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Direct evidence for a role of intramitochondrial Ca^{2+} in the regulation of oxidative phosphorylation in the stimulated rat heart

Studies using ^{31}P n.m.r. and Ruthenium Red

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1. The concentrations of free ATP, phosphocreatine (PCr), P_i, H^+ and ADP (calculated) were monitored in perfused rat hearts by ^{31}P n.m.r. before and during positive inotropic stimulation. Data were accumulated in 20 s blocks. 2. Administration of 0.1 μM-(−)-isoprenaline resulted in no significant changes in ATP, transient decreases in PCr, and transient increases in ADP and P_i. However, the concentrations of all of these metabolites returned to pre-stimulated values within 1 min, whereas cardiac work and O_2 uptake remained elevated. 3. In contrast, in hearts perfused continuously with Ruthenium Red (2.5 μg/ml), a potent inhibitor of mitochondrial Ca^{2+} uptake, administration of isoprenaline caused significant decreases in ATP, and also much larger and more prolonged changes in the concentrations of ADP, PCr and P_i. In this instance values did not fully return to pre-stimulated concentrations. Administration of Ruthenium Red alone to unstimulated hearts had minor effects. 4. It is proposed that, in the absence of Ruthenium Red, the transmission of changes in cytoplasmic Ca^{2+} across the mitochondrial inner membrane is able to maintain the phosphorylation potential of the heart during positive inotropic stimulation, through activation of the Ca^{2+}-sensitive intramitochondrial dehydrogenases (pyruvate, NAD^+–isocitrate and 2-oxoglutarate dehydrogenases) leading to enhanced NADH production. 5. This mechanism is unavailable in the presence of Ruthenium Red, and oxidative phosphorylation must be stimulated primarily by a fall in phosphorylation potential, in accordance with the classical concept of respiratory control. However, the full oxidative response of the heart to stimulation may not be achievable under such circumstances.

INTRODUCTION

The functioning mammalian heart is able to match closely its energy requirements for activities such as contraction and Ca^{2+} pumps with energy production by oxidative metabolism (Williamson, 1975; Randle & Tubbs, 1979). Therefore, when increased work is demanded of the heart, an increase in O_2 consumption is necessary to produce ATP at enhanced rates. According to the classical theory of the control of mitochondrial respiration and oxidative phosphorylation, an increased ATP utilization results in a decline in the phosphorylation potential (i.e. [ATP]/[ADP]·[P_i] ratio) and it is this, or a component, which elicits the stimulation of ATP production (see, e.g., Nicholls, 1984; Gibbs, 1985; Chance *et al.*, 1986).

However, there have been a number of reports of studies on the intact mammalian heart under a variety of different conditions in which no correlations between the tissue concentrations of high-energy phosphates and rates of work or oxidative metabolism could be observed (see, e.g., Matthews *et al.*, 1982; Allen *et al.*, 1986; Balaban *et al.*, 1986; From *et al.*, 1986; Katz *et al.*, 1988*a*, 1989). Indeed, the most notable feature of these studies was the remarkable stability of the phosphorylation potential in the face of varying workloads. Obviously these findings suggest that additional factors may be involved in the control of oxidative phosphorylation in the mammalian heart, and perhaps also in other tissues.

An attractive alternative hypothesis for the promotion of oxidative phosphorylation in stimulated tissues during maintenance of the phosphorylation potential is that some degree of regulation of the respiratory rate is achieved through increases in NADH supply to the respiratory chain (Denton & McCormack, 1980, 1985; Hansford, 1985; Brand & Murphy, 1987; Katz *et al.*, 1988*a*, 1989). It can be shown that increases in the NADH/NAD^+ redox potential can promote increases in respiration and ATP production in isolated mitochondria without any change in extramitochondrial phosphorylation potential (see, e.g., McCormack, 1985; McCormack & Denton, 1986*a*; Koretsky & Balaban, 1987). Such a mechanism would be of obvious advantage to cells. In support of this idea, Katz *et al.* (1987) were able to demonstrate increases in NAD(P)H when glucose-perfused rat hearts were subjected to increases in work.

It has been proposed that the increases in intramitochondrial NADH could be achieved by the relay of

Abbreviations used: RR, Ruthenium Red; PDH, the pyruvate dehydrogenase complex; PDH_a, the active, non-phosphorylated, form of PDH; f.i.d.s., free-induction decays; PCr, phosphocreatine.

