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APPEARANCE OF ADENOSINE TRIPHOSPHATE IN THE CORONARY SINUS EFFLUENT FROM ISOLATED WORKING RAT HEART IN RESPONSE TO HYPOXIA

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SUMMARY

1. A working rat heart preparation was used to study the release of adenosine-5′-triphosphate (ATP) into the coronary sinus effluent in response to hypoxia.

2. The left ventricle was set to pump against an hydrostatic pressure of 65 cm water; the left atrial filling pressure was kept constant at 10 cm water. The power output of the heart at these pressures was estimated to be approximately one half of the maximum power development.

3. Samples for ATP assay were collected (*a*) 30 sec before onset of hypoxia, (*b*) 60–90 sec after onset of hypoxia, (*c*) 5 min after restoration of oxygenated buffer solution. Respective concentrations of ATP were (nM ± S.E.) 0·63 (±0·18), 4·70 (±0·39) and 0·63 (±0·06). The total amounts of ATP detected were (p-mole/min) 5·9 (±0·9), 46·1 (±6·0) and 5·5 (±1·2) respectively.

4. Viability of the hearts was judged to be satisfactory on the following grounds. Alterations in left atrial filling pressure produced typical Frank-Starling responses of the left ventricle. Oxygen extraction from the perfusate increased in response to increased workload. Coronary blood flow increased immediately upon introduction of hypoxic conditions and mechanical recovery from hypoxia was always complete within 5 min of restoring oxygen.

5. In view of the marked extracellular ATPase activity it is concluded that significant vasodilatory concentrations of ATP are released into the myocardial extracellular space in response to hypoxia. A scheme is proposed describing the possible role of adenine nucleotides in the local control of myocardial blood flow.

INTRODUCTION

Adenosine-5′-triphosphate (ATP) has long been known as a powerful dilator of coronary arteries (Fleisch & Weger, 1937; Folkow, 1949). It is 4 times more potent in this respect than adenosine monophosphate and adenosine (Winbury, Papierski, Hemmer & Hambourger, 1953; Wolf & Berne, 1956). Much evidence has accumulated

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to indicate that ATP is released from electrically active frog skeletal muscle (Abood, Koketsu & Miyamoto, 1962; Boyd & Forrester, 1968), human skeletal muscle (Forrester & Lind, 1969; Forrester, 1972; Parkinson, 1973) and nervous tissue (Holton, 1959; Abood *et al.* 1962; Kuperman, Volpert & Okamoto, 1964; Pull & McIlwain, 1972; Burnstock, 1972). A particularly clear demonstration of ATP release from (or through) an electrically excited membrane has been provided by Israel, Lesbats, Meunier & Stinnakre (1976). They monitored the post-synaptic release of ATP from electric organ of *Torpedo* directly, using the sensitive luciferin-luciferase assay technique. In a similar way the release of ATP from brain synaptosomes depolarized by elevated extrasynaptosomal potassium or veratridine has been directly measured (White, 1977). Some indirect evidence has led to the suggestion that ATP may cross the cardiac cell membrane (Fedelešová, Ziegelhöffer, Krause & Wollenberger, 1969; Ziegelhöffer, Fedelešová & Šiška, 1971) and also skeletal muscle membrane (Chaudry & Gould, 1970) as the intact molecule.

Paddle & Burnstock (1974) have demonstrated the presence of ATP in the coronary sinus effluent from isolated guinea-pig hearts in response to hypoxia. A similar finding was noted by Stowe, Sullivan, Dabney, Scott & Haddy (1974). These hearts were perfused in the Langendorff mode and hence the amount of work performed by the musculature was very low and difficult to assess. The source of ATP was undetermined, but Paddle & Burnstock discussed the possibility that 'purinergic' nerves might give rise to such concentrations in the coronary sinus effluent. Release of ATP from isolated heart cells in response to very brief periods of hypoxia has been recently measured (Forrester & Williams, 1977), indicating that one source of ATP was the hypoxic myocardial cell.

The purpose of the present experiments was to test the coronary effluent for ATP from a *working* heart preparation. Such a preparation was developed by Neely, Liebermeister, Battersby & Morgan (1967) and is particularly suited for the study of myocardium beating against a normal load. Perfusion of the coronary vascular bed also becomes similar to the situation *in vivo*. The work of the heart can be readily calculated and the metabolic state can be assessed by measurement of the oxygen extraction by the myocardium.

A preliminary account of some of these results has already appeared (Clemens & Forrester, 1979).

METHODS

ATP assay. The ATP concentration of the coronary sinus effluent was determined by the firefly luminescence technique described by Strehler & McElroy (1957). Two modifications of this procedure were used in different series of experiments. In all Ca^{2+} paradox experiments the assay was carried out as modified by Forrester (1972). In the experiments dealing with hypoxia the procedure described by Silinsky (1974) was adopted in order to assay very low concentrations and also to allow for any alterations of fluid composition in its intravascular passage through the myocardium. Silinsky showed that this method gives reproducible results despite differences in firefly enzyme sensitivity and levels of inhibition by buffer constituents. The firefly luminescence was continuously monitored before, during and 15 sec after addition of the sinus effluent sample by automatic pipette; this makes it unnecessary to assume that the magnitude of the background signal is a constant. Aliquots of 1 ml. of coronary sinus effluent were removed from the collecting tube and placed in a chilled microfuge tube kept on ice. Samples were stored at 0 °C until assayed for ATP. Several vials of firefly lantern extract (Sigma FLE 50) were each reconstituted with 5 ml. deionized distilled water; all vials were pooled to give an homogeneous suspension of extract.

