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The response of isolated cardiac muscle to acute anoxia: protective effect of adenosine triphosphate and creatine phosphate

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A standardized and reproduceable preparation is described that enables the effect of drugs to be examined on the response of isolated cardiac muscle to acute anoxia. There was a linear relation ($r = 0{\cdot}963$) between tension developed in isolated, electrically-driven guinea-pig atria and the oxygen tension of the fluid surrounding the muscle. For any one atrial preparation the time taken, from the beginning of the anoxic period, for tension to be reduced by 50% was constant for consecutive anoxic periods. ATP and creatine phosphate significantly increased this time and protected cardiac muscle against the consequences of anoxia; in concentrations that were without direct cardiac effects, a greater degree of protection was possible with creatine phosphate.

Cardiac muscle, unlike skeletal muscle, is primarily adapted to work aerobically and an acute diminution of oxygen supply to the myocardium leads to a rapid, though reversible, decrease in contractility. A determination of the factors which limit the functional integrity of the myocardium during periods of acute anoxia may be of therapeutic importance in the management of conditions, such as coronary insufficiency, where the heart may be deprived of its oxygen supply for varying periods of time. There have been a number of investigations along these lines. Some workers (Winbury, 1956; Penn, 1965, 1970; Jewitt, Skelton & Sonnenblick, 1973) have examined the *recovery* of myocardial contractility after acute anoxia, whilst others (e.g. Setnikar & Ravasi, 1960; Siess, 1961; McInnes & Parratt, 1969) have been more concerned with the question of possible *protection* of cardiac muscle from the consequences of oxygen lack. Much of this work can be criticized on methodological grounds. In some cases isolated whole heart preparations have been used (with the associated problem of adequate oxygenation in the control state) whilst the use of isolated, spontaneously beating, atrial preparations is complicated by the interrelation between rate and developed contractile force. In no study have adequate safeguards been taken to ensure a controlled degree of anoxia as assessed by measurements of oxygen tension. The purpose of this paper is to describe a simple, yet controlled, preparation for the assessment of the possible effect of drugs on the ability of cardiac muscle to withstand acute anoxia. It also describes, by way of example, the effects of adenosine triphosphate (ATP) and of creatine phosphate (CP) in this model.

METHODS

Male guinea-pigs (Dunkin Hartley strain), 300–500 g, were killed by stunning and bleeding, and the heart quickly excised and placed in physiological salt solution

(PSS) containing (mM litre^{-1}) Na^+ 143·3, K^+ 5·9, Ca^{2+} 2·6, Mg^{2+} 1·2, Cl^- 128·3, $H_2PO_4^-$ 2·2, HCO_3^- 24·9, SO_4^{2-} 1·2 and glucose 10, bubbled with 95% O_2 and 5% CO_2. The left atrium was dissected free of ventricular muscle and connective tissue and was impaled on a thin platinum wire electrode. The atrium (mean weight 106 ± 12 mg) was placed in a 20 ml organ bath maintained at 32·0 ± 0·4° containing PSS bubbled with 95% O_2 +5% CO_2 and was attached by means of a thin thread to a strain-gauge transducer (Nihon Kohden, Tokyo). The atrium, placed under a resting tension of 1 g, was stimulated supramaximally (usually 5–10V) with square wave pulses of 5 ms duration at a frequency of 2 Hz. In some experiments the rate of rise of developed tension (dT/dt) was also measured, simultaneously with tension, using a Devices differentiating circuit and the second channel of a Devices M2 recorder.

Anaerobic samples of bath fluid (0·5 ml) were taken and were analysed for Po_2 and pH using suitably calibrated Radiometer electrodes thermostatically controlled at 37°. If the Po_2 of a solution is measured, as in these experiments, at a higher temperature than that of the fluid surrounding the muscle then the measured Po_2 will be greater than the actual Po_2 of the solution in the organ bath. Any applied correction factor will depend on the temperature and composition of the fluid and on the barometric pressure. As no attempt has been made to correct for this considerable (5°) temperature difference between the measuring electrodes and the solution in the organ bath, the values in the text overestimate bath fluid Po_2 by between 8 and 12%.

