–g] $P<0.05$.

4. Discussion

A number of calcium antagonists reduce high-energy phosphate breakdown during hypoxia. Although these slow-channel blockers vary in their mode of action (Opie, 1980; Henry, 1980; Fleckenstein, 1980), nifedipine probably acts in a similar way. However, there are only limited data supporting this view (Nayler et al., 1980; Higgins et al., 1980; but see Ichihara et al., 1979). We decided to study the effect of nifedipine on the myocardial release of the AMP catabolites adenosine, inosine and (hypo)xanthine. For that purpose we made isolated rat hearts ischemic for a period of a length comparable to the duration of an atrial pacing stress test in patients. We showed that breakdown of adenine nucleotides was indeed prevented by nifedipine. The evidence is based on measurements of purine nucleosides and oxypurines (figs. 2, 4 and 5), and adenylate energy charge (table 2).

In addition we found that the release of lactate, a marker of myocardial ischemia (see, for instance, Brachfeld, 1976; Stam and De Jong, 1977) was reduced by nifedipine (figs. 3 and 6). Ichihara et al. (1980) were unable to detect an effect of nifedipine on myocardial lactate release during ischemia in the dog. The reductions found in our experiments were not due to the negative inotropic action of nifedipine, because the drug did not affect contractility during ischemia (fig. 1 and table 1). Similarly, Perez et al. (1980) found no

nifedipine-induced depression of the mechanical activity of ischemic canine heart but, instead, they observed a significant enhancement of its performance. With the highest dose of nifedipine we found an increase in apex displacement (table 1), concomitant with a decrease in purine release (fig. 5).

Atkinson (1977) has reviewed the activities of important regulatory enzymes in vitro as a function of energy charge. With an increase in energy charge from 0 to 1, the regulatory enzymes in ATP-using sequences increase in activity and those which regenerate ATP decrease in activity. Nifedipine increased the energy charge to the levels necessary for the proper functioning of the heart (table 2).

It is of interest that rat heart produces relatively large amounts of xanthine during ischemia (fig. 2; see De Jong, 1982). Xanthine oxidase, which converts hypoxanthine to xanthine (and xanthine to urate) is reported to be absent from rat heart (Gandhi and Ahuja, 1979), or present in low amounts (Maguire et al., 1972). Because xanthine oxidase in heart and blood vessels has been implicated as a causative factor in atherosclerosis (Carr et al., 1975), further studies are clearly needed.

Nayler et al. (1980) showed that hearts from rabbits treated with nifedipine were protected against the ischemia-induced decline in the ATP-generating and O_2-utilizing capacity of the mitochondria. According to Nayler and Poole-Wilson (1981) no simple explanation for the protection afforded by calcium antagonists to hypoxic and ischemic heart muscle can be given at present. From our experiments we conclude that nifedipine prevents myocardial adenine nucleotide breakdown in ischemic rat heart, but the precise mechanism remains to be solved.

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APPENDIX PAPER 6

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SYNERGISTIC EFFECT OF NIFEDIPINE AND PROPRANOLOL ON ADENOSINE (CATABOLITE) RELEASE FROM ISCHEMIC RAT HEART

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Both nifedipine a calcium antagonist, and propranolol a β-adrenergic blocker, are used as protective agents of the ischemic myocardium. In the clinical setting, the combination of the two drugs is used succesfully although several case reports indicate potential dangers of the combination. For this reason we decided to study the combined effect of nifedipine and DL-propranolol in the isolated rat heart made ischemic for a short period of time. Apex displacement was taken as a measure of contractility. Release of the AMP catabolites adenosine, inosine, (hypo)xanthine and uric acid was used as a marker of ATP breakdown. Contractility during ischemia was not affected by the drugs. DL-Propranolol (30 or 150 μg/l) had no effect on ischemic myocardial purine release, while nifedipine (15 μg/l) reduced purine release during ischemia by 33% ($P < 0.02$). The combination of 15 μg/l nifedipine and 150 μg/l DL-propranolol decreased purine release by 53% ($P < 0.005$ vs. nifedipine). We conclude from these results that propranolol has a synergistic effect, adding to the beneficial action of nifedipine on ischemic myocardium.

Calcium antagonist β-Blocker High-energy phosphates Oxypurine Ischemia Heart
Purine nucleoside

1. Introduction

Pretreatment with nifedipine, a calcium antagonist, prevents excessive calcium accumulation in the myocardial cell during ischemia and reperfusion. This probably ensures good ATP and creatine phosphate (CrP) levels and proper functioning of the cell (Henry, 1980; Nayler et al., 1980; Fleckenstein and Fleckenstein-Gruen, 1980; Watts et al., 1980; Magovern et al., 1981). Propranolol blocks the β-adrenoceptors of the heart, protecting the heart from adrenergic stimulation which causes O_2 wastage and ATP and CrP depletion (Opie et al., 1979; Manning et al., 1981). Both nifedipine and propranolol are used in the clinic for treatment of angina pectoris, hypertension, and prevention of myocardial infarction (see, for instance, Hugenholtz et al., 1981). In the last few years, the combination of the two drugs has also been used for several reasons: (1) The vasodilatation and subsequent hypotension caused by nifedipine induces reflex baroreceptor activation and provokes β-adrenergic stimulation, which can be blocked with propranolol (Lederballe Pedersen et al., 1980; Joshi et al., 1981; Landmark et al., 1982). (2) When nifedipine and propranolol are combined, the doses of each drug can be lowered, thus diminishing the side-effects (Ekelund and Oro, 1979; Imai et al., 1980; Lynch et al., 1980; Dargie et al., 1981; Tweddel et al., 1981). (3) Cases with complicated angina are likely to be best treated with the combination, because nifedipine seems indicated for spasm-induced angina, while propranolol is more suitable for treating stable angina (Fox et al., 1981; Moses et al., 1981).

However, a number of case reports indicate potential dangers, especially an excessive fall in

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blood pressure, resulting in shock or even myocardial infarction (Motte et al., 1980; Opie and White, 1980; Staffurth and Emery, 1981; Robson and Vishwanath, 1982). To our surprise the combination of these drugs had not been tested in ischemic animal heart. We studied the effect of nifedipine and DL-propranolol, alone or in combination, on ATP metabolism in isolated rat hearts. In our model, relatively mild ischemia during a short period of time was introduced to mimic the clinical situation of exertional angina. The release of purine nucleosides and oxypurines was used as a measure of ATP catabolism. This has proven useful in the isolated rat heart (Stam and De Jong, 1977), the open-chested pig heart (De Jong et al., 1977) and patients with ischemic heart disease (Remme et al., 1977; Fox et al., 1979; Kugler, 1979).

Recently we have shown that nifedipine could depress purine release in ischemic hearts in a dose-dependent way (De Jong et al., 1982). The present report shows that nifedipine decreases the release of ATP breakdown products, with DL-propranolol acting synergistically.

2. Materials and methods

2.1. Ischemic heart preparation

Isolated rat hearts from male Wistar rats (292 ± 32 (S.D.) g) were retrogradely perfused according to De Jong (1972) and De Jong et al. (1982). In short: the perfusion buffer (pH 7.4) contained 10 mM glucose, 125 mM NaCl, 4.7 mM KCl, 1.35 mM $CaCl_2$, 20 mM $NaHCO_3$, 0.4 mM NaH_2PO_4, 1 mM $MgCl_2$, and was equilibrated with 95% O_2 + 5% CO_2. Perfusion pressure was 72 mmHg (Statham pressure transducer, model P23D6, Gould Inc., Oxnard, CA). The temperature, measured in the cannula tip, was maintained at 37.0 ± 0.5°C. Ventricles were paced at 4 V with 2 ms duration at 360 beats/min. To induce ischemia, flow was restricted with a model 2132 peristaltic pump (LKB, Stockholm, Sweden) to 2.5 ml/min.

2.2. Chemicals

All chemicals were analytical grade. Water was purified with the Milli-R04/MilliQ system (Millipore Co., Bedford, MA). Nifedipine (in ampoules containing 0.1 g/l, supplied by Bayer AG, Leverkusen, FRG) and DL-propranolol.HCl (in ampoules containing 0.1 g/l, purchased from ICI Chemicals, Macclesfield, UK) were dissolved in the perfusion buffer. Nifedipine-containing solutions were made and stored in alumina-wrapped glassware. The perfusion apparatus was made of brown-colored glass to prevent photolytic degradation (Clark et al., 1981).

2.3. Apex displacement

Apex displacement was used as a measure of contractility (Stam and De Jong, 1977). It was measured with a displacement transducer (model 7-DCDT-1010, Hewlett-Packard, Palo Alto, CA), fitted with a tampon steel hooked to the apex of the ventricle, and monitored with a Brush 2800 recorder (Gould, Cleveland, OH). The displacement observed 5 min after the start of the perfusion before any drug was added was taken as 100%.

2.4. Protocol

The hearts were allowed to equilibrate with the perfusion fluid for 5 min plus an additional 10 min with medium containing nifedipine, DL-propranolol or both (preperfusion). The perfusate was then collected for 10 min (control) and 10 min of ischemia. The hearts were freeze-clamped at the end of the ischemic period. Two sets of experiments were performed. In the first set, purine release from ischemic hearts was compared with the release from ischemic hearts treated with 30 μg/l nifedipine, 30 μg/l DL-propranolol, or 30 μg/l nifedipine and 30 μg/l DL-propranolol. The second set of experiments was identical to the first, except for the concentrations of nifedipine and DL-propranolol, which were 15 and 150 μg/l, respectively.

2.5. Assay of purines

Purines were assayed in a slightly modified version of the high-performance liquid chromatography (HPLC) described by Harmsen et al. (1981). Two-hundred μl of perfusate was injected on a μBondapak C_{18} column. The column was eluted at a rate of 3 ml/min with 0.07 M KH_2PO_4, pH 4.5, with methanol (30 ml added to 1 l buffer). Samples of perfusate were collected on ice. NaN_3 (0.02% final concentration) was added to prevent bacterial purine breakdown. Analysis took place within 2 h.

2.6. Analysis of myocardial adenine nucleotides

The freeze-clamped heart was ground with

liquid N_2 and about 400 mg used for dry weight determination. The other part, about 600 mg, was mixed with 3.0 ml 0.75 M $HClO_4$ at liquid N_2 temperature, thawed, centrifuged and the supernatant fluid stored on ice. Analysis of myocardial high-energy phosphates in the extracts neutralized with KOH was also done by HPLC (Harmsen et al., 1982). The elution profile of ATP, ADP and AMP from the ion-exchange column was used to calculate adenylate energy charge (ATP +0.5 ADP)/(ATP + ADP + AMP). Analysis took place within 6 h.

2.7. *Statistical analysis*

Statistical analysis was performed with Student's t-test. $P > 0.05$ (two-sided) was considered not significant (NS). Results are given as means ±S.E.M.