Throughout the duration of assays the pooled suspensions were kept on ice to prevent any loss of activity: 0·4 ml. of extract was pipetted into quartz microcuvettes which were placed in a cuvette carrier and then into the light-tight sample housing of the photomultiplier (P.M.) tube. After positioning the cuvette in front of the tube windows and rendering the housing light-tight, 1400–1600 V DC were delivered across the P.M. tube. The background light signal was recorded. 0.25 ml. of sinus effluent was then added to the firefly extract through an injection port in the lid of the housing. This results in an immediate fall in light signal intensity, presumably caused by

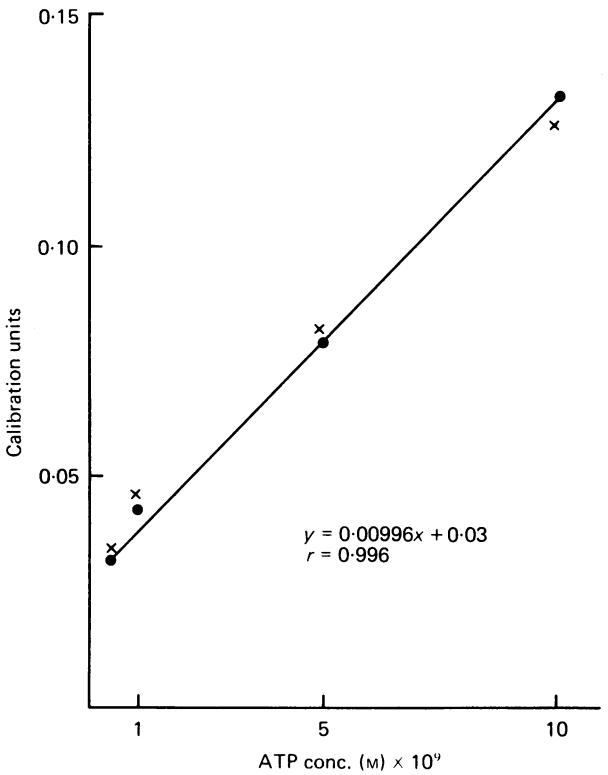

Fig. 1. Dose–response relationship for ATP using firefly assay. (●), values before assay of test solutions; (×), values after assay procedure (about 4 hr): Calibration units = $(S_{10}-S_i)/S_B$, where S_{10} = height of signal 10 sec after injection of sample, S_i = height of inhibited signal and S_B = height of background signal. Abscissa scale shows range between 10^{-9} and 10^{-8} M.

some inhibition of the luciferin-luciferase reaction. Then follows a rise in light signal caused by the ATP-dependent light production (see Silinsky, 1974, Fig. 2). The actual signal intensity was obtained by subtracting the level of inhibition immediately following sample injection from the light emission signal 10 sec after injection. If any variation in background signal occurred during an assay run, all values were standardized by dividing by the intensity (height) of the background signal. Calibration standards with ATP concentrations of 0·5, 1·0, 5·0 and 10 nM were run at the beginning and end of each assaying session. Sample concentrations were determined from a standard dose–response curve (Fig. 1). Concentrations down to 5×10^{-10} M can be reliably detected by this method.

Working heart preparation. A modification of that described by Neely *et al.* (1967) was used. This model differs from the Langendorff perfused heart in that the left ventricle is required to perform work against a controlled afterload (Fig. 2). The left atrial filling pressure is set by the height of

an overflow chamber. Fluid is hydrostatically propelled into the atrium and passes through the opening of the mitral valve, resulting in left ventricular diastolic filling and subsequent systolic ejection against a column of fluid set at a pressure determined by the height of the upper overflow chamber. The apparatus contained parallel overflow chambers and perfusion lines on both the atrial and aortic sides (not shown in Fig. 2). The atrial and aortic perfusion systems could be rapidly changed by stopcocks situated close to the heart. Both atrial overflow chambers and one aortic chamber were filled by a peristaltic pump from reservoirs kept in a water-bath set at 38 °C. The overflow from each chamber was returned to its respective perfusate reservoir. The overflow from the aortic chamber could be collected to quantify aortic output. Glass microfibre fillers with an effective retention of 1·6 μm were placed in each perfusion system to remove any particles (i.e. $CaCO_3$ precipitate) that might compromise coronary flow. In the Ca^{2+} paradox experiments the pulmonary artery was cut and the coronary sinus effluent allowed to run down the epicardial surface of the heart for sample collection. In the hypoxia experiments and in all experiments in which O_2 consumption was measured, the pulmonary artery was cannulated to avoid contamination by damaged tissue or room air. The cannula was secured directly over a fraction collector for collection of coronary sinus effluent.

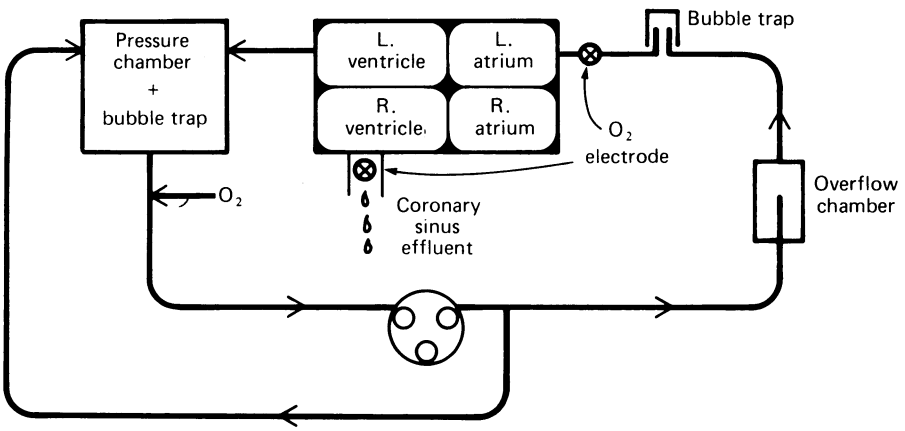

Fig. 2. Simplified scheme of working heart preparation modified after Neely *et al.* (1967). Left ventricular afterload set by height of overflow chamber. Coronary sinus effluent collected from cannulated pulmonary trunk. O_2 electrodes (⊗) placed in cannulae to left atrium and from pulmonary trunk. O_2 delivered to perfusate just below pressure chamber + bubble trap.