After a stabilization period, the gas aerating the organ bath was changed to 97% N_2 + 3% CO_2 and the time course of the decrease in tension followed. When the developed tension had decreased to about 20–30% of control, a sample was again taken for Po_2 and pH analysis. The organ bath was then washed out and bubbled with 95% O_2 + 5% CO_2. The time taken for the developed tension to reach 75, 50 and 25% of the pre-anoxic control value was noted. This procedure was followed at least twice and was then repeated 15 min after the administration of the test drug to the bath. After drug treatment, care was taken that the sample of bath fluid removed for analysis was withdrawn at the same time after the start of the anoxia as in the control runs. This ensured that any protective effect was due to the drug and not due to differences in time course of the fall in Po_2. Thirty minutes were allowed to elapse between anoxic runs.

In a further series of experiments, the preparations were set up as described above and, to study more closely the time course of anoxia, samples were taken for Po_2 measurements at 60 s intervals. The bath fluid Po_2 was then plotted against the decrease in developed tension. As before, at least two control runs were carried out and then the procedure repeated 15 min after the test drug.

All concentrations in the text refer to the free base or acid. The results were treated statistically using Student's *t*-test for paired data.

The drugs used were creatine phosphate (CP), creatine, adenosine triphosphate (ATP) all obtained from Centro Ricerche Schiapparelli, Turin, creatinine phosphate (Farmaceutici Midy, Milan) and sodium dihydrogen phosphate (Merck A.G., Darmstadt).

RESULTS

Subjecting isolated atria to acute anoxia led to a gradual reduction both in developed tension and in the maximum rate of tension development (dT/dt). During anoxia, both developed tension, and the maximum rate of tension development were highly

dependent upon the oxygen tension of the bath fluid surrounding the atria. This is illustrated in Fig. 1 which shows the results of taking samples of bath fluid for Po_2 measurement every 60 s from the commencement of the anoxic period. There was no

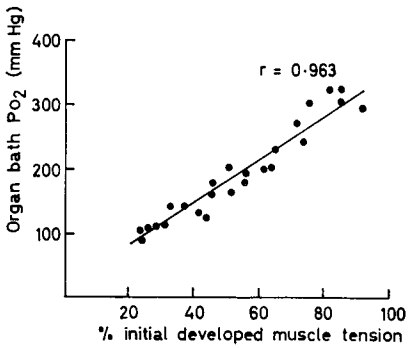

FIG. 1. The relation between organ bath oxygen tension (Po_2 mmHg) and developed muscle tension. There is a good correlation over the range examined (300 to 80 mmHg):— $Po_2 = 3{\cdot}33$ developed tension $+14{\cdot}0$.

reduction in tension until the Po_2 had fallen from the control level (505 ± 12 mmHg) to about 300 mmHg; thereafter there was a gradual decrease in tension with the reduction in bath fluid Po_2. A 50% reduction in tension occurred with a Po_2 of about 180 mmHg and a 75% reduction with a Po_2 of about 90 mmHg. Tension developed by the muscle could be readily restored on washing and reoxygenation.

In a total of 50 separate experiments the mean times taken to reach 75, 50 and 25% of the initial tension before anoxia were 146 ± 12, 281 ± 16 and 441 ± 17 s respectively. The results obtained with any one atrial preparation were highly reproduceable. Thus in Table 1 the results are given for two consecutive anoxic periods (with a 30 min rest period between) using 25 separate atria. Results obtained during the two consecutive anoxic periods were similar and this reproducibility of response could be demonstrated with up to seven or eight such periods of anoxia. The main value of this method lies in using the same atrial preparation for both control and test (drug) anoxic periods. The reason for this is that although there is uniformity of response in the same preparation there is, as expected, considerable variation between individual atria. This is best illustrated in Table 2 which shows the detailed results for control and test (in this case creatine) anoxic runs in six separate atrial preparations.

Table 1. *Comparison of bath fluid* Po_2 *and the times for tension to be reduced by 25, 50 and 75%, during two consecutive periods of anoxia.* There was a 30 min rest period between the two runs; $n = 25$.