3. Results

3.1. *Flow*

Fig. 1 demonstrates the vasodilator effect of nifedipine (15 μg/l or 30 μg/l) during aerobic perfusion. Flow increased two-fold ($P < 0.005$ vs. non-treated hearts). Apparently 15 μg/l nifedipine was enough to dilate the coronary arteries maximally. DL-propranolol had no effect on flow in both concentrations used. Ischemia was induced with a roller pump and resulted in a coronary flow of 2.5 ml/min (13.5 ml/min per g dry weight i.e. flow reduction in the non-treated heart was 75% ($P < 0.005$)).

3.2. *Apex displacement*

Fig. 2 shows the negative inotropic properties

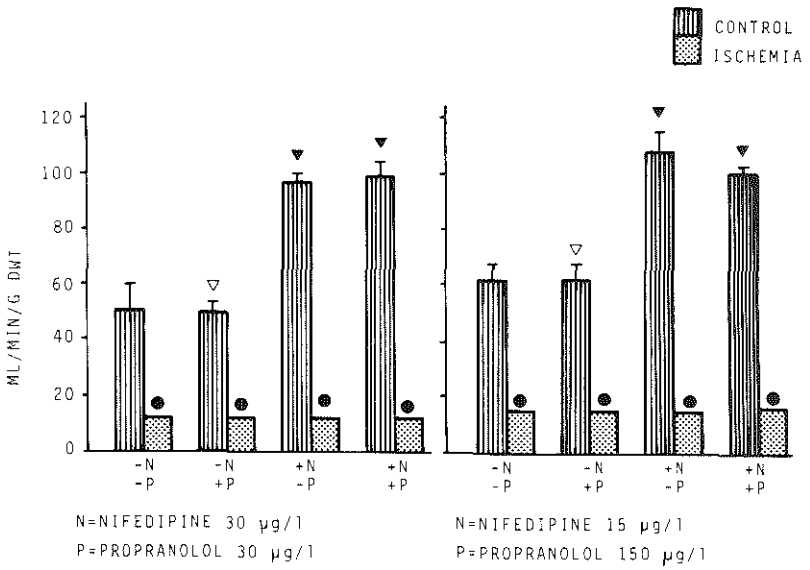

Fig. 1. Influence of nifedipine and DL-propranolol on coronary flow during control period and ischemia. The drug was added 5 min after starting the perfusion. The average flow for 10 min fractions is presented. Ischemia was induced by means of a roller pump with a fixed flow of 2.5 ml/min. Heart rate was 360 bpm, perfusion pressure 72 mmHg. Means ±S.E.M. (n = 5-9). ▼ $P < 0.05$ vs. non-treated rat hearts (−N, −P); ▽ $P < 0.05$ vs. nifedipine-treated rat hearts (+N, −P); ● $P < 0.005$ vs. control period.

of nifedipine during aerobic perfusion. Fifteen μg/l nifedipine induced a 25% decrease in apex displacement ($P < 0.005$ vs. non-treated hearts) and 30 μg/l nifedipine a 60% decrease ($P < 0.005$ vs. non-treated). DL-propranolol showed no effect on aerobic contractility. When the the combination was infused, the negative inotropic effects found during the control period seemed only due to nifedipine. During ischemia, apex displacement was about 40% of the aerobic values, in both treated and untreated hearts. This means that ischemia induced a 60% decrease in the non-treated and propranolol-perfused hearts ($P < 0.005$). With 15 μg/l nifedipine (alone or with propranolol), ischemia caused the apex displacement to decrease by half (from 75 to 40%, $P < 0.05$) and with 30 μg/l nifedipine (alone or with propranolol) no change in apex displacement due to ischemia was seen.

3.3. Purine release

The release during aerobic perfusion was 0.4 μmol/10 min per g dry weight. During ischemia, purine release was about 1.2 μmol/10 min per g dry weight ($P < 0.005$, see fig. 3). Nifedipine produced a dose-dependent decrease of the ischemic purine release (to 67 and 44% of this value for 15 and 30 μg/l, respectively). DL-Propranolol had no influence on purine release. However, the combination of 30 μg/l nifedipine and 30 μg/l Dl-propranolol seemed to suppress the ischemic purine release better than either drug alone (NS). When the nifedipine concentration was decreased to 15 μg/l and the propranolol concentration increased to 150 μg/l, the suppression of ischemic purine release was significantly greater than with nifedipine ($P < 0.05$) or propranolol ($P < 0.005$) alone. Fig. 4 shows that nifedipine alone decreased

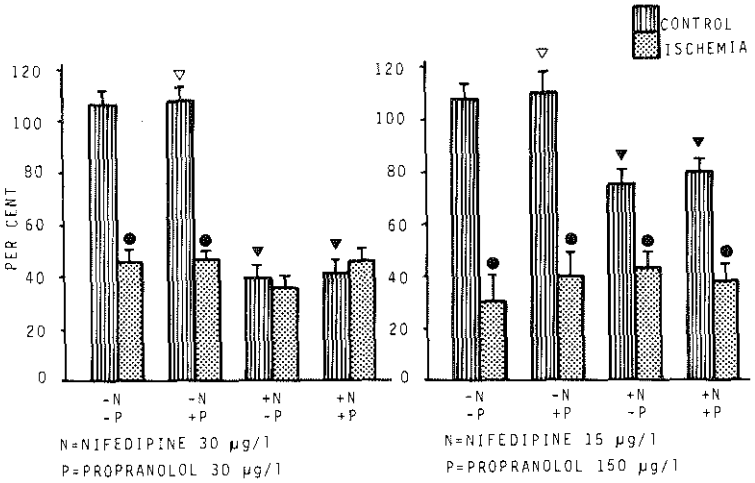

Fig. 2. Effect of nifedipine and DL-propranolol on apex displacement during aerobic perfusion and after 10 min of ischemia. The displacement before any drug was added was taken as 100%. Means ± S.E.M. (n = 5-9). Experimental conditions, see legend to fig. 1. ▼ $P < 0.05$ vs. non-treated rat hearts (−N, −P); ▽ $P < 0.05$ vs. nifedipine-treated rat hearts (+N, −P); ● $P < 0.005$ vs. control period.

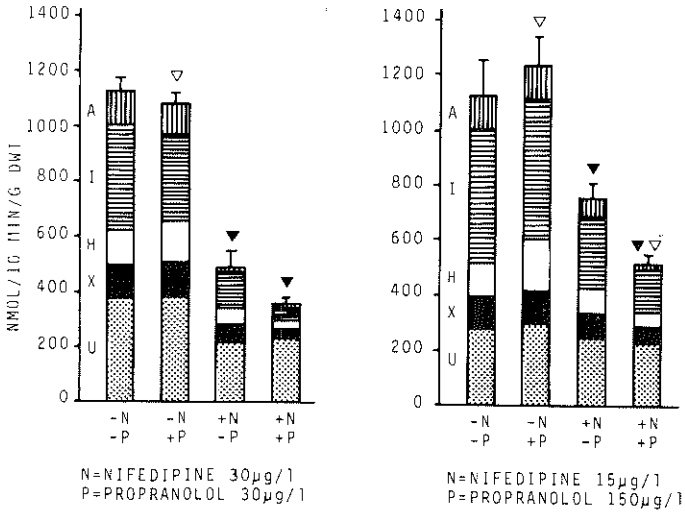

Fig. 3. Reduction of purine release by nifedipine and DL-propranolol during myocardial ischemia. Means ± S.E.M. (n = 5-9). Conditions, see legend to fig. 1. A = adenosine, I = inosine, H = hypoxanthine, X = xanthine and U = uric acid. ▼ $P < 0.05$ vs. non-treated rat hearts (−N, −P); ▽ $P < 0.05$ vs. nifedipine-treated rat hearts (+N, −P).

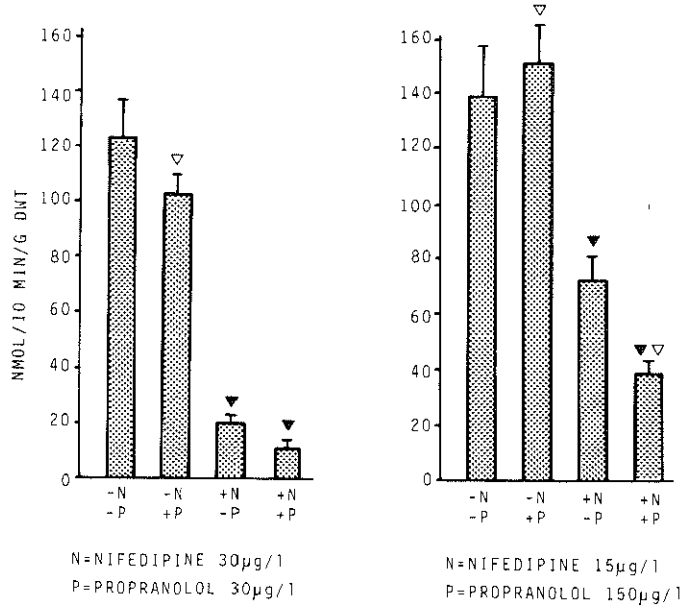

Fig. 4. Reduction of adenosine release during ischemia. Means ± S.E.M. (n = 5-9). ▼ $P < 0.05$ vs. non-treated rat hearts (−N, −P); ▽ $P < 0.05$ vs. nifedipine-treated rat hearts (+N, −P).

adenosine release (with 15 μg/l nifedipine by 47%, with 30 μg/l by 85%). Propranolol alone had no effect on adenosine release, whereas 150 μg/l propranolol and 15 μg/l nifedipine decreased adenosine release by 72% ($P < 0.05$ vs. non-treated; $P < 0.05$ vs. nifedipine; $P < 0.05$ vs. propranolol).

3.4. *High-energy phosphates*

It can be seen in table 1 that nifedipine (30 μg/l) significantly increased the ATP and CrP content, which seemed further augmented with 30 μg/l propranolol. Nifedipine 15 μg/l failed to protect high-energy phosphate levels but 150 μg/l propranolol increased CrP, although the energy charge did not change. Addition of nifedipine to the propranolol-containing buffer improved the energy charge to aerobic values. The latter were: ATP and CrP, 23.0 ± 0.4 and 22.9 ± 2.1 μmol/g dry weight, respectively, and energy charge 0.902 ± 0.003 ($n = 4$).

The ischemic ATP and CrP values of non-treated rat hearts were somewhat lower in the first set of experiments than in the second set (see table 1). The only difference we noticed between these two groups was between the dry weight of the hearts (200 ± 4 vs 167 ± 4 mg, respectively, $P < 0.005$). Rat body weights were comparable (289 ± 8 vs. 298 ± 5 g). Seasonal variations between groups of animals could not be excluded. Because the effect of various concentrations of nifedipine and propranolol was evaluated within the sets of experiments, conclusions may be drawn from each series of experiments.

4. Discussion

Ca antagonists e.g. nifedipine, and β-blockers e.g. propranolol, are thought to protect the heart under ischemic conditions. This supposedly ensures proper ATP and CrP levels in the heart, with good functioning of subcellular structures. The mode of action of these drugs is still rather obscure. We studied their effect on myocardial ATP metabolism in a model in which the hemodynamic variables were kept as constant as possible.