The perfusion solution used for all experiments was a modified Krebs-Henseleit bicarbonate buffer of the following composition (mM): NaCl, 118; KCl, 4·7; $MgSO_4$, 1·2; K_2HPO_4, 1·2; $CaCl_2$, 2·5; $NaHCO_3$, 25; glucose, 11·1. The buffer was equilibrated with 95 % O_2:5 % CO_2 and the pH adjusted to 7·4 ± 0·01 with 1 N-NaOH.

Measurement of left ventricular work. The mechanical work performed by the left ventricle was determined from the *cardiac output* and *aortic pressure*. The cardiac output was measured by collecting the output from the aortic overflow chamber in a graduated cylinder for 60 sec intervals. To this amount was added the coronary flow to give total cardiac output. Aortic pressure was monitored with a Statham pressure transducer displayed on a Grass polygraph. Heart rates were determined by visual counting of pulse pressure waves at a rapid chart speed. Since preload and afterload were held constant, aortic pulse pressure was normally used as an indication of left ventricular 'contractility'. In some experiments where heart rate altered, a pressure-rate index (Kobayashi & Neely, 1979) was used to eliminate error from the Frank-Starling effect of increased diastolic filling time during bradycardia. Left ventricular rate of work production (power) was calculated using the formulae of Kannengiesser, Opie & Van der Werff (1979). This method computes *pressure* power and *kinetic* power by the following equations:

$$\text{pressure power} = 0{\cdot}002222 \times P_S \times CO,$$

$$\text{kinetic power} = \frac{1}{432 \times 10^7} \times \frac{\rho CO^3}{A^2} \times \frac{T}{T_E},$$

where P_S = peak systolic pressure, CO = cardiac output, ρ = density (1 gm/ml.), A = cross-sectional area of aortic cannula, T = cardiac cycle time and T_E = ejection time.

Measurement of coronary flow. The output of the right ventricle was taken as an indication of approximately 95 % of the total coronary artery flow. It does not take into account that portion of coronary flow draining directly into the left ventricle via Thebesian veins. This was collected from a cannula securely placed in the cut end of the pulmonary artery in timed fractions with a Gilson escargot type fraction collector.

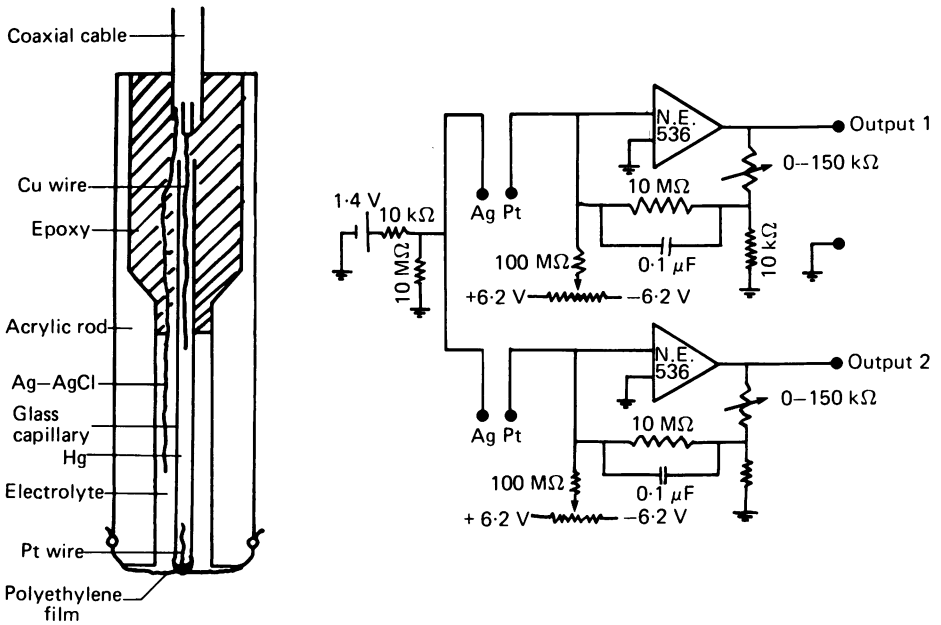

Fig. 3. Construction and circuitry of O_2 electrode (see text).

Oxygen electrode construction. A modification of the method of Clark (1956) and Staub (1961) was used. The housing contained both the platinum cathode and the Ag/AgCl reference anode (Fig. 3). Approximately 1 cm of 25 μm diameter Pt wire (Medwire Inc.) was stripped of its Teflon coating and placed in the end of a glass capillary tube. The Pt wire tip was then sealed into a glass bead by heating in a burner flame. The capillary tube was then filled with mercury and a fine copper wire then inserted, making electrical contact with the platinum wire through the mercury. This copper wire was then soldered to the centre strand of a coaxial cable. Approximately 5 cm Ag wire which had previously been coated with AgCl by electrolysis was then soldered to the outer sheath of the coaxial cable. This whole assembly was then inserted into the milled core of an acrylic rod so that the glass bead containing the platinum wire protruded about 1 mm beyond the tip of the rod. The base portion of the rod was then filled with acrylic softened with ethylene dichloride. Extreme caution was taken to ensure no electrical communication between the central and outer strands of cable. The glass bead portion was then gently abraded on no. 400 emery paper wetted with a 3 M solution of KCl until the resistance between the central strand and any probe placed in the KCl solution was no longer infinite. The cavity in the tip of the acrylic rod was then filled with saturated KCl solution and the tip covered with polyethylene film (Glad Wrap-Union Carbide). With the electrodes filled with electrolyte, a resistance between the strands of cable of about 60 kΩ indicated intact electrical connexion between the Pt cathode and the Ag/AgCl anode, with no low

resistance short circuits in the base of the electrode. Only the O_2-sensitive tip of the electrode came in contact with the perfusion fluid which flowed past the electrode tip. The output of the electrode was amplified by a two-channel operational amplifier current-to-voltage circuit similar to that described by LaForce (1967). It consisted of a field effect transistor operational amplifier (input impedance 10^{12} Ω) with zero set by a 'bucking' current at the input, and the output set by a variable resistance in the feed-back loop. The cathode is polarized to −0·7 V by a 1·4 V H_g cell and a voltage divider circuit (Fig. 3). The amplifier circuit produced an output of 0·2–0·8 V for 95 % O_2 saturated with water vapour at 37 °C.