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the increases in cytosolic Ca^{2+} (which are directly responsible for promoting the increased work) into the mitochondrial matrix. This would result in the activation of three key intramitochondrial dehydrogenases, namely pyruvate (PDH), NAD^+–isocitrate and 2-oxoglutarate dehydrogenases (see Denton & McCormack, 1985; Hansford, 1985; McMillin & Pauly, 1988). It is well established that the administration of positive inotropic agents to the heart increases the amount of active, non-phosphorylated, PDH (PDH_a) (Hiraoka *et al.*, 1980; McCormack & Denton, 1981). Moreover, McCormack & England (1983) showed that these increases in PDH_a could be completely blocked by perfusion with Ruthenium Red (RR), a potent inhibitor of mitochondrial Ca^{2+} uptake (Moore, 1971), whereas stimulated cytosolic Ca^{2+}-dependent processes such as contraction and phosphorylase *a* levels were unaffected. Hansford (1987) also showed RR blocked the activation of PDH in quin-2-loaded myocytes subjected to various treatments designed to elicit increases in cytosolic Ca^{2+}, whereas the measured increases in cytosolic Ca^{2+} were largely unaffected. Further supporting evidence was provided by studies employing the rapid isolation of mitochondria from adrenaline-treated hearts, under conditions where Ca^{2+} uptake or loss by the mitochondria was minimized. Thus it was shown that, compared with controls, these mitochondria had persistently elevated PDH_a content, elevated 2-oxoglutarate dehydrogenase activity (when assayed at a sub-saturating concentration of 2-oxoglutarate; see McCormack & Denton, 1979), and an elevated total calcium content (Crompton *et al.*, 1983; McCormack & Denton, 1984). Depletion of mitochondrial Ca content by the addition of Na^+ to promote Ca^{2+} egress (see Crompton, 1985) led to the diminution of these persistent elevations (see McCormack & Denton, 1984). In addition, studies using X-ray probe microanalysis of intact heart have shown increased mitochondrial total Ca content (within the range required to regulate these enzymes) in hearts subjected to positive inotropic stimulation (see Wendt-Gallitelli, 1986).

^{31}P n.m.r. represents a powerful non-invasive technique for monitoring the concentrations of high-energy-phosphate-containing metabolites in the perfused heart, and the effects of various inotropic interventions on these (see Garlick *et al.*, 1977; Jacobus *et al.*, 1977; Hoerter *et al.*, 1986; He *et al.*, 1987; Camacho *et al.*, 1988; Kusuoka *et al.*, 1988). In the present study we have taken advantage of our ability to acquire rapid ^{31}P-n.m.r. data in 20 s blocks. We have examined transient changes in cardiac energy metabolism, and also used the apparent ability of RR to inhibit mitochondrial Ca^{2+} uptake *in situ*, to test the above hypothesis in rat hearts subjected to positive inotropic stimulation by isoprenaline. A small part of the present work has been reported briefly as a meeting abstract (Unitt *et al.*, 1988).

MATERIALS AND METHODS

Materials

All bichoemicals and chemicals were of the highest grade commercially available and were purchased from either Sigma Chemical Co. (Poole, Dorset, U.K.) or BDH Chemicals Ltd. (Poole, Dorset, U.K.) except *p*-(*p*-aminophenyl)azobenzenesulphonic acid, which was from Pfaltz & Bauer, Standford, CT 06902, U.S.A. Ruthenium Red (batch no. 124F 0673) was obtained from Sigma, and was quoted as being 48–50% pure.

Heart perfusions

Male Wistar rats (280–320 g) were killed and their hearts excised and placed briefly in ice-cold saline. The hearts were perfused by the Langendorff technique (drip-through, without re-circulation) at a constant flow rate (12 ml/min) at 37 °C in a fluid-filled glass chamber. All hearts were perfused with bicarbonate-buffered medium (after Krebs & Henseleit, 1932) gassed with O_2/CO_2 (19:1). Unless otherwise stated in Figures and legends, this contained 1.5 mM-$CaCl_2$, 1.2 mM-$MgSO_4$, 0.5 mM-EDTA, 0.2 mM-KH_2PO_4 and 11 mM-glucose.

Contractile force, PDH activity, O_2 uptake and lactate and CO_2 production by the perfused rat hearts were measured in separate experiments from the n.m.r. measurements, but under similar conditions as described by England (1976), McCormack & Denton (1981) and Unitt *et al.* (1988) respectively. Further perfusion details are given in the Figure legends.