4.1. *Nifedipine*

Nifedipine is suggested to act by selective inhibition of Ca influx through the cell membrane. It supposedly uncouples electromechanical processes, resulting in a negative inotropic action. Nayler et al. (1980) found that during ischemia and reperfusion, nifedipine prevented excessive Ca overload, maintained ATP levels (but see Ichihara et al., 1979), and preserved mitochondrial function and membrane structures. However, Church and Zsoter (1980) found no decrease of Ca influx in the mesenteric vein of the rabbit during nifedipine administration. They supposed an intracellular action of nifedipine. Other authors suggested that beneficial effects of nifedipine were only due to its negative inotropic action (by lowering O_2 consumption through reduction of heart work and vasodilatation; see e.g., Fleckenstein and Fleckenstein-Gruen, 1980).

In our model, during *aerobic* conditions, nifedipine acted as a negative inotropic agent on

TABLE 1

ATP and creatine phosphate content, and energy charge of freeze-clamped perfused rat hearts. The hearts were perfused for 25 min aerobically and for 10 min of ischemia, as described in the Material and Methods section. The drugs nifedipine (Nif) and/or DL-propranolol (Prop) were added to the perfusion fluid 5 min after starting the perfusion. I and II refer to the first and second set of experiments, respectively. Means ± S.E.M. ($n = 5$-10). [a] $P < 0.05$ vs. none.

Drugs	Concentration μg/l	ATP μmol/g dwt	CrP μmol/g dwt	Energy charge
I				
None		16.5 ± 1.2	13.6 ± 2.2	0.832 ± 0.022
Nif	30	20.2 ± 0.6 [a]	25.4 ± 0.9 [a]	0.866 ± 0.016
Prop	30	18.5 ± 0.8	15.7 ± 1.1	0.823 ± 0.016
Nif/Prop	30/30	22.4 ± 2.0 [a]	28.6 ± 3.6 [a]	0.880 ± 0.013
II				
None		20.7 ± 0.8	16.6 ± 1.3	0.880 ± 0.013
Nif	15	21.3 ± 1.4	16.0 ± 1.8	0.866 ± 0.013
Prop	150	22.1 ± 0.7	21.5 ± 1.6 [a]	0.875 ± 0.007
Nif/Prop	15/150	21.9 ± 0.6	19.6 ± 0.5 [a]	0.901 ± 0.010 [a]

the heart (see fig. 2). The effect was dose-dependent and confirmed our earlier results (De Jong et al., 1982). Vasodilatation seemed maximal at the lowest concentration used (15 μg/l, see fig. 1), whereas doubling the nifedipine concentration further decreased contractility. This indicates that arterial smooth muscle cells are more sensitive to nifedipine than heart cells (see fig. 1). This is in accordance with findings of other authors (see, e.g., Fleckenstein and Fleckenstein-Gruen, 1980).

During *ischemia*, nifedipine had no effect on contractility in our preparation. Probably it cannot interfere with electromechanical processes during ischemia. However, despite its lack of negative inotropy during ischemia, nifedipine reduced adenosine (catabolite) release (figs. 3 and 4), with concomitant higher myocardial ATP and CrP levels (table 1). Furthermore the adenylate energy charge was better maintained (table 1).

Recently, Verdouw et al. (1982) found that, after nifedipine treatment, there was a decrease of myocardial O_2 consumption in pig hearts while O_2 demand was unchanged. Nayler et al. (1980) proposed that mitochondrial Ca overload was prevented, resulting in better preservation of function and preservation of myocardial ATP and CrP levels. Other possibilities are also conceivable. Nifedipine is likely to cause a shift from fatty acid to glucose oxidation (Ichihara et al., 1979). Also several Ca (calmodulin)-dependent ATP-ases and -phosphorylases are possibly inhibited (for a review, see Stocklet, 1981). We conclude from our experiments that during ischemia, nifedipine shows ATP-sparing properties which are independent of the negative inotropic action of the drug. Whether this effect is caused by better or more economical ATP production or by a decrease in ATP catabolism, or both, is still unknown. The loss of the negative inotropic properties of nifedipine remains intriguing. Recently Weintraub et al. (1982) reported increases in contractility during nifedipine administration to ischemic dog heart without giving a satisfactory explanation.

4.2. Propranolol

DL-Propranolol protects the heart from the stimulatory actions of catecholamines. The latter increase oxygen consumption, and cause oxygen wastage, e.g., due to a shift from carbohydrate to fatty acid oxidation. Cyclic AMP is thought to mediate this action (Opie et al., 1979). It is thought that D- and L-propranolol stabilize membranes (Arnim and Welman, 1981).

In our model, DL-propranolol showed no effect on coronary flow and contractility (see figs. 1 and 2). This suggests the absence of catecholamine release. Also no effect on ischemic purine release was seen, although a concentration of 150 μg/l seemed to reduce CrP breakdown. The energy charge, however, was comparable with that in non-treated hearts. This is in accordance with findings of Edoute et al. (1980) and Lubbe et al. (1980), who did not see a significant improvement of ATP levels during ischemia after propranolol infusion, although mitochondrial function improved slightly. Nayler et al. (1980) found pretreatment of rabbits with propranolol effective for protection. However, in the intact animal β-blockade seems much more important. In our model DL-propranolol did not show any effect on purine release.

4.3. Nifedipine and propranolol

The effects of the combination of drugs on flow and contractility can be ascribed to nifedipine. Although propranolol alone shows no effect on ischemic purine release, 150 μg/l propranolol added to 15 μg/l nifedipine gave a significantly lower adenosine (catabolite) release than did 15 μg/l nifedipine alone (see figs. 3 and 4). Also ATP, CrP and energy charge (table 1) returned to their aerobic control levels. We conclude from this observation that the ATP-sparing effect of nifedipine can be augmented by propranolol. Also this synergistic effect of propranolol seems independent of contractility, i.e., independent of electromechanical coupling. We therefore assume that the ATP-sparing effect is the result of a more economical ATP metabolism. Especially ATP catabolism (via ATP-ases and phosphorylases) is controlled by Ca (via activated calmodulin) or cyclic AMP. It seems possible that nifedipine, by lowering the internal free Ca concentration, activates cyclic AMP-dependent processes via compensatory processes which are blocked with propranolol.

We conclude from our experiments that nifedipine alone or in combination with propranolol prevents adenine nucleotide breakdown in ischemic rat heart. This effect seems independent of electromechanical coupling. Therefore these results suggest a direct effect of nifedipine on energy metabolism, which can be intensified by propranolol. The precise mechanism remains to be elucidated. Part of the beneficial effect of nifedipine and propranolol administration in patients could be due to the effect we described. The negative side-effects of the combination of the drugs reported in the literature seem to be due

more to an excessive fall in blood pressure than to a direct negative effect on the metabolism of (ischemic) myocardium.

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Diltiazem Administered Before or During Myocardial Ischemia Decreases Adenine Nucleotide Catabolism

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J. W. de Jong, E. Harmsen and P. P. de Tombe. Diltiazem Administered Before or During Myocardial Ischemia Decreases Adenine Nucleotide Catabolism. *Journal of Molecular and Cellular Cardiology* (1984) 16, 000–000. Calcium antagonists potentially prevent ATP breakdown, but literature data on this subject are conflicting. We studied the effect of diltiazem on ATP catabolism in rat heart, perfused according to Langendorff. Administration of the drug took place either before or during ischemia, induced by lowering the perfusion pressure. The reduction in flow without diltiazem was 85%. We observed a significantly ($P < 0.001$) lower production of purine nucleosides and oxypurines by the ischemic heart, when we gave diltiazem in a dose range of 1 to 100 μM *before* ischemia. The highest drug concentration reduced purine release by 85%. Due to ischemia, myocardial adenine nucleotide content decreased by 40% ($P < 0.001$); this was partially prevented by 5 μM diltiazem ($P = 0.04$ *v.* untreated hearts). The drug also effectively reduced purine release, when applied five minutes *after* the induction of ischemia, but higher concentrations were needed.

Key Words: ATP catabolism; Calcium antagonist; Creatine phosphate; Diltiazem; Ischemia; Inotropic effect; Purine nucleosides; Oxypurines.

Introduction

Adenosine 5'-triphosphate plays a central role in the maintenance of cellular integrity. A reduction in flow leads to increased cytosolic calcium concentrations and gives rise to reduced ATP levels with loss of structure and function as a result [*23*]. Blockers of slow calcium channels presumably prevent this sequence of events [*7*]. Under a number of conditions, related to myocardial ischemia and reperfusion, diltiazem is capable to preserve the ATP-generating capacity [*3, 20*] or to slow down ATP catabolism of the heart [*3, 36*]. Diltiazem protected (partially) the ATP content of hearts during the calcium paradox [*1*]. In some other studies diltiazem did not improve ATP metabolism, when an ischemic insult had already taken place [*2, 22, 35, 37*]. We studied therefore in perfused rat heart the effect of diltiazem on nucleotide breakdown, when the drug was administered either before or during ischemia. Purine efflux was routinely used as an index of ATP catabolism [*4, 26, 33*].

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Materials and Methods

Preparation of ischemic heart

Male Wistar rats (240 to 350 g), with free access to food and water, were anesthetized with 30 mg sodium pentobarbital i.p. Hearts were rapidly removed and cooled in ice-cold saline. Retrograde perfusion of the aorta was immediately started with a modified Tyrode buffer as described before [*5*]. The buffer consisted of 128 mM NaCl, 4.70 mM KCl, 1.35 mM $CaCl_2$, 20.2 mM $NaHCO_3$, 0.42 mM NaH_2PO_4, 1.0 mM $MgCl_2$ and 10 mM D-glucose. The mixture was passed through a 0.45 μm filter and equilibrated with 95% O_2 to 5% CO_2. This resulted in a buffer pH of 7.35 ± 0.05. Pacing frequency was 300 beats/min (4 V stimulation for 2 ms). Perfusion temperature (37°C) was measured in the

aortic canula with a A-KC1 probe connected to a DU-3 monitor (Ellab, Copenhagen, Denmark). Ischemia was induced by lowering the perfusion pressure from 72 mmHg to 12 mmHg.

Chemicals

All chemicals were analytical grade. Water was purified with the Millipore-Ro4/Milli-Q system (Millipore Co., Bedford, MA). Synthélabo (Brussels, Belgium) supplied diltiazem, d-3-acetoxy-cis-2,3-dihydro-5-[2-(dimethylamino)ethyl]-2-(p-methoxyphenyl)-1,5-benzothiazepin-4(5H)-one HCl, as a solid.

Myocardial function

Apex displacement [*33*] or developed tension [*24*] was used as an indicator of myocardial performance. The former was measured as described before [*33*]. The displacement, observed 5 min after the start of the perfusion, was taken as 100%. Alternatively, tension development was monitored with a P23Db transducer (Statham, Hato Rey, Puerto Rico), connected to the apex of the heart. Before ischemia the hearts were adjusted to a resting tension of about 12 g.