Measurement of O_2 consumption. The consumption of O_2 by the working heart was determined from the difference in p_{O_2} between buffer entering the heart (arterial perfusion line) and that of the coronary sinus effluent. O_2 tension was constantly monitored by the Clark-type platinum O_2 cathode described above. This was mounted in a flow-through chamber kept at 38 °C with a water jacket. The current resulting from the reduction of O_2 at the cathode was amplified and converted to a voltage signal by the laboratory constructed high impedance (10^{12} Ω) electrometer amplifier (Fig. 3). The amplifier output was calibrated with standard gases and recorded using a potentiometric recorder (Fisher Recordall). $\dot{V}_{O_2}$ was calculated by multiplying the difference between inflow p_{O_2} (arterial) and coronary sinus p_{O_2} (venous) by the solubility of O_2 in water at 37 °C ($2{\cdot}7 \times 10^{-5}$ ml. O_2 . ml. H_2O^{-1} . $mmHg^{-1}$). This gives the total volume of O_2 consumed per ml. coronary flow. Total oxygen consumed per minute is obtained by multiplying by the coronary flow per minute. $\dot{V}_{O_2}$ for each heart was expressed as ml. min^{-1} . 100 g wet wt^{-1}.

Setting up the heart preparation. Sprague-Dawley rats (150–300 g, either sex) were given 500 u. Na heparin I.P. 15–30 min before sacrifice. The rats were anaesthetized with Na pentobarbitone (0·4–1·0 ml., depending on size of rat and body fat content). As soon as the rat no longer showed an abdominal stretch reflex, a skin incision was made and the heart was exposed by cutting out a sternal flap. The heart was lifted manually and freed with one scissor cut. This freed portion consisted of both atria and ventricles plus thymus and portions of lung tissue. The heart was immediately dropped into a beaker of ice-chilled Krebs-Henseleit bicarbonate buffer solution. Time from initial thoracotomy incision to placing heart in cold buffer was usually 10–15 sec. After the heart had cooled and contractions ceased, it was then cleaned from excess tissue and the aortic and pulmonary stumps identified and isolated. The aorta was cannulated (polyethylene flared cannula) with a ligature placed around the aorta proximal to the origin of the brachiocephalic artery and perfusion of the coronary artery bed begun immediately at 37 °C (Langendorff mode) from the overflow chamber set 65 cm above the heart. The chamber was supplied with buffer by a peristaltic pump. Once the heart resumed spontaneous beating at a stable rate it was prepared for atrial cannulation. After further cleaning, the inferior vena cava and the boundary between left and right atrium were identified to avoid damage to the right atrium. A small hole in the left atrium was produced by cutting the junction between one of the pulmonary veins and the left atrium. The left atrial cannula was then inserted. It was usually necessary to rotate the aorta on its cannula to position the left atrium to receive the cannula. This prevented excessive torsion on the aorta which might obstruct the openings of the coronary arteries. The atrium was then secured to the cannula with several ligatures which included any remaining lung tissue and associated pulmonary veins. Competency of the cannulation was verified by opening the stopcock in the atrial perfusion line and observing distension of the left auricular appendage and a sudden increase in aortic pulse pressure. When the pulmonary artery was cannulated competence of the left atrial cannulation was further verified by the absence of perfusate leaking from the heart. Each heart was allowed to beat for at least 15 min in the non-working mode before left atrial filling pressure was set at 10 cm H_2O.

RESULTS

When the perfusate of an isolated working rat heart was changed from an oxygenated buffer to one made hypoxic by bubbling with 95 % N_2 : 5 % CO_2, there was a typical rapid decline of aortic pulse pressure and often a bradycardia (Fig. 4). Occasionally there was a complete loss of pulse pressure. In all cases cardiac output was severely compromised to the extent that the aortic overflow chamber had to be supplemented by a parallel reservoir to maintain a constant coronary perfusion

pressure. Upon reversion to an oxygenated perfusate after a 90 sec period, the mechanical activity steadily improved to the control level. This was the case even in hearts that had completely lost the aortic pulse during the hypoxic period.

Samples of coronary sinus effluent for ATP assay were taken (1) in the 30 sec control period before the onset of hypoxic perfusion, (2) 60–90 sec after the onset of hypoxia, and (3) 5 min after the restoration of oxygenated buffer (Fig. 4*A*). In four