	Control		Anoxia		Time (s) for tension to be reduced by		
	Po_2	pH	Po_2	pH	25%	50%	75%
First anoxic period	516 ± 24	$7{\cdot}35 \pm 0{\cdot}04$	70 ± 5	$7{\cdot}42 \pm 0{\cdot}04$	172 ± 31	281 ± 24	412 ± 23
Second anoxic period	530 ± 19	$7{\cdot}33 \pm 0{\cdot}04$	61 ± 4	$7{\cdot}42 \pm 0{\cdot}04$	173 ± 22	264 ± 21	380 ± 25

$P > 0{\cdot}1$

The time taken to reach 50% of the pre-anoxic developed tension varied between 230 and 440 s (mean 303 ± 31 s) and there was a similar variation in the time taken to reach 75 and 25% of the tension developed during oxygenation. The possibility that this variation between atria was dependent upon the initial developed tension was examined in a further sixteen atrial preparations. There was in fact a very poor correlation (r = 0·17) between tension developed by individual atria during oxygenation (which varied between 210 and 860 mg) and the time taken for tension to fall to 50% of control (which varied, in these 16 preparations between 128 and 374 s; mean 280 ± 23 s).

Table 2. *The effect of creatine (4·74mM) on the anoxia-induced reduction in developed tension of guinea-pig isolated driven left atria. (Mean ± s.e.).*

	Control							In presence of creatine (4·74 mM)						
	Pre-anoxia		Anoxia*		Time (s) for tension to be reduced by:—			Pre-anoxia		Anoxia*		Time (s) for tension to be reduced by:—		
Expt No.	Po_2	pH	Po_2	pH	25%	50%	75%	Po_2	pH	Po_2	pH	25%	50%	100%
25	610	7·423	60	7·530	184	259	380	690	7·438	70	7·525	133	203	311
26	510	7·578	50	7·680	166	230	360	450	7·550	60	7·635	166	240	400
27	530	7·460	90	7·500	190	260	395	550	7·475	100	7·510	200	270	370
28	570	7·420	85	7·465	200	310	440	580	7·435	80	7·470	190	320	490
29	500	7·683	50	7·680	184	320	460	450	7·595	75	7·600	195	300	440
30	540	7·515	140	7·558	380	440	540	550	7·460	120	7·506	350	410	500
	543	7·499	79	7·568	217	303	429	545	7·492	84	7·541	206	291	419
	±17	±0·063	±14	±0·062	±33	±31	±27	±37	±0·033	±10	±0·026	±31	±29	±30

* Sample taken when tension had been reduced by 70–80%.

The effect of organic phosphates on the ability of isolated atria to withstand the negative inotropic effects of acute anoxia

The substances examined were ATP, CP and creatinine phosphate.

(i) *The effect of ATP.* In concentrations between 0·02 and 1·98 mM ATP caused a dose-dependent decrease in developed tension, readily reversible on washing. In a concentration of 0·118 mM, ATP considerably protected atria against the effects of acute anoxia. The time taken for the atria to reach 50% of the pre-anoxic tension was 402 ± 32 s compared with 323 ± 33 s for the same preparations in the absence of ATP ($P < 0{\cdot}05$; n = 6). The times taken for tension to be reduced by 25 and by 75% were also significantly prolonged. They were 218 ± 26 s (control i.e. in the absence of ATP) and 264 ± 22 s (in the presence of ATP) for a 25% reduction in tension and 489 ± 43 s (control) and 611 ± 52 s (ATP) for a 75% reduction in tension. These results also show that it makes little difference whether the time for a 25, 50 or 75% reduction in tension is taken as the end-point. ATP increased the time taken for a 25% reduction in tension by 21%, the time taken for a 50% decrease in tension by 26% and the time taken for a 75% reduction in tension by 22% (See Fig. 2).