Coronary flow

Mean coronary flow was measured by collection of perfusate during the last minute of normoxia, ischemia and reperfusion.

Assay of purines

Adenosine, inosine, hypoxanthine, xanthine and urate were assayed with a slightly modified version of the HPLC method described before [*10*]. A μBondapak column (30 × 0.4 cm, Waters Assoc., Milford, MA) was eluted at a rate of 3.0 ml/min with 75mM KH_2PO_4, pH 4.5, to which methanol (30 ml/l) had been added. The samples were mixed with NaN_3 (0.02% final concentration) to prevent bacterial purine breakdown. Perfusates were kept on ice until analysis, which took place within 12 h.

Assay of high-energy phosphates

In a limited number of hearts, adenine nucleotides and creatine phosphate were determined as described before [*11*]. Data from these hearts are expressed per gram dry weight. The conversion factor to wet weight is 0.15 to 0.20.

Statistical analysis

Analysis of variance was employed with two-way classification; further evaluations were made using Scheffé's method for multiple comparisons [*32*]. $P > 0.05$ (two-tailed) was considered not significant (NS). Results are expessed as means ± S.E.M.

One experiment out of 7 (0 μM diltiazem) was rejected, because during ischemia and reperfusion apex displacement was nil, and purine release was about 37 nmol/min/g wet wt. These values were completely outside the range observed in the other experiments. According to the rejection rule of Anscombe and Tukey [*32*], with a 5% premium, the values of the experiment mentioned earlier were considered gross errors and rejected.

Results

Coronary flow

Diltiazem increased coronary flow in control (normoxic) hearts dose-dependently ($P < 0.001$; Fig. 1). One hundred micromolar diltiazem augmented coronary flow about twofold. The change in perfusion pressure from 72.0 ± 0.1 to 12.4 ± 0.7 mmHg caused myocardial ischemia: coronary flow in hearts without diltiazem was reduced by 85%. Flow in the presence of diltiazem during ischemia was comparable to that without drug (see Fig. 1). Reperfusion pressure was 72.2 ± 0.1 mmHg. It increased flows, but they were somewhat lower than the normoxic values ($P < 0.001$, see Fig. 1). During reperfusion diltiazem increased coronary flow dose-dependently ($P < 0.001$). The time of diltiazem administration (before or during ischemia) did not affect the coronary flow observed during reperfusion.

Myocardial function

Diltiazem caused a decrease in apex displacement in normoxic control hearts ($P < 0.001$; Fig. 2). Analysis of variance showed that hearts treated with the two highest concentrations had a significantly smaller apex displacement than hearts perfused with

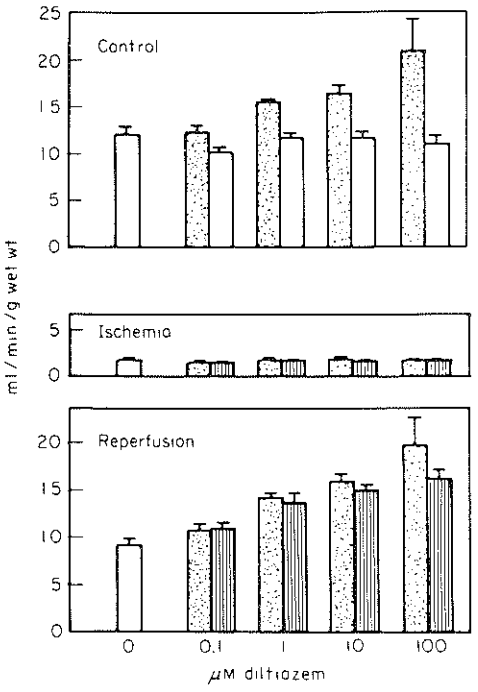

FIGURE 1. Influence of diltiazem on coronary flow in isolated hearts, perfused at a pressure of 72 mmHg (normoxic control and reperfusion) or 12 mmHg (ischemia). Open bars: no diltiazem; shaded bars: diltiazem administered 10 min before ischemia; hatched bars: diltiazem given 5 min after the induction of ischemia. Normoxic control perfusion, ischemia, and reperfusion took place for 15 min each. Measurements were made at the end of each period. Means ± S.E.M. ($n = 6$ for 0 μM diltiazem, $n = 4$–5 for other experiments). For analysis of variance, see text.

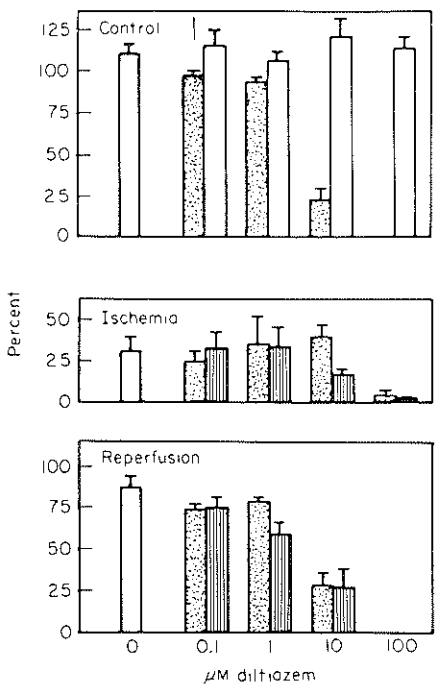

FIGURE 2. Effect of diltiazem on apex displacement of isolated perfused hearts. The value found 5 min after the start of the perfusion (before any drug was given) was taken as 100%. Open bars: no diltiazem; shaded bars: diltiazem administered 10 min before ischemia; hatched bars: diltiazem given 5 min after the induction of ischemia. For other details, see legend to Fig. 1.

the other concentrations. Due to ischemia, apex displacement in hearts without the drug decreased from 107 ± 6 to 27 ± 8% ($P < 0.002$). In hearts treated with diltiazem up to 10 μM, we found that the ischemic values were comparable for the various doses, regardless of the application time. The higher concentrations, however, depressed the hearts significantly. Apex displacement in untreated hearts recovered partially during reperfusion; there was no significant difference with normoxic control hearts (Fig. 2). With 10 and 100 μM diltiazem, applied before ischemia, no significant differences were found during control, ischemia and reperfusion. With the latter concentration, regardless of the application time, apex displacement throughout the experiment was virtually zero.

The variation in apex displacement, observed during ischemia, was considerable. In a second series of experiments, we used therefore developed tension as a measure of myocardial function. Ten minutes after the start of the perfusion, developed tension was about 28 g. Five micromolar diltiazem decreased this by 52% ($P < 0.005$, upper panel of Fig. 3). When the flow was reduced by 88%, developed tension decreased rapidly; hearts without diltiazem became arrhythmic and ceased to beat after about 15 min of ischemia. Hearts, pretreated with the drug, did not show arrhythmias and developed 1.2 ± 0.2 g tension until they were freeze-clamped (after 30 min of ischemia, see Fig. 3). The differences between treated and untreated

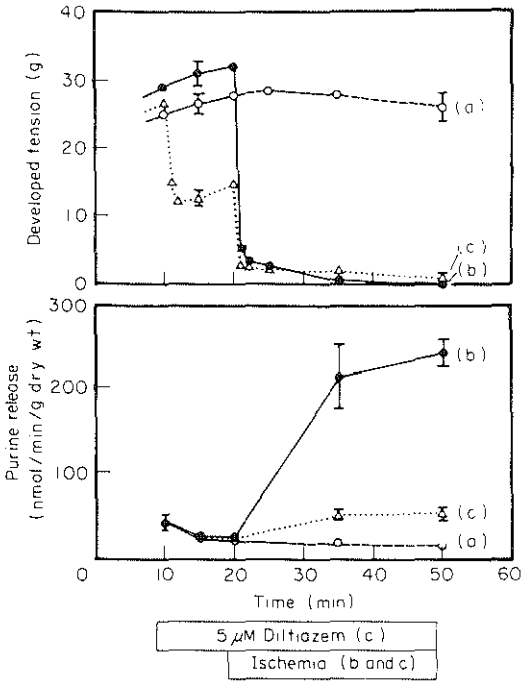

FIGURE 3. Developed tension and purine release in normoxic control hearts (a), and hearts made ischemic after 20 min perfusion (b and c). Hearts in group C received 5 μM diltiazem 10 min prior to ischemia. Means ± S.E.M. ($n = 4$).

ischemic hearts were not statistically significant, however.

Purine release

In normoxic control hearts, diltiazem caused a dose-dependent release of purine nucleosides. With analysis of variance we found a significant ($P < 0.001$) difference for adenosine and inosine production with the various diltiazem concentrations (Fig. 4). With 100 μM this release was two to three times higher than in the untreated hearts. Diltiazem also increased the release of oxypurines [(hypo)xanthine and urate], but we did not observe statistically significant differences. Figure 5 depicts total purine release. The hearts produced somewhat more purines during normoxia, when diltiazem was used ($P = 0.008$), but no significant dose-dependency was found. The drug effectively suppressed purine release during ischemia ($P < 0.001$). We observed an almost complete decrease in adenosine and inosine production with 100 μM diltiazem, administered before ischemia (Fig. 6). Also the release of hypoxanthine, xanthine and urate diminished with the higher diltiazem doses. Total purine release decreased 85% with the highest dose of diltiazem (Fig. 5). A similar picture emerged with diltiazem given when ischemia had already been present for some time, although the protective effect was less ($P = 0.02$ *v.* pre-

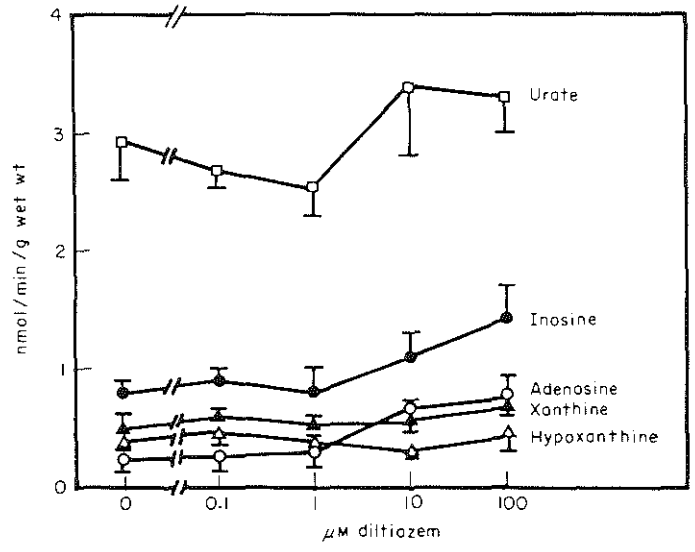

FIGURE 4. Release of purine nucleosides and oxypurines from isolated hearts, after 5 min preperfusion and 10 min of perfusion with diltiazem at 72 mmHg (normoxic controls). Means ± S.E.M. ($n = 6$ for 0 μM diltiazem; $n = 5$ for other experiments).