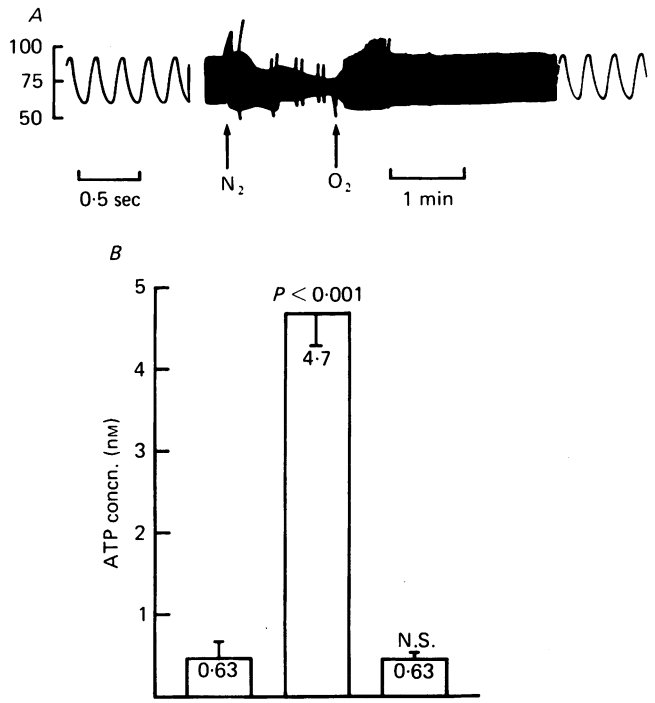

Fig. 4. *A*, recording of aortic pulse pressure from a working heart preparation exposed to hypoxia for a period of 90 sec. Samples for ATP assay collected 30 sec before hypoxia, 60–90 sec after onset of hypoxia and 5 min after restoration of oxygenated buffer solution. Note change in time scale. Vertical scale, mmHg *B*, concentrations (±S.E.) of ATP in the coronary sinus effluent from four hearts. *P* value indicates significance from control and recovery samples. N.S., not significantly different from control (one way analysis of variance test used).

hearts, ATP was detected in the coronary effluent during the 30 sec before the onset of the hypoxic period. A mean concentration of 0·63 (±S.E. 0·18) nM-ATP was detected in the coronary effluent during the period of 30 sec before the onset of hypoxia; samples collected during the last 30 sec of the hypoxic period contained ATP, 4·7 (±S.E. 0·39) nM. Restoration of oxygenated buffer after the 90 sec hypoxia resulted in a decline in ATP concentration and by 5 min the level of ATP had returned to control levels of 0·63 (±S.E. 0·06) nM (Fig. 4*B*).

Total amount of ATP released. The total amount of ATP released into the coronary sinus effluent is equal to the concentration of ATP multiplied by the coronary sinus flow. The coronary flow was measured throughout the periods of sampling and hypoxia and Fig. 5 shows the increase in flow produced during hypoxic perfusion.

In eight hearts the mean coronary flow was significantly increased during hypoxia ($P < 0.05$). In two hearts the coronary flow during hypoxia actually decreased. However, in those cases the aortic pulse pressure became unrecordable; there was failure of ventricular emptying and distension of both ventricles was evident. It is likely that this resulted in a combination of increased intramural pressure from ventricular distension, and increased coronary venous pressure from the deficiency in right ventricular emptying. In four hearts, the total amounts of ATP before, during and after the hypoxic period were (p-mole/min): 5·9 (±S.E. 0·9), 46·1 (±S.E. 6·0) and 5·5 (±S.E. 1·2), respectively.

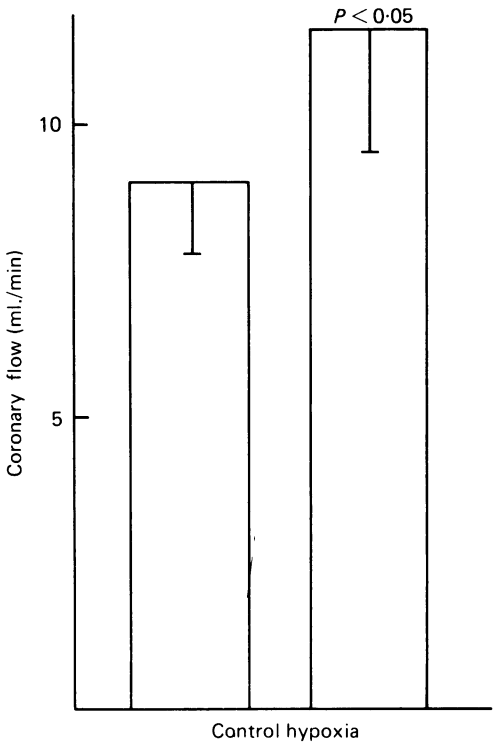

Fig. 5. Coronary flow (±S.E.) of eight working heart preparations during oxygenated and hypoxic periods. Significantly greater flow in hypoxia ($P < 0.05$, Student paired t test).

It is noteworthy that in four preliminary experiments in which the pulmonary artery was not cannulated, but rather the coronary effluent was allowed to drip from the heart through the cut pulmonary trunk, the ATP contents during control, hypoxia, and recovery periods were (p-mole/min) 26·8 (±S.E. 6·6), 63·6 (±S.E. 10·3) and 25·3 (±S.E. 10·0), respectively. This resulted in an increased total loss of ATP; nevertheless, the *difference* in ATP content between control and hypoxic coronary effluent samples is virtually the same for both sets of experiments (40·2 p-mole/min *vs.* 42·9 p-mole/min for uncannulated).

Assessment of heart viability

While the correlation between the rise of ATP concentration in the sinus effluent and the development of hypoxia seems clear-cut, nevertheless the question of cellular damage, and hence leakage of ATP through incompetent membranes, must be addressed. All hearts used in this study fully regained control levels of aortic pulse pressure, but this cannot discount the possibility of membrane damage during the hypoxic period. It may simply indicate that the damage was *reversible* and that membrane reconstitution was dependent upon a renewed supply of O_2.

Various experiments were carried out which together indicate that these heart preparations were at an acceptable level of viability when compared to those of other workers.