For the n.m.r. experiments, the standard procedure involved a stabilization period of about 20 min to ensure that the energy metabolites in the perfused hearts had reached a steady state, and to allow time for optimizing magnetic-field homogeneity in the sensitive volume of the n.m.r. probe. The hearts were then perfused for 10 min in the absence or presence of 2.5 μg of RR/ml, followed by a 10 min period with or without the addition of 0.1 μM-(−)-isoprenaline. This resulted in a total perfusion time of approx. 40 min.

^{31}P n.m.r. spectroscopy was performed in a wide-bore (89 mm) 8.43 T vertical superconducting magnet (Oxford Instruments) at a resonant frequency of about 145.8 MHz. Langendorff-perfused rat hearts were mounted in a probe, constructed at Smith, Kline & French's Research Engineering Laboratories, in which the organ could be maintained at 37 °C. Data acquisition and processing were performed on a Bruker AM-360 spectrometer equipped with an ASPECT 3000 computer.

Phosphorus spectra were acquired by co-adding 16 free-induction decays (f.i.d.s.) generated by a 60 μs 90° pulse, with an acquisition time of 0.13 s and subsequent relaxation delay of 1 s. The f.i.d.s. were multiplied by a 40 Hz signal-to-noise enhancement function, before Fourier transformation. Relative concentrations were determined by integrating the relevant peaks, and pH was measured from the chemical shift of P_i relative to PCr, and a standard titration curve (Morris, 1987). Possible effects of saturation on relative peak intensities were checked by running a spectrum with 10 s inter-pulse delays. Any discrepancies were found to be negligible within the constraints imposed by the signal-to-noise ratio.

Calculations

Free concentrations of ADP were calculated from the values obtained for ATP, PCr and internal pH, and the value for the equilibrium constant for creatine kinase ($[ATP][creatine]/[ADP][H^+][PCr] = 1.66 \times 10^9$ M^{-1}; Lawson & Veech, 1979), by using the method of From *et al.* (1986), which assumes a free $[Mg^{2+}]$ of 1 mM. For this a value of 68.5 μmol/g dry wt. was used for the total PCr plus creatine content, and a value for the PCr/creatine ratio of 0.8 in unstimulated hearts. Both of these values were determined by h.p.l.c. analyses (Sellevold *et al.*,

Table 1. Effects of Ruthenium Red on the activation of pyruvate dehydrogenase by isoprenaline in the perfused rat heart

Rat hearts were perfused in the absence or presence of RR (2.5 μg/ml) as described in the Materials and methods section under conditions designed to be analogous to those for the n.m.r. experiments (see Fig. 3 below). Hearts were pre-perfused for 15–20 min and then perfused, with or without L-isoprenaline (0.1 μM), for the periods indicated before being freeze-clamped for subsequent extraction and assay of PDH as described by McCormack & Denton (1981). Results are expressed as the percentage of total PDH activity existing as PDH_a. Total PDH activity was unaffected by the conditions tested and gave values in the expected range (around 3–5 units/g wet wt. of tissue, where a unit catalyses the conversion of 1 μmol of substrate/min at 30 °C; McCormack & Denton, 1981): *** indicates a significant effect of isoprenaline ($P \leqslant 0.001$), and †, ††, ††† indicates a significant effect of RR ($P \leqslant 0.05$, 0.01 and 0.001 respectively).

Treatment	Time (min)	PDH_a (% of total PDH activity) in heart perfused: Without RR	With RR
Control	2	11.6±1.3 (3)	12.7±2.5 (3)
Isoprenaline	2	41.3±1.1 (3)***	16.7±1.9 (4)†††
Control	10	11.2±1.6 (4)	6.8±0.8 (5)†
Isoprenaline	10	40.8±3.1 (5)***	24.2±1.0 (6)***††

1986) of freeze-clamped hearts (at least 20 in each case to provide mean values) extracted as described by Sharps & McCarl (1982) (results not shown). These values are similar to those found previously (see, e.g., Williamson, 1965; Sellevold *et al.*, 1986). RR did not appreciably affect the above values in unstimulated hearts. Where P_i was not detectable (see, e.g., Fig. 4) and pH could thus not be measured, a value of 7.15 was used for the calculations; this value was taken from previous reports under similar conditions (Ellis & Thomas, 1976; Katz *et al.*, 1988*b*). It should also be noted that the low concentration of extracellular P_i (see above) used in the present study meant that this did not interfere with the intracellular P_i estimations.