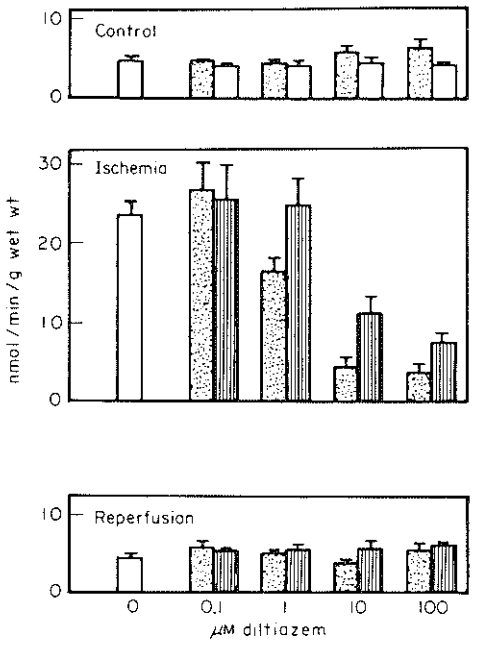

FIGURE 5. Effect of diltiazem on the release of purines (adenosine, inosine, hypoxanthine, xanthine and urate) from isolated perfused hearts. Open bars: no diltiazem; shaded bars: diltiazem administered 10 min before ischemia; hatched bars: diltiazem given 5 min after the induction of ischemia. For other details, see legend to Fig. 1.

treatment, Figs 5 and 6). Diltiazem did not significantly affect the release of purine nucleosides and oxypurines during reperfusion.

High-energy phosphates

Myocardial ATP content decreased by 56% ($P < 0.001$, Scheffé's method) due to 30 min ischemia (Fig. 7). If 5 μM diltiazem had been present before the ischemic insult, this decrease was only 22% ($P = 0.04$ *v.* untreated hearts). Due to ischemia myocardial creatine phosphate decreased by 66% in untreated hearts ($P < 0.001$), and by 48% when treated with 5 μM diltiazem ($P < 0.001$; NS *v.* decrease in untreated hearts). The myocardial energy charge, (ATP + 0.5 ADP)/(ATP + ADP + AMP), decreased from 0.914 ± 0.003 to 0.778 ± 0.14 due ischemia ($P < 0.001$). Pretreatment of the ischemic hearts with 5 μM diltiazem improved the energy charge

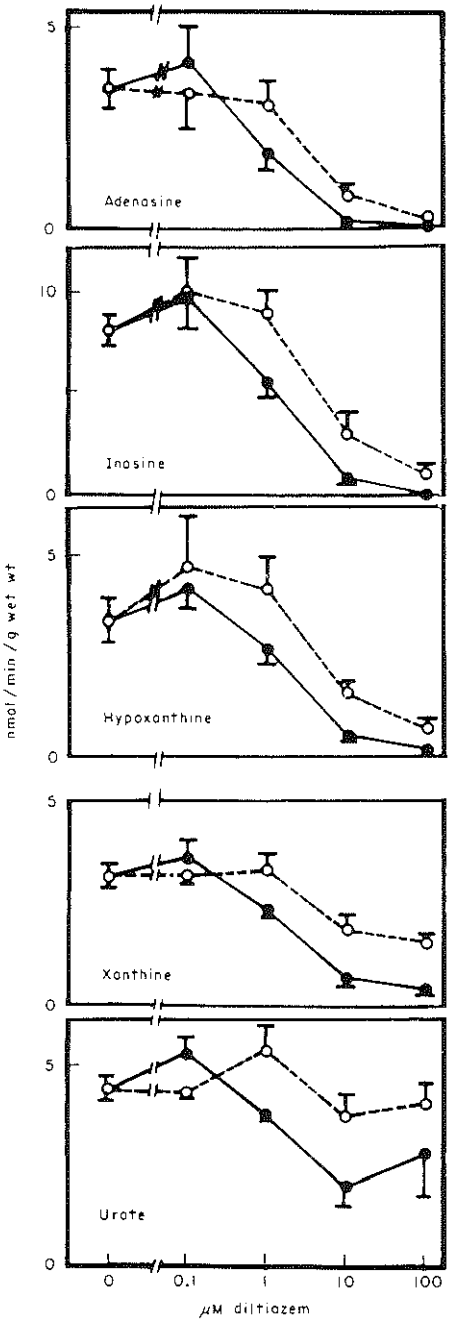

FIGURE 6. Reduction of purine nucleoside and oxypurine release from ischemic hearts by diltiazem. ●——● diltiazem administered 10 min before ischemia; ○ – – – ○ diltiazem added 5 min after induction of ischemia. Means ± S.E.M. ($n = 6$ for 0 μM diltiazem; $n = 5$ for other experiments). Other details are given in the legend to Fig. 1.

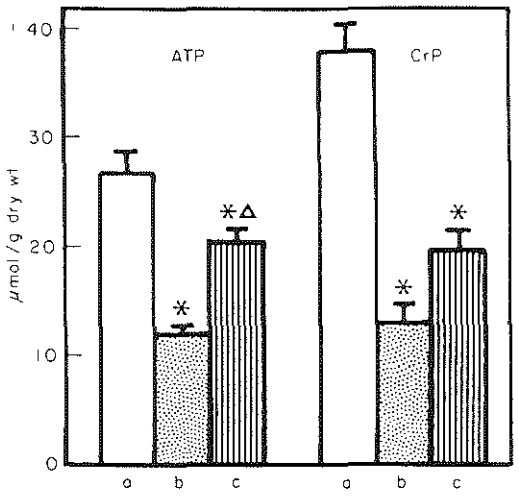

FIGURE 7. Content of ATP and creatine phosphate (CrP) in normoxic control hearts (a), ischemic hearts (b), and ischemic hearts, pretreated with 5 μM diltiazem (c). *$P < 0.005$ *v.* (a); Δ$P < 0.04$ *v.* (b). For details, see Fig. 3.

found in ischemic hearts significantly to 0.881 ± 0.007 ($P < 0.001$). This value is not significantly different from the energy charge in normoxic control hearts.

Figure 8 shows that there was a good negative correlation between the myocardial content of adenine nucleotides and purine release. In the ischemic samples a low nucleotide content was found with a high release of purine nucleosides and oxypurines. With 5 μM diltiazem the values approached those of normoxic controls.

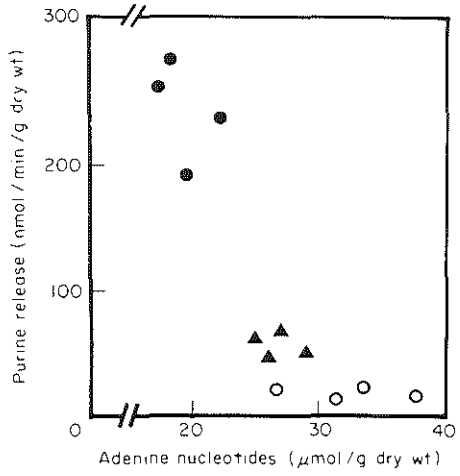

FIGURE 8. Negative correlation between myocardial adenine nucleotide content (ATP + ADP + AMP) and release of purines (adenosine + inosine + hypoxanthine + xanthine + urate). ○ normoxic control hearts; ● hearts, made ischemic for 30 min; ▲ ischemic hearts, pretreated with 5 μM diltiazem.

Discussion

When, due to lack of oxygen, the myocardial ATP content falls rapidly, AMP is formed, which can be broken down to adenosine, inosine, and hypoxanthine. These compounds leave the cell before cell death occurs. They have been used as markers for myocardial ischemia [*4, 8, 9, 17, 26, 33*]. Quite often the sum of these purines is taken to calculate myocardial adenosine production [*14, 19, 28, 31*]. However, the rat heart produces also substantial amounts of xanthine and urate [*27, 30*], because it contains xanthine oxidase [*18, 30*]. Therefore, we decided to monitor the myocardial release of the adenosine catabolites up to urate, as an index of myocardial ATP breakdown; the relationship between adenine nucleotide content and purine release from the ischemic heart is clearly depicted in Figure 8. From this study it is clear that xanthine and urate comprise a substantial fraction of the purines leaving the heart. Urate makes up 62% of the purines released by normoxic tissue (Fig. 4), but the value drops to 18% during ischemia (Fig. 6). This could indicate that xanthine oxidase operates under saturating conditions in the ischemic myocardium.

We showed earlier [*5*] that nifedipine, administered before ischemia, could prevent adenine nucleotide catabolism. In a preliminary study [*6*], a relatively low dose of this drug applied during ischemia was without effect. Weishaar and Bing [*37*] were unable to show a protective effect of diltiazem (administered 5 min prior to reperfusion) on myocardial ATP, although the creatine phosphate content increased. In this study early administration of diltiazem dimished ischemic ATP breakdown considerably, but creatine phosphate levels remained relatively low (Fig. 7). Diltiazem protected the myocardial energy charge against an ischemia-induced decrease. In addition, we observed that diltiazem decreased dose-dependently

myocardial purine release most effectively, when the drug was given before ischemia (Figs 5 and 6). Similarly, Takeo and Takenaka [*34*] demonstrated that the isoproterenol-induced breakdown of high-energy phosphates was prevented dose-dependently by diltiazem. From the nifedipine study mentioned above [*5*], with somewhat different ischemic conditions, and the present data, we estimate that nifedipine is 30 times more potent than diltiazem as a protective agent.

The increased purine production during normoxia due to diltiazem (Fig. 4) is puzzling. Diltiazem does not affect normal functioning mitochondria [*20*]. We would have expected the ATP-generative capacity to be adequate, especially if one considers the decreased myocardial function. Possibly, vasodilatation is responsible for increased washout of the purines. In view of the fact that the higher diltiazem doses exceed those observed in human plasma [*16*], one might take the posture that the enhanced release of ATP metabolites during normoxia represents in part a non-therapeutic or toxic effect.

The vasodilatory and negative inotropic properties of diltiazem, given to normoxic hearts, are clear from Figures 1 to 3. These have been well-established by other studies [*15, 21, 29*]. We found no correlation between myocardial function and purine production during ischemia (see, for instance, Fig. 3), so that it seems doubtful that the ATP-sparing effect of diltiazem, administered before ischemia, is due to a reduction in contractility. In anesthetized dogs, diltiazem nor nifedipine depressed mechanical activity of ischemic heart; they in fact significantly enhanced its performance [*25*]. Henry and Wahl [*13*] recently demonstrated that diltiazem acts directly on cardiac muscle in the absence of rhythmic electrical and mechanical activity. We conclude, like we did from earlier studies with nifedipine alone [*5, 6*], or nifedipine plus propranolol [*12*], that diltiazem presumably does not protect by negative inotropy during myocardial ischemia. We speculate that calcium antagonists reduce myocardial oxygen demand, thereby diminishing the effect of flow impairment.