TABLE 1. Response to incremental change in left atrial pressure

Preload (cm H_2O)	S_p (mmHg)	CO (ml./min)	T_c/T_e	W_P	W_K	Power (mW)
10	88 (82)	19·2 (22·3)	1·86 (2·2)	3·75 (4·06)	0·0116 (0·025)	3·76 (4·09)
15	90 (87)	27·8 (30·6)	2·40 (2·2)	5·56 (5·92)	0·059 (0·066)	5·62 (5·98)
20	92 (90)	37·1 (39·3)	2·40 (2·2)	7·58 (7·86)	0·14 (0·139)	7·72 (8·00)
10	85 (85)	19·8 (22·0)	3·99 (2·2)	3·74 (4·16)	0·033 (0·024)	3·77 (4·18)

Second heart results in parentheses. Diameter of aortic cannula = 1·68 mm.
S_p = peak systolic pressure; CO = cardiac output; T_c/T_e = ratio of cardiac cycle time to ejection time; W_p = pressure power; W_k = kinetic power.

Display of Frank-Starling mechanism. The preparations rapidly responded to a change in left atrial filling pressure. Table 1 shows the results from two preparations in which the left atrial pressure was rapidly changed in increments of 5 cm H_2O. The pulse pressure and stroke volume increased to new levels within 3–4 beats (heart rate = 200/min). It can be seen that while the systolic pressure was only slightly altered, the cardiac output faithfully reflected the change in preload, with kinetic work (W_K) being greatest at the greatest value of cardiac output.

Measurement of myocardial O_2 consumption. The O_2 consumption of two working heart preparations was measured with the left atrial filling pressure set at 10 and 15 cm H_2O and the aortic pressure set at 65 cm H_2O. Advantage was taken of the fact that the hearts are set up in the Langendorff (non-working) mode of perfusion before insertion of the left atrial cannula and imposition of an afterload on the left ventricle (see Methods). Thus a comparison between the O_2 consumption in the non-working and working mode could be made on the same hearts. Table 2 gives a summary of the results obtained from two hearts first set up in the non-working mode and then set to work at two left atrial pressures, 10 and 15 cm H_2O. The coronary sinus p_{O_2} ranged from 123 to 209 mmHg, indicating an O_2 extraction much less than the maximum that can be achieved, since *in vivo* coronary sinus p_{O_2} is usually less than 20 mmHg (Keele & Neil, 1971). Also, the hearts were able to increase the O_2 extraction in response to an increase in left atrial preload of 5 cm H_2O. It is thus likely that at the lower workload (10 cm H_2O), at which the ATP experiments were performed, the hearts were adequately oxygenated.

TABLE 2. Response in two hearts to change in left atrial pressure at zero and 10 and 15 cm H_2O

L. atrial filling pressure (cm H_2O)	Non-working (Langendorff)	Working	
	0	10	15
Coronary flow (ml. min^{-1})	7·2 (8·6)	11·4 (11·7)	12·2 (11·4)
Coronary sinus p_{O_2} (mmHg)	170 (209)	157 (152)	123 (140)
$\dot{V}_{O_2}$ (ml. min^{-1} . 100 g^{-1})	8·3 (10·3)	12·9 (16·1)	14·9 (19·4)

Coronary flow during perfusion with aqueous buffer. In the control period of perfusion the coronary flow was 9 ml./min. Despite this high control flow, however, the introduction of hypoxic buffer perfusion increased the flow to 11·6 ml./min. (Fig. 5). The quantitative aspect of the adequacy of oxygen supply to the working heart will be discussed later (see Discussion, p. 153).

Computation of the power output. Two experiments were performed to calculate the power output of hearts working at different preload levels. The left atrial filling pressure (cm H_2O) was set at the usual 10, then increased to 15, 20, and back to 10. The two equations derived by Kannengiesser *et al.* (1979) were used to compute the power outputs (see Methods, p. 147). Pressure can be approximated either as the average systolic pressure (Neely *et al.* 1967) or as the peak systolic pressure (Kannengiesser *et al.* 1979), as done here. The former gives an underestimation of the pressure power whereas the latter gives an over-estimation.

These results can be compared with those obtained by Kannengiesser *et al.* (1979) who found a mean power output (mW) of 13·3 (±0·5) and 14·0 (±0.8) after 2 and 9 min respectively. The peak systolic pressure in their experiments was 163 as against 85 mmHg in the present work. The significance of this comparison is that ATP has been identified in the coronary sinus effluent from hearts which are probably not exerting their full power output. It is noteworthy (see Discussion) that the working heart preparation produced almost eight times the concentration of ATP in the sinus effluent in response to hypoxia than that demonstrated by Paddle & Burnstock (1974) in the non-working heart, and yet still has the capacity for further power output.

Exhibition of Ca paradox. In 1966 Zimmerman & Hülsmann described the occurrence of sarcolemmal damage when Ca was restored to a heart previously deprived of Ca. This they termed the 'Ca paradox'. Experiments in this work have shown that deprivation of Ca from the working myocardium with subsequent restoration abolished irreversibly the ability of the heart to contract. In six heart preparations working at the usual load (preload, 10 cm H_2O; afterload, 65 cm H_2O), switching from the normal perfusion buffer to Ca-free buffer with 1 mM-EGTA caused loss of mechanical activity within 30 sec. No activity was possible after restoration of Ca in 10 min. In two hearts ATP, 7 and 10 nM, appeared in the coronary sinus effluent after 30 and 60 sec of Ca-free perfusion, respectively. These findings indicate that ATP may be lost from the cell (or membrane) during the period of Ca restoration. The main point to be made is that these heart preparations responded like other myocardial preparations to Ca-free perfusion, probably indicating similar healthy membrane structure.

DISCUSSION

ATP has been detected in the coronary sinus effluent from the working heart preparation; upon rendering the heart hypoxic the concentration in the effluent increased eightfold. The total amounts released during the hypoxic period were calculated to be 46·1 p-mol/min. Ruigrok, Boink, Spies, Blok, Maas & Zimmerman (1978) report tissue ATP levels of 18·8 μmole . g dry wt^{-1} for oxygenated isolated rat hearts; assuming 0·2 g dry wt. per heart, the total tissue ATP would amount to approximately 4 μmole. The value of 46·1 p-mole thus represents a loss of less than 0·001 % of the total tissue ATP per minute. These rates are far below the reported rates of *de novo* synthesis of adenine nucleotides in rat heart (Zimmer, Trendelenberg, Kammermeier & Gerlach, 1973).