(ii) *The effect of creatine phosphate (CP).* In the presence of CP (0·1 to 1·0 mg ml^{-1}; 0·47 to 4·74 mM) atrial muscle was protected against the decrease in contractility induced by anoxia. This protection was particularly marked with the higher concentration used (4·74 mM). The times taken for tension to be reduced by 25% of

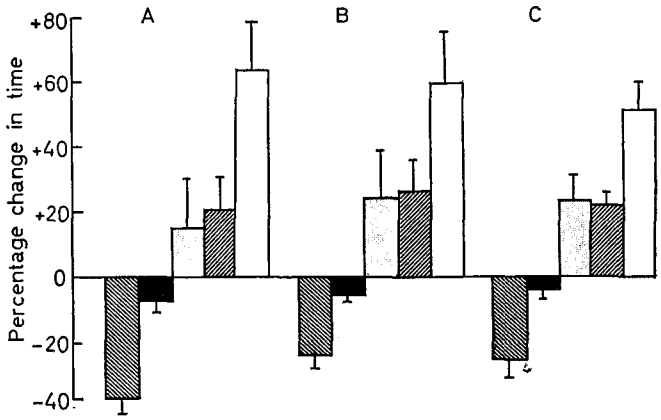

FIG. 2. Change in time taken for tension to be reduced during anoxia by (A) 25, (B) 50 and (C) 75% of the initial developed tension. Creatine phosphate (open columns), ATP (hatched columns Rt—L) and creatinine phosphate (stippled columns) enabled atria to withstand anoxia for a longer period of time. Sodium hydrogen phosphate (hatched columns L—Rt) had a detrimental effect and creatine (solid columns) was ineffective. All drugs in concentrations of 4·34mM except ATP (0·118mM).

control were 198 ± 21 s (control) and 325 ± 36 s (in the presence of CP), for a 50% reduction in tension, 293 ± 29 s (control) and 469 ± 46 s (CP) and for a 75% reduction in tension 407 ± 29 s (control) and 596 ± 47 s (CP). All these increases (of 64, 60 and 51% respectively) were significant ($P < 0{\cdot}005$; n = 11).

(iii) *The effect of creatinine phosphate.* Similar, though less marked, protection was observed with creatinine phosphate (4·74 mM). In these experiments creatinine phosphate prolonged the time taken to reach 50% of resting tension by a mean of 56 ± 22 s (a mean increase of 24%), and the time taken to reach 25% of the resting tension by a mean of 78 ± 17 s (i.e. 23%).

(iv) *The effect of creatine and of sodium dihydrogen phosphate.* In view of the protection observed with creatine phosphate, the effects of creatine and of inorganic phosphate alone were examined. As is clear from Table 2 creatine (4·74 mM) had no significant effect on the decline in developed tension that occurred during the anoxic phase. It actually slightly decreased the time taken to reach 75% of the initial developed tension (by a mean of 12 ± 10 s i.e. −6%), the time taken to reach 50% of the initial tension by a mean of 13 ± 11 s (−4%) and the time taken to reach 25% of the initial tension by a mean of 11 ± 19 s (−3%). The results obtained with sodium dihydrogen phosphate were complicated by the reduction in pH which resulted from its addition to the bath fluid. At the chosen test concentration (4·74 mM) there was a mean reduction in tension of oxygenated driven atria of 19 ± 3% and a significant decrease in the pH of the bath fluid (from 7·423 ± 0·038 to 7·324 ± 0·045; $P < 0{\cdot}005$). The times taken to reach 75, 50 and 25% of the initial resting tension after the commencement of anoxia were decreased by 43 ± 11, 42 ± 13 and 74 ± 28 s respectively (i.e. 37, 24 and 25%). This meant that sodium dihydrogen phosphate accelerated the negative inotropic effect of anoxia. These results are summarized in Fig. 2.

DISCUSSION

There have been a number of attempts to modify, with drugs, the ability of isolated

cardiac muscle to withstand the effects of anoxia. Penn (1965), for example, using spontaneously beating rabbit atria, demonstrated that dipyridamole, iproniazid and sodium nitrite enhanced the recovery of both contractility and rate following a period of anoxia. McInnes & Parratt (1969) found that, in the presence of the antianginal drug hexobendine, spontaneously beating atria were protected from the consequences of anoxia; that is, the times taken for contractile force to be reduced to 80, 50 and 25% of the pre-anoxic levels were considerably longer in the presence of the drug. These relatively simple methods can be criticized on two grounds. Firstly, the results presented in this paper show that the developed tension of atrial preparations depends on the oxygen tension (Fig. 1). It is important therefore to monitor Po_2, and probably also pH, to ensure that similar levels of oxygenation are achieved in the absence and in the presence of the drug under test. Secondly, it is known that the strength of contraction of isolated cardiac muscle is influenced by the frequency of contraction (Koch-Weser & Blinks, 1963). It is therefore also important to use an electrically driven atrial preparation.