Acknowledgements

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Enhanced ATP and GTP synthesis from hypoxanthine or inosine after myocardial ischemia

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HARMSEN, EEF, PETER P. DE TOMBE, JAN WILLEM DE JONG, AND PETER W. ACHTERBERG. *Enhanced ATP and GTP synthesis from hypoxanthine or inosine after myocardial ischemia.* Am. J. Physiol. 246 (Heart Circ. Physiol. 15): H37–H43, 1984.—Increasing therapeutic use is made of purines for the treatment of ischemic heart disease, but little is known about regulatory mechanisms involved. Therefore we perfused isolated rat hearts with 0.02 mmol/l [8-^{14}C]hypoxanthine or inosine. Under normoxic conditions about 1% is taken up by the heart and partially used for synthesis of ATP and GTP at a rate of 0.4 and 0.1 nmol·min^{-1}·g dry wt^{-1}, respectively. After relatively mild ischemia (coronary flow reduction of 70% for 20 min), no increase in myocardial purine uptake is observed, but ATP and GTP synthesis rates are doubled ($P < 0.001$). D-Ribose stimulates the hypoxanthine incorporation rate in normoxic perfused rat hearts to 1.1 and 0.5 nmol·min^{-1}·g dry wt^{-1} for ATP and GTP, respectively, which is further increased during postischemic perfusion. About 80% of the [8-^{14}C]inosine or [8-^{14}C]hypoxanthine passes through the heart unchanged, while 15% is converted to (hypo)xanthine and uric acid. We conclude from these experiments that inosine and hypoxanthine incorporation into ATP and GTP is at least partly regulated by the availability of 5-phosphoribosyl-1-pyrophosphate.

nucleotide biosynthesis; salvage; oxypurine; nucleoside; ribose; heart; rat

IN THE AEROBIC HEART a critical balance exists between production of ATP and its utilization. If the heart becomes ischemic, this delicate balance is disturbed, and energy-rich phosphates are broken down to the nucleosides and purine bases adenosine, inosine, hypoxanthine, xanthine, and uric acid. These apolar compounds can pass the cellular membrane and enter the bloodstream (2, 9). Because of this loss of purines, ATP levels will remain below control levels after reoxygenation (see, e.g., Ref. 18). To restore these adenine nucleotide pools three main pathways exist (Fig. 1). *1*) In adenosine phosphorylation, adenosine is directly phosphorylated to AMP by adenosine kinase. The maximal incorporation rate in rat heart amounts to about 50 nmol·min^{-1}·g dry wt^{-1} (16). This pathway is energetically economical. However, the adenosine concentration in blood is low, and this could be a restricting factor for adenosine phosphorylation. *2*) In hypoxanthine salvage, hypoxanthine can be phosphorylated with 5-phosphoribosyl-1-pyrophosphate (PRPP) to form IMP (5, 12, 14, 22). IMP is a crucial branching point between adenine and guanine nucleotide synthesis. In rat heart the hypoxanthine incorporation rate into ATP can amount to 3 nmol·min^{-1}·g dry wt^{-1} (14). *3*) In de novo synthesis, purine nucleotides (adenine and guanine nucleotides) are also synthesized from small precursor molecules (among others, glycine, CO_2). In a 10-step synthesis from PRPP, IMP is formed. De novo production rate of IMP in rat heart is about 0.1 nmol·min^{-1}·g dry wt^{-1} (24). This process is stimulated after anoxia, ischemia, and hypertrophy. As an explanation for this phenomenon, an enhanced synthesis of PRPP by acceleration of the hexose-monophosphate shunt has been proposed (24). To bypass this shunt, ribose (a precursor of PRPP) can also be supplied (25).

We decided to study the incorporation of [8-^{14}C]hypoxanthine into normoxic or postischemic rat hearts in the presence of D-ribose to investigate whether hypoxanthine incorporation is also PRPP dependent. Because inosine, a precursor of hypoxanthine, is used as a cardioprotective and vasodilatory agent (4, 10, 11, 21, 23), [8-^{14}C]inosine incorporation rates in aerobic and postischemic rat hearts were also determined. Mammalian cells lack inosine kinase (22); therefore inosine is converted to hypoxanthine and ribose-1-phosphate. Subsequently, part of the hypoxanthine is converted to IMP; ribose-1-phosphate can serve as a precursor of PRPP.

Hypoxanthine and inosine are incorporated not only into adenine nucleotides but also into guanine nucleotides. To our knowledge no data about purine incorporation into myocardial guanine nucleotides have been published, although Swain et al. (20) recently investigated the incorporation of 5-aminoimidazol-4-carboxamide riboside (AICAR, an intermediate of the de novo pathway) in dog heart and found repletion of ATP and GTP levels.

MATERIALS AND METHODS

Perfusion protocol. Male Wistar rats [315 ± 34 (SD) g] were used. Hearts were removed and perfused as described earlier (3). The basic perfusion buffer (pH 7.4) consisted of (in mM) NaCl 125, KCl 4.7, $CaCl_2$ 1.35, $NaHCO_3$ 20, NaH_2PO_4 0.4, $MgCl_2$ 1.0, and D-glucose 10 and was equilibrated with 95% O_2-5% CO_2. The perfusion pressure was 72 mmHg, measured with a Statham pressure transducer (model P2306, Gould, Oxnard, CA). The hearts were paced at 300 beats/min, and the temperature

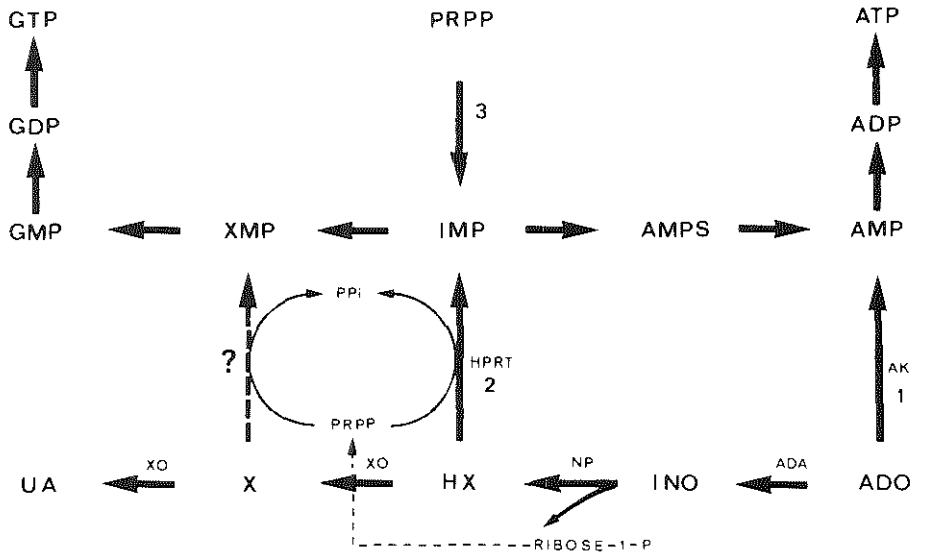

FIG. 1. Biosynthesis of ATP and GTP. *Pathway 1*, adenosine phosphorylation; *pathway 2*, hypoxanthine salvage; *pathway 3*, de novo synthesis. ADA, adenosine deaminase (EC 3.5.4.4); ADO, adenosine; AK, adenosine kinase (EC 2.7.1.20), AMPS, adenylosuccinate; HPRT, hypoxanthine phosphoribosyltransferase (EC 2.4.2.8); INO, inosine; NP, purine-nucleoside phosphorylase (EC 2.4.2.1); PP_i, pyrophosphate; PRPP, phosphoribosyl pyrophosphate; UA, uric acid; XAN, xanthine; XMP, xanthine-5'-monophosphate; XO, xanthine oxidase (EC 1.2.3.2).

was 37.0 ± 0.5°C.

The hearts were equilibrated with the buffer for 20 min (*period 1*: aerobic perfusion). Then global ischemia was induced with a roller pump at a fixed flow of 2.5 ml/min (flow reduction by 65–73%) for 20 min (*period 2*: ischemia), followed by 5 min of reperfusion (*period 3*: reactive hyperemia). Hereafter perfusion took place for an additional 15 min (*period 4*: reperfusion) with 0.02 mM [8-^{14}C]hypoxanthine, 0.02 mM [8-^{14}C]inosine, or 0.02 mM [8-^{14}C]hypoxanthine + 0.5 mM ribose (all dissolved in the perfusion fluid, radioactivity 5 μCi/l). After *period 4*, hearts were perfused with basic perfusion medium for 1 min to wash out the labeled substrates from the blood vessels. In the control group no ischemia was induced during *period 2*.

Chemicals. All chemicals were analytic grade. Water was purified with the Milli-Ro4/Milli-Q system (Millipore, Bedford, MA). [8-^{14}C]hypoxanthine and [8-^{14}C]inosine were supplied by Radiochemical Centre (Amersham, Bucks., UK; sp act 50–60 Ci/mol, diluted to 0.25 Ci/mol before use).

Analytic methods. Samples were collected and stored on ice until analysis. No changes in purine composition of the perfusates were seen within the storage period, which was 12 h at the most. Adenosine(catabolites) were assayed by a slightly modified version of the high-performance liquid chromatography (HPLC) method described by Harmsen et al. (6). A μBondapak C_{18} column (Waters Associates, Milford, MA) was eluted at a flow rate of 3.0 ml/min with 75 mM KH_2PO_4, pH 4.50, to which methanol (30 ml/l) had been added. Two hundred microliters of the coronary effluent were injected on the system. Peaks were integrated (Varian CDS-111, Varian, Palo Alto, CA) and compared with standards. From radioactive samples the various HPLC peaks were collected. Ten milliliters of Instagel (Packard Instruments, Downers Grove, IL) were added, and radioactivity was determined with a Packard Tricarb 2650 liquid scintillation counter.

The perfusion experiment was terminated by freeze clamping the heart between two aluminum blocks precooled in liquid N_2 (−190°C). After being weighed, the heart was ground in a mortar, and about 0.5 g was mixed with 3.0 ml 0.8 N $HClO_4$ at −190°C. The other half was freeze dried to determine dry weight. The acid homogenate was thawed and centrifuged at 4°C. Two milliliters of the supernatant fluid were neutralized with about 200 μl 6 N KOH. After centrifugation, 20 μl of the supernatant fluid were used for the determination of nucleotides and creatine phosphate with HPLC according to Harmsen et al. (8).

To determine ^{14}C radioactivity in the ATP and GTP fractions, a fast, isocratic HPLC separation was developed. A Partisil-10-SAX column (Whatman, Maidstone, UK) was eluted at 3.0 ml/min with 0.42 M KH_2PO_4, pH 4.20. One milliliter of neutralized heart extract was injected. Fractions were collected and counted (Fig. 2). In addition 50 μl of this extract were counted to determine total ^{14}C-radioactivity uptake in the heart. Values were corrected for blanks. ^{14}C-counting efficiency was 85–90%.

Coronary flow measurements. Mean coronary flow was measured by timed collection of perfusate in the periods described above.