Results are generally expressed as means ± S.E.M. for the numbers of observations indicated in parentheses or in legends; where appropriate, statistical significance was assessed by using Student's *t* test.

RESULTS AND DISCUSSION

Activation of PDH

McCormack & England (1983) have previously shown that 2.5 μg of RR/ml was sufficient to block the activation of PDH in the perfused rat heart by positive inotropic agents present for 2 min. These observations are confirmed in Table 1, and also extended to the longer perfusion times which are used in the n.m.r. experiments reported below. This minimally active RR concentration was chosen because RR also has some negative inotropic effects on the heart (McCormack & England, 1983) (see Fig. 2 below), possibly owing to its interaction with sarcoplasmic-reticulum membranes (Chamberlain *et al.*, 1984). At the longer time point (Table 1) there is evidently some activation of PDH in the presence of RR, although this is still much diminished compared with the control in the absence of RR, and this is also exacerbated by an apparent decrease in PDH_a by RR at this time point in the unstimulated hearts (Table 1). However, it is possible that, even here, mitochondrial Ca^{2+} uptake is still being inhibited, as PDH_a content can also be raised by a fall in intramitochondrial ATP/ADP ratio (see below and, e.g., McCormack & Denton, 1979). It should also be noted, as found by McCormack & England (1983), that RR did not affect the activation of phosphorylase by isoprenaline under the conditions used in Table 1 (results not shown). Therefore the results of Table 1 indicate that this system is appropriate for testing the hypothesis that regulation of the respiratory chain may in part reside in the supply of NADH.

Effects of RR and isoprenaline on metabolism and contraction

The results given in Fig. 1 show the effects of RR on lactate and CO_2 production, and O_2 uptake, by the hearts before and during a positive inotropic intervention by isoprenaline. Fig. 2 shows the effects of RR on contractile force, contractile frequency and the total work output of the hearts in the same type of experiment. It can thus be seen from Fig. 2 that, before the administration of isoprenaline, RR caused about a 20% decrease in contractile force but very little effect on beating frequency, such that overall (Fig. 2*c*) the work output was depressed by around 20%. This coincides well with the slight decreases in all of the parameters shown in Fig. 1, indicating that the metabolism of the heart is suppressed to an approximately similar degree as work output. However, after the positive inotropic intervention, the contractile force developed by the hearts was actually slightly higher in the presence of RR (Fig. 2*a*). As again in this circumstance RR had little effect on the stimulated chronotropic response to isoprenaline (Fig. 2*b*), this means that overall the work output actually was higher in the presence of RR and isoprenaline (Fig. 2*c*). In contrast with this, however, the stimulated metabolic parameters (Fig. 1) remained depressed by RR throughout the positive inotropic intervention, by an approximately similar amount in each case. Thus the response of oxidative metabolism (Fig .1*c*) to isoprenaline is slightly repressed by RR, whereas the work output of the heart is slightly increased by RR in the presence of the positive inotropic stimulus. There is also a report that RR slightly depresses the oxidative response of isolated myocytes to depolarization (Moreno-Sanchez & Hansford, 1988).

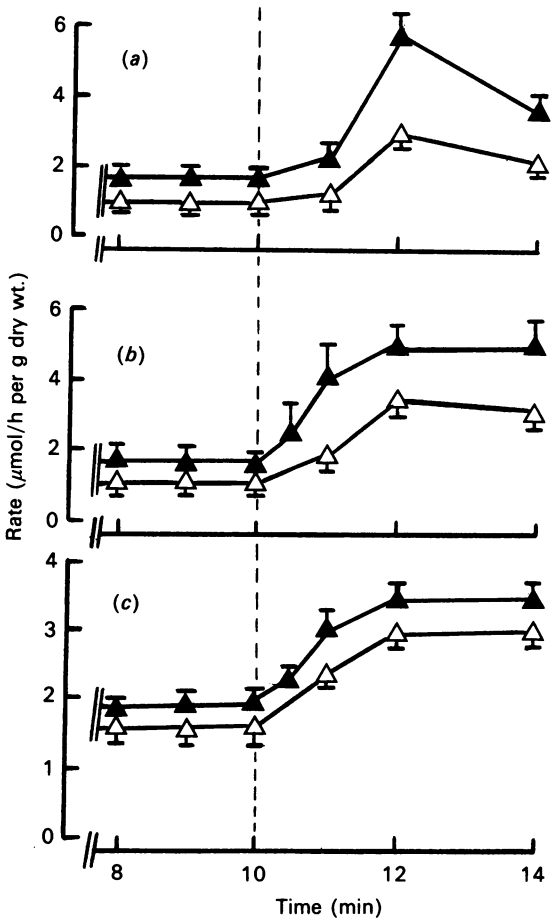

Fig. 1. Effects of Ruthenium Red on the rates of (*a*) lactate production, (*b*) CO_2 production and (*c*) O_2 uptake by the perfused rat heart in the absence and presence of isoprenaline