A number of other factors presumably influence the ability of cardiac muscle to withstand anoxia and some of these (sex, age and species of donor) were investigated by Penn (1970). Presumably the thickness of the muscle is a major factor determining adequate oxygenation and the state of the high-energy phosphate stores at the commencement of the experiment (which will depend partly upon the time taken to set up the preparation), the resting metabolic rate and the temperature are probably also important. It is mandatory therefore to use the same muscle preparation for both the control (pre-drug) anoxic periods and for the anoxic stimulus in the presence of the drug. There is a remarkable uniformity in the response of the same atrial preparation to consecutive anoxic stimuli (Table 1 and McInnes & Parratt, 1969), the reduction in contractility is readily reversible on reoxygenation and each preparation can hence be used for more than one test drug. This is partly due to the relatively short total period of anoxia used (usually less than 8 min). Other workers (Gardner & Farah, 1954; Winbury, 1956; Penn, 1965; Jewitt & others, 1973) have found that with longer periods of anoxia (30–60 min) recovery of myocardial contractility on reoxygenation is seldom complete, a result which implies impaired viability. Clearly, the total period of anoxia must be kept below 30 min if full contractile recovery is to be achieved and if the same preparation is to be used to examine any modifying influence of drugs.

Considerable protection was obtained in the present experiments with ATP and creatine phosphate and, to a lesser extent, with creatinine phosphate. There are isolated reports in the literature that ATP can improve contractility in hypodynamic hearts (Robb, and Farah & Gardner, quoted by Gardner & Farah, 1954) although it has apparently no effect when administered during an actual anaerobic phase (Gardner & Farah, 1954). Although there is some evidence that ATP can be released from intact cells, in association with depolarization (Abood, Koketsu & Miyamoto, 1962), secretion (Douglas, 1968) and muscular activity (Forrester & Lind, 1969), it is probably doubtful whether it can penetrate into the cell from an external fluid medium, unless the permeability characteristics of the membrane are drastically altered by anoxia. Hopkins (1973a) however has shown that ATP, added to McEwen solution that has passed through the coronary circulation of perfused guinea-pig hearts, is broken down to AMP, ADP and ultimately to adenosine. This destruction of ATP is extremely rapid; in these experiments only 10% of the added ATP was present after

10 s. These results show that ATP-ases, and probably also 5'-nucleotidase, are leached out of the cardiac muscle by the perfusing fluid. It is likely that this will be even more pronounced under anoxic conditions. Adenosine itself is taken up by cardiac muscle by a process of facilitated diffusion (Olsson, Snow & others, 1972) and perhaps, in the presence of high extracellular nucleoside levels, also by simple diffusion (Schrader, Berne & Rubio, 1972). Under normal conditions this adenosine is rapidly converted into ATP and retained in this form (Hopkins, 1973b). There may also be an increased production of 3,5-AMP leading to increased myocardial glycolysis and lipolysis (Raberger, Kraupp & others, 1970). These findings may explain the results described in the present paper that, in the presence of ATP, the anoxia-induced decrease in myocardial contractility is less marked. Creatine phosphate had a similar protective action though in a much higher concentration. Thus, in a concentration of 0·118 mM, ATP increased the time, during anoxia, taken to reach 50% of control tension by a mean of 25%; CP in a concentration of (4·74 mM) increased it by about 60% and, in a concentration of 0·474 mM, by about 14%. ATP is therefore at least 10–12 times more active than CP. However, since higher concentrations of CP can be given without causing a direct negative inotropic effect (which unfortunately is a characteristic of higher concentrations of ATP), a more substantial degree of protection can ultimately be achieved with this substance. The protective effect of CP is more difficult to explain but as creatine alone was ineffective and sodium dihydrogen phosphate made the muscle even more susceptible to anoxia, the presence of a high-energy phosphate bond is, in some way, probably implicated.

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