Statistical analysis. For statistical evaluation, analysis

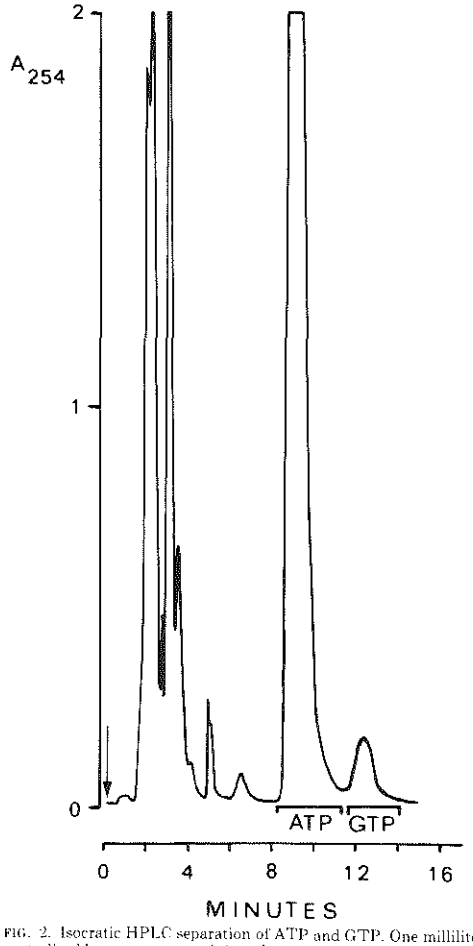

FIG. 2. Isocratic HPLC separation of ATP and GTP. One milliliter of neutralized heart extract was injected on a Partisil-SAX column and eluted with a flow of 3.0 ml/min with 0.42 mM KH_2PO_4, pH 4.20.

of variance was used (2-way classification); further comparisons were made using Scheffé's method for multiple comparisons (19). When only two means were compared, Student's unpaired t test was used. $P > 0.05$ was considered not significant (NS). Results are given as means ± SE. Three treatments (hypoxanthine, inosine, or hypoxanthine + ribose) were tested during two conditions (normoxia or reperfusion). Analysis of variance revealed that one experiment (hypoxanthine + ribose during normoxia), in which the ATP and GTP biosynthesis rates were 4.43 and 0.83 nmol·min^{-1}·g dry wt^{-1}, respectively, contributed the major part of variance. The means ± SD for all experiments ($n = 26$) were 1.06 ± 0.71 and 0.31 ± 0.11 nmol·min^{-1}·g dry wt^{-1}, respectively. According to the rejection rule of Anscombe and Tukey (see Ref. 19) with a premium of 5%, the values of the experiment mentioned earlier were considered as gross errors and rejected. The recalculated biosynthesis rates for ATP and GTP for all experiments were 0.92 ± 0.19 and 0.29 ± 0.06 nmol·min^{-1}·g dry wt^{-1}, respectively ($n = 25$).

RESULTS

In Fig. 3 mean coronary flows during the various periods are presented. During *period 2*, flow was restricted to 13 ml·min^{-1}·g dry wt^{-1} (flow reduction of 80%, $P < 0.001$ vs. control). In the first 5 min of reperfusion the average flow increased to above control level (*level 3*, reactive hyperemia; $P < 0.001$ vs. control), but in *period 4* flows in control and postischemic hearts were comparable. In preliminary experiments we investigated the time dependency of hypoxanthine and inosine release during and after myocardial ischemia (Fig. 4). After induction of ischemia, inosine and hypoxanthine appeared in the coronary effluent. However, within the first 5 min of reperfusion (reactive hyperemia, *period 3*) inosine and hypoxanthine returned to control levels (<0.1 μM).

Total normoxic purine release (i.e., adenosine, inosine, hypoxanthine, xanthine, and uric acid) was about 20 nmol·min^{-1}·g dry wt^{-1} (Fig. 5; *period 2*, control). This purine release was increased about six times during ischemia ($P < 0.001$ vs. control) and nine times during reactive hyperemia ($P < 0.001$ vs. control). During normoxic perfusion 64% of the purines released consisted of uric acid, but during ischemia this percentage fell to 28%. The percentages of the other purine compounds in the perfusate collected during normoxia were (with ischemic values in parentheses) adenosine 5 (11), inosine 17 (38), hypoxanthine 8 (13), and xanthine 5 (9), respectively.

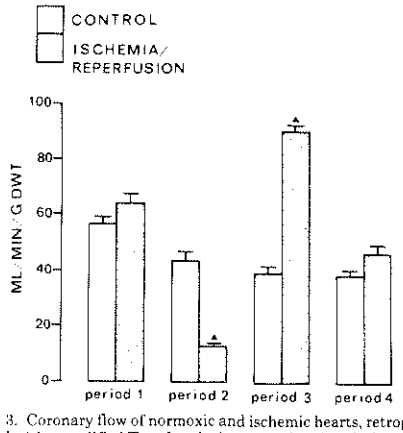

FIG. 3. Coronary flow of normoxic and ischemic hearts, retrogradely perfused with a modified Tyrode solution, gassed with 95% O_2–5% CO_2, at a perfusion pressure of 72 mmHg and a heart rate of 300 beats/min. Ischemia was introduced with a roller pump at a fixed flow of 2.5 ml/min. *Period 1*, aerobic perfusion (0–20 min); *period 2*, ischemic period (20–40 min); *period 3*, reactive hyperemia (40–45 min); and *period 4*, reperfusion (45–60 min). In control hearts no flow reduction was used. Values are means ± SE ($n = 4$–6). ▲, $P < 0.001$ vs. control (t statistics).

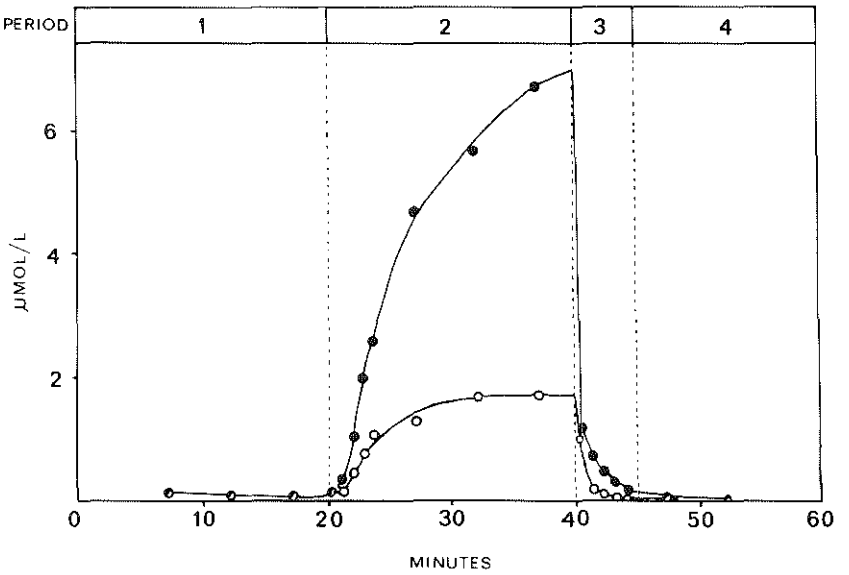

FIG. 4. Myocardial release of hypoxanthine (○—○) and inosine (●—●). Both compounds were determined in effluent with HPLC (see METHODS). *Periods 1–4* are defined in legend to Fig. 3. Average of at least 3 experiments.

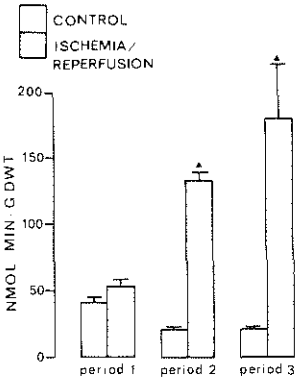

FIG. 5. Total purine release (adenosine + inosine + hypoxanthine + xanthine + uric acid) from isolated rat hearts. *Periods 1–3* are defined in legend of Fig. 3. Values are means ± SE (n = 4–6). ▲, $P < 0.001$ vs. control (t statistics).

TABLE 1. *Uptake and incorporation rates of hypoxanthine and inosine*

Addition	Total Uptake	ATP	GTP
		Normoxia	
Hypoxanthine	84 ± 12 (100%)	6.4 ± 0.9 (8%)	2.0 ± 0.4 (2%)
Hypoxanthine + ribose	93 ± 21 (100%)	16.0 ± 1.3 (18%)	5.3 ± 0.4 (7%)
Inosine	107 ± 8 (100%)	7.3 ± 1.3 (7%)	2.9 ± 0.4 (3%)
		Reperfusion	
Hypoxanthine	96 ± 7 (100%)	14.6 ± 0.5 (15%)	3.9 ± 0.3 (4%)
Hypoxanthine + ribose	91 ± 14 (100%)	22.3 ± 1.3 (26%)	7.2 ± 0.7 (9%)
Inosine	123 ± 14 (100%)	16.3 ± 2.5 (14%)	4.9 ± 0.6 (4%)

Values are means ± SE in nmol·15 min^{-1}·g dry wt^{-1} (n = 3–6). Figures in parentheses are percents of total ^{14}C-radiolabel uptake by tissue. [8-^{14}C]hypoxanthine or [8-^{14}C]inosine conversion to ATP and GTP and uptake of radioactivity were determined in acid extracts of freeze-clamped hearts. For statistical analysis, see text.

In Table 1, the biosynthesis of ATP and GTP from 0.02 mM hypoxanthine or inosine is presented. Analysis of variance for hypoxanthine and inosine incorporation rates in the ATP fraction showed no significant interaction (P = 0.65) between the conditions normoxia and reperfusion on the one hand and the three treatments (infusion of hypoxanthine, of hypoxanthine + ribose, and of inosine) on the other hand. Thus the pattern for the three treatments was similar for normoxia compared with reperfusion. Incorporation rates during reperfusion were significantly ($P < 0.0001$) increased compared with the normoxic controls. Comparison of the three treatments proved them to be statistically different ($P < 0.0001$). In addition, by Scheffé's method of multiple comparison the incorporation rates in the group treated with hypoxanthine + ribose were found to be different from the groups treated with hypoxanthine or inosine, the latter two not being different. Also the GTP incorporation rates showed no significant interaction (P = 0.97) between normoxia/reperfusion and the three treatments. The significant differences paralleled those of the ATP incorporation rates.