It is not possible to attribute the source of ATP to any single tissue in a perfusion experiment such as the one performed in this work, although the formed elements in the blood can be discounted since perfusion was with a saline solution. Apart from the myocardial cell, ATP could be released from nerves, vascular endothelium, vascular smooth muscle or some other unknown source. Paddle & Burnstock (1974) suggested 'purinergic' nerves as a possibility, although to date there is no evidence for the existence of such nerves associated with heart muscle. Pearson & Gordon (1979) have demonstrated selective release of adenine nucleotides, including ATP, from cultured vascular endothelial and smooth muscle cells in response to 'potentially damaging stimuli' such as trypsin and physical trauma. While this release of nucleotides was not accompanied by perceptible cell damage, it is likely that these stimuli are considerably more severe than 90 sec hypoxia. ATP is known to be released along with catecholamines (Douglas, Poisner & Rubin, 1965), and hypoxia may exacerbate this release from adrenergic nerve terminals. The previous finding that ATP is released from isolated adult rat heart cells in response to very brief periods of hypoxia (Forrester & Williams, 1977) clearly indicates the myocyte as one definite source. So although the proportion of ATP released into the coronary sinus effluent from the myocyte is unknown at the present time, it is probable that the contribution is a significant one.

The coronary flow rate in the present experiments was markedly elevated at 500 ml. 100^{-1} g . min^{-1} in the control period, suggesting that these preparations might be hypoxic from the outset. The rate of perfusate flow required to satisfy the myocardial O_2 demand can be readily assessed. Since blood is approximately 3·6 times more viscous than aqueous solution, and flow is inversely proportional to the first power of the viscosity (for straight tubes), for any given vascular resistance and perfusion pressure the flow should be at least 3·6 times greater for an aqueous solution than for blood. If we assume an *in vivo* O_2 consumption rate of 15 ml. min^{-1} . 100 g^{-1} for an atrial filling pressure of 10 cm water, and 70 % O_2 extraction from blood (Rushmer, 1976), then a coronary blood flow of 1·7 ml. min^{-1} would be necessary to supply the oxygen requirements. In fact, the mean coronary perfusion flow during the control periods was 9·6 ml. min^{-1}. The vascular resistance required for this flow rate of *aqueous medium* would give a *blood flow* of 2·3 ml. min^{-1}. Other observations demonstrate that the hearts were satisfactorily oxygenated throughout the control periods. First, introduction of hypoxic conditions caused an immediate increase in

coronary flow. Secondly, the coronary sinus p_{O_2} ranged from 123 to 209 mmHg, indicating that a considerable proportion of oxygen in the perfusate was not being utilized. Thirdly, the hearts were able to increase O_2 extraction in response to an increase in left atrial preload (Table 2). It is probable that at the low workload (10 cm water) imposed, the hearts received sufficient O_2 throughout the control period. Thus, although the coronary vasculature is more dilated when perfused with aqueous buffer solution, the extent of this dilatation is moderate and allows further dilatation to take place in the face of increased O_2 demand.

The amounts of ATP appearing in the coronary sinus effluent from working heart can be compared to the values found by Paddle & Burnstock (1974) using the non-working guinea-pig heart preparation. Although the guinea-pig heart is probably larger than the rat heart, for simplicity the same size will be assumed. A further assumption is that extracellular ATPase activity remains constant in both working and non-working preparations. The mean flow rate for the non-working preparations was 5·8 ml. min^{-1}; the mean concentration of ATP in the coronary effluent before hypoxia (90 sec duration) was 0·225 nM and after hypoxia was 0·67 nM (Paddle & Burnstock, 1974, Table 1). Thus the average total amount of ATP increased from 1·3 to 3·9 p-mole . min^{-1}. In the working heart the average total amount in the coronary effluent increased from 5·9 to 46·1 p-mole . min^{-1} for the same duration of hypoxia. Thus, the working heart releases more ATP than the non-working heart in the oxygenated state, a strong indication that the release of ATP is coupled in some way to O_2 demand. It is evident that the imposition of hypoxia upon the working heart provides a strong stimulus for release of ATP.

When the role of ATP in the dilatory response is considered, it becomes necessary to assess the actual amounts that are released into the extracellular space. Paddle & Burnstock (1974) showed that only 1 % of infused ATP was recovered in the sinus effluent. Thus we can suppose that the concentrations detected in the effluent from hypoxic myocardium represent only 1 % of the actual amounts present in the perivascular space. This assumes no change in the extracellular ATPase activity with increased myocardial work or hypoxia and also no account is taken of the surface ATPase activity of the myocardial cells (Williamson & DiPietro, 1965). McCruden (1970) demonstrated that the threshold of coronary vasodilatation in response to ATP lay between 6×10^{-8} and $5{\cdot}6 \times 10^{-7}$ M entering rabbit coronary arteries; no allowance could be made for any attenuation of ATP by the vascular endothelium. This would indicate that only a threshold effect would occur with $4{\cdot}7 \times 10^{-7}$ M concentration perivascularly. However, recent work has shown that cat pial vessels have a threshold response to ATP at 10^{-10} M applied to the adventitial side (Forrester, Harper, MacKenzie & Thomson, 1979). Obviously $4{\cdot}7 \times 10^{-7}$ M would profoundly affect the caibre of the resistance vessels, provided the sensitivity of the two types of vessel to ATP were the same.