Hearts were perfused as described in the Materials and methods section in a 6 ml air-tight fluid-filled chamber at a constant flow rate (12 ml/min) for a total of 14 min in either the absence (▲) or the presence (△) of RR (2.5 μg/ml); 0.1 μM-(−)-isoprenaline was added after 10 min (as indicated by the vertical dashed line). Points and error bars represent means ± S.E.M. for at least three hearts in each case.

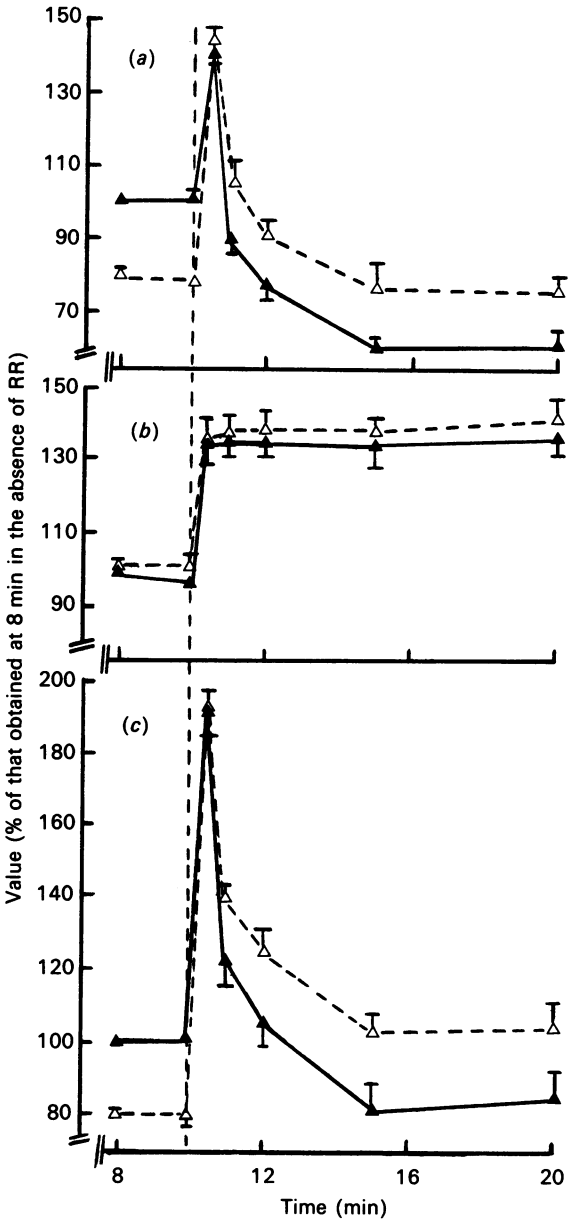

Fig. 2. Effects of Ruthenium Red on (*a*) contractile force, (*b*) beating frequency and (*c*) work output of the perfused rat heart in the absence and presence of isoprenaline

Hearts were perfused as described in the Materials and methods section and Fig. 1 legend, but without the chamber, and the contractile force and the cardiac frequency were measured at the peak of the length–tension curve as described by England (1976); work output was estimated as the product of these two parameters. Conditions and additions and expression of results are as in Fig. 1, except that data obtained in the presence of RR are also indicated by dashed lines for clarity.

This apparent anomaly may be due to the decrease in energy-dissipating Ca^{2+} cycling by RR at either the sarcoplasmic reticulum (Chamberlain *et al.*, 1984) or the mitochondria, although in the latter case this is likely to be too slow to contribute significantly (Crompton, 1985).

Effects of RR and isoprenaline on phosphorylated metabolites and pH

Fig. 3 illustrates typical ^{31}P-n.m.r. spectra obtained over 20 s time periods from hearts perfused in the absence (*a–c*) or presence (*d–f*) of RR. The effects of the presence of isoprenaline for two different time periods are also shown in Fig. 3. From such data, the free concentrations of ATP, PCr and P_i in the sarcoplasm can be calculated, as well as the pH and ADP concentration (see the Materials and