Besides incorporation into nucleotides, the other met-

TABLE 2. *Metabolic fate of [8-^{14}C]hypoxanthine and [8-^{14}C]inosine*

Addition	Percent of Label Infused					
	Myocardial uptake	Adenosine	Inosine	Hypoxanthine	Xanthine	Uric acid
			Normoxia			
Hypoxanthine	0.9 ±0.2	0	0	82.7 ±0.7	5.5 ±0.2	2.7 ±0.6
Hypoxanthine + ribose	0.9 ±0.1	0	0.5 ±0.2	83.8 ±2.9	5.7 ±0.8	4.5 ±0.4
Inosine	1.4 ±0.1	0	81.1 ±2.9	3.8 ±0.2	2.4 ±0.2	7.3 ±0.1
			Reperfusion			
Hypoxanthine	0.8 ±0.1	0	0.2 ±0.1	83.1 ±0.6	6.1 ±0.2	2.6 ±0.5
Hypoxanthine + ribose	0.9 ±0.1	0	0.5 ±0.2	83.2 ±2.2	7.0 ±1.1	4.7 ±0.2
Inosine	1.5 ±0.1	0	82.4 ±1.4	3.8 ±0.2	2.4 ±0.2	7.1 ±0.1

Values (means ± SE) represent radioactivity recovered in various high-pressure liquid chromatography fractions of perfusate. Uptake is calculated from ^{14}C-radiolabel measured in extracts of freeze-clamped hearts. Data are expressed as percent of [8-^{14}C]hypoxanthine or [8-^{14}C]inosine offered to hearts (n = 4–6). For statistical analysis, see text.

abolic fate of inosine and hypoxanthine was conversion to xanthine and uric acid. We determined [8-^{14}C]inosine or hypoxanthine conversion in the coronary effluent (Table 2). About 80% of both purines passed the heart unchanged and 10–15% were metabolized during a single passage through the coronary vasculature. About 0.8% of hypoxanthine was taken up by the heart, whether or not ribose was added. On the other hand, [8-^{14}C]inosine uptake was about 1.5%, which is twice the hypoxanthine uptake ($P < 0.001$). Incorporation rates were comparable for hypoxanthine and inosine. During reperfusion, the uptake figures for these purines were comparable to the control values. In the statistical analysis of the uric acid data neither the interaction nor the reperfusion versus normoxic data were statistically significant ($P = 0.76$; $P = 0.60$, respectively). The three treatments, however, were significantly different ($P < 0.0001$); by Scheffé's method of multiple comparison a significant difference ($P < 0.001$) existed between the group treated with inosine and the groups perfused with hypoxanthine in the presence or absence of ribose. Furthermore, perfusion with a mixture of ribose and hypoxanthine increased the uric acid release significantly ($P < 0.05$) compared with hypoxanthine infusion.

Table 3 shows the data on energy-rich phosphates determined in freeze-clamped hearts. During ischemia, total adenine nucleotides (TAN), ATP, and creatine phosphate (CrP) fell (20, 30, and 50%, respectively). Two-way analysis of variance showed a borderline significant interaction between normoxic and reperfused hearts with respect to TAN ($P = 0.13$) and ATP ($P = 0.06$), i.e., the decrease of TAN and ATP due to ischemia and reperfusion disappeared when 0.02 mM inosine was added to the reperfusion buffer. CrP during reperfusion showed an overshoot in all groups studied ($P < 0.001$, interaction 0.71).

DISCUSSION

Theoretically nucleotide synthesis rates from radiolabeled purines should be calculated on the basis of specific activity of the intracellular purine compounds. However, we were unable to measure intracellular hypoxanthine and inosine concentrations. To overcome this problem, we assumed that the hypoxanthine and inosine concentrations in the heart cell were reflected by the concentrations in the coronary effluent. As is shown in Fig. 4, 5 min after ischemia inosine and hypoxanthine concentrations returned to normal values (<0.1 μM). Therefore we concluded that within 5 min after ischemia, inosine and hypoxanthine concentrations in the heart cell had also returned to control values, which are low in heart (<1 μM, see, e.g., Ref. 20). To minimize isotopic dilution, 0.02 mM [8-^{14}C]hypoxanthine or -inosine were used.

Hypoxanthine and inosine incorporation rates were about 0.4 nmol·min^{-1}·g dry wt^{-1} into the ATP and 0.1 into the GTP pool (8 and 2% of the ^{14}C radioactivity accumulated in the heart, respectively; see Table 1). In earlier experiments we showed that about 75% of [U-^{14}C]inosine (also labeled in the ribose ring) recovered in nucleotides was incorporated into the combined ATP and GTP pool both in normoxic and in reperfused hearts (7). Therefore we estimated that the total hypoxanthine and inosine incorporation into adenine and guanine nucleotides amounts to about 0.7 nmol·min^{-1}·g dry wt^{-1} during aerobic perfusion. After ischemia a twofold increase in the incorporation rates of these purines into ATP and GTP is seen. The total amount of radioactivity taken up by the heart is not changed, however. Thus the percentage of hypoxanthine and inosine in heart, con-

TABLE 3. *Concentrations of high-energy phosphates in freeze-clamped rat hearts*

Addition	TAN	ATP	ADP	AMP	GTP	CrP
			Normoxia			
None	28.2 ±1.0	22.9 ±1.2	4.8 ±0.3	0.5 ±0.2	0.9 ±0.1	27.5 ±2.8
Hypoxanthine	27.4 ±1.7	22.1 ±1.2	4.9 ±0.6	0.3 ±0.1	1.0 ±0.3	33.0 ±2.6
Hypoxanthine + ribose	28.6 ±1.6	22.9 ±1.3	5.3 ±0.4	0.5 ±0.1	0.9 ±0.1	38.1 ±3.8
Inosine	26.3 ±2.1	21.1 ±1.6	4.6 ±0.6	0.7 ±0.2	0.9 ±0.1	22.3 ±1.5
			Ischemia			
None	23.0 ±2.1	15.8 ±2.8	5.8 ±1.1	1.4 ±0.6	0.7 ±0.2	13.4 ±1.9
			Reperfusion			
None	21.5 ±1.3	17.6 ±1.2	3.4 ±0.3	0.5 ±0.2	0.7 ±0.2	44.9 ±7.5
Hypoxanthine	23.7 ±1.0	19.6 ±1.0	3.8 ±0.1	0.3 ±0.1	0.8 ±0.1	44.3 ±3.7
Hypoxanthine + ribose	24.5 ±2.0	19.5 ±2.1	4.5 ±0.3	0.5 ±0.1	0.7 ±0.1	44.7 ±9.2
Inosine	26.7 ±1.2	22.3 ±0.9	4.0 ±0.2	0.4 ±0.1	0.9 ±0.1	37.5 ±1.7

Values are means ± SE (n = 3–6) in μmol/g dry wt. Hearts were perfused as described in legend to Fig. 3. Ischemia refers to hearts preperfused for 20 min, with an additional 20 min of ischemia. For statistical analysis, see text. TAN, total adenine nucleotides.

verted to ATP and GTP, is increased. The rest of the ^{14}C label is found in the fraction containing the nucleosides and oxypurines (see Ref. 8). We have not measured myocardial nucleosides and oxypurines, but we assume that more than 80% of the myocardial purine fraction consists of hypoxanthine (during hypoxanthine infusion) or inosine (during inosine infusion), as reflected by the perfusate concentration (see Table 2). If hypoxanthine and inosine were evenly distributed over the myocardial cells and extracellular space, intracellular hypoxanthine or inosine concentrations are estimated to be minimally 15 μM. These concentrations are in the same order of magnitude as found in the perfusate. Therefore ATP and GTP biosynthesis is not limited by purine transport but is regulated intracellularly. According to Zimmer et al. (24), the NADP-to-NADPH ratio decreases during and after ischemia, and the hexose-monophosphate shunt is accelerated. As a result of this process, ribose-5-phosphate levels are increased, which in turn increases the PRPP concentration. Because hypoxanthine phosphorylation is PRPP dependent, this could give a reasonable explanation for increased salvage rates after ischemia. It is possible to bypass the hexose-monophosphate shunt by supplying ribose as a precursor for PRPP (25). Indeed after ribose infusion, a significant increase in salvage rates is seen, which is further enhanced after ischemia.

Namm (14) found that during normoxic perfusion 0.01 mM inosine or hypoxanthine is incorporated into isolated rat hearts at a rate of about 3 nmol·min^{-1}·g dry wt^{-1}. Using myocardial homogenates of the same species, Maguire et al. (13) measured hypoxanthine phosphoribosyltransferase (HPRT) activities with a maximal initial velocity (V_{max}) of about 40 μmol·min^{-1}·g dry wt^{-1}. This is about 10,000 times higher than the salvage rates reported by Namm (14) or found in our experiments. Although an in vitro enzyme activity measurement can hardly be compared with in vivo activity, it seems that the hypoxanthine incorporation is not limited by the potentially available HPRT activity. Raivio et al. (15) have found a striking correlation between PRPP synthesis and cellular phosphate concentration in human fibroblasts. Myocardial PRPP synthesis could be regulated by phosphate concentration as well.

During hypoxanthine perfusion (with or without ribose) after ischemia, a slight (NS) increase of TAN is seen. During postischemic inosine infusion, however, TAN and ATP are about 5 μmol/g dry wt higher. ATP biosynthesis from [8-^{14}C]inosine during reperfusion is 1.1 nmol·min^{-1}·g dry wt^{-1} (Table 1), so TAN synthesis will be about 1.5. This is insufficient by far to account for the observed total restoration. There are two other known pathways that could account for ATP biosynthesis, namely adenosine phosphorylation and de novo synthesis. Adenosine phosphorylation accounts maximally for 50 nmol·min^{-1}·g dry wt^{-1} (14, 16). However, the adenosine concentration in the perfusate at the end of the ischemic period is about 1.2 μmol/l and is lowered within 5 min of reperfusion to 0.04 μmol/l. Therefore, under these experimental conditions, adenosine phosphorylation will be low because of lack of substrate. De novo synthesis in isolated rat hearts is 0.1 nmol·min^{-1}·g dry wt^{-1} (23). This synthesis rate is PRPP dependent and is accelerated after ischemia. Whether de novo synthesis is stimulated by inosine is unknown at this moment, but it seems unrealistic to assume that the restoration of TAN and ATP is due to de novo synthesis. Bates et al. (1) postulated the existence of a pool of nonacid-extractable ATP, which under certain conditions can be converted to acid-extractable ATP. It remains to be seen whether this explanation is applicable to our results.

During reperfusion an overshoot of creatine phosphate is seen (Table 3). In the model of Saks et al. (17) CrP serves as an "energy carrier" from mitochondria to sites of utilization. In this scheme it is possible for the mitochondria to start producing energy after mild ischemia, without direct utilization. For that reason, no CrP overshoot would be expected after severe ischemia, when the mitochondria are damaged (see Ref. 18).

To our knowledge, this is the first report describing purine incorporation into the myocardial GTP pool, although Swain et al. (20) recently reported restoration of GTP levels by de novo synthesis in dog heart. It is interesting to see that the purine incorporation rate into GTP is about 25% of that of ATP, although the GTP content in heart is only about 5% of the ATP content. This indicates a faster recovery of GTP after ischemia.

Our final conclusions are that hypoxanthine and inosine are incorporated in both the ATP and GTP pool in the heart. This process is stimulated after ischemia and by ribose perfusion and is thereby dependent on myocardial PRPP concentrations. Inosine especially seems to restore ATP levels, and this could (partly) explain its beneficial action.

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