The eightfold rise in the amounts of ATP released from the working heart during the hypoxic period probably signifies a major role for ATP in the vasodilatory response of the coronary arteries to hypoxia. The levels of ATP found in the effluent during the control periods indicate that ATP was being continuously released, even when the myocardium was oxygenated. This may contribute to the typical high 'resting' flow rate found in myocardium. Continuous release of ATP has already been

observed in the soleus muscle (Forrester & Hamilton, 1975) which also has a high resting flow rate.

The comparative roles of ATP, ADP, AMP, and adenosine in the control of coronary flow are unclear at the present time. Although adenosine has been implicated in the dilator response of coronary arteries to hypoxia (Berne, 1964), the

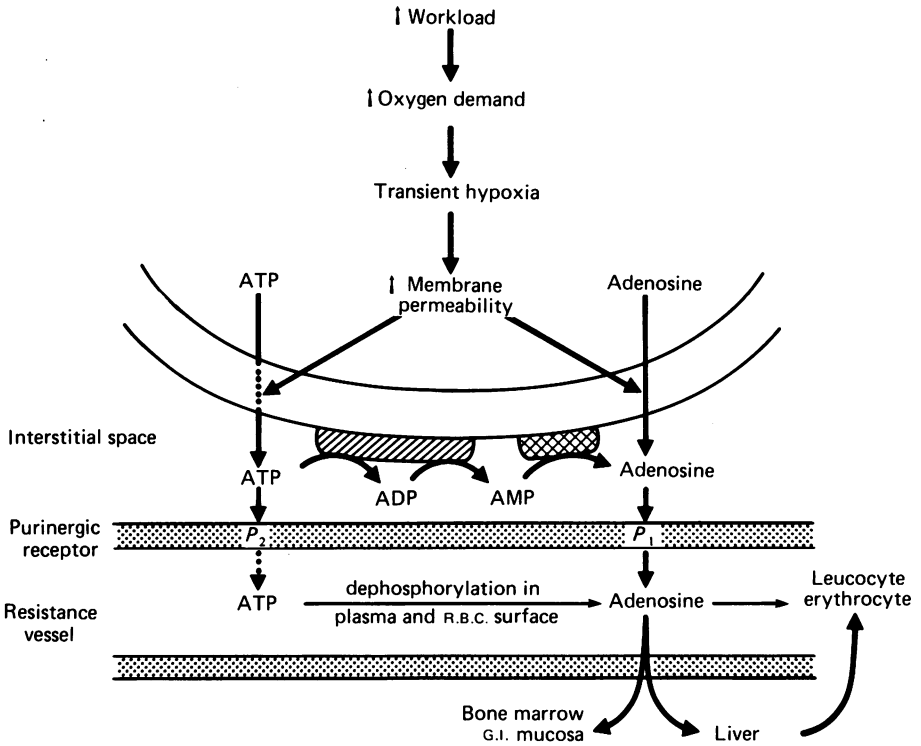

Fig. 6. Scheme showing proposed link between an increase in myocardial workload and dilatation of local resistance vessels. Increase of membrane permeability to adenosine and ATP is triggered by some unknown means through the effect of transient hypoxia on the cell membrane. Although permeability of membrane to adenosine is greater, the potency of ATP as a vasodilator in the coronary circulation is greater than that of adenosine (Wolf & Berne, 1956). Interstitial space contains ectoATPases which dephosphorylate (▨) (▩) ATP to adenosine (Pearson & Gordon, 1979). Not depicted are ATPases which face outward from the vascular smooth muscle. Adenosine and ATP act upon purine receptors P_1 and P_2 respectively (Burnstock, 1978). Residual ATP is slowly dephosphorylated in the blood plasma and more rapidly degraded (or taken up) by the erythrocyte. Adenosine is finally distributed to tissues unable to synthesize the purine ring moiety *de novo* (Pritchard *et al.* 1975). Not shown is the further degradation of adenosine to inosine and hypoxanthine in the interstitial and vascular compartments.

hyperaemia associated with myocardial hypoxia is not blocked by aminophylline, whereas the action of adenosine is (Afonso, Ansfield, Berndt & Rowe, 1972). Furthermore, Giles & Wilcken (1977) demonstrated that the vasodilatory action of ATP upon the coronary circulation is not blocked by aminophylline.

The recent characterization of outward facing 5′-nucleotidase in the perfused rat heart (Frick & Lowenstein, 1976), the demonstration of surface ATPase activity in

rat myocardial cells (Williamson & DiPietro, 1965) and the ecto-ATPase activity described in porcine vascular endothelium and smooth muscle (Pearson & Gordon, 1979) allow the following proposal with regard to the role of adenine nucleotides in the local control of myocardial blow flow (Fig. 6).

Adenine nucleotides and adenosine can all act as vasodilators, ATP being the most potent compound. Inosine and hypoxanthine do not have any vasodilatory action (Wolf & Berne, 1956). The presence of extracellular ATPase activity suggests that ATP released from hypoxic myocardium is rapidly broken down by stepwise dephosphorylation to adenosine (see discussion by Pearson & Gordon, 1979). That the vasodilatory action of adenosine, but not that of hypoxia or ATP, is blocked by aminophylline makes it more likely that the principal vasodilator involved is ATP. Degradation products can be taken up locally by the myocardial cell or distributed to those cells that cannot synthesize the purine ring *de novo*, such as erythrocytes, leucocytes, bone marrow cells and cells of the gastrointestinal mucosa. Such distribution can take place either directly in the bloodstream or via the liver (Pritchard, O'Connor, Oliver & Berlin, 1975).

Such findings once again emphasize the fundamental problem of how ATP is released from the hypoxic cell. It is plausible to regard the intrinsic membrane protein as a source of ATP (see Discussion in Forrester & Williams, 1977). This would mean that ATP in the first instance came *from* the membrane rather than through it and implies some compartmentalization of ATP in the cytoplasm for purposes of replenishment. In any event, elucidation of the release mechanism may further our understanding of the behaviour of heart cell membranes exposed to low O_2 tension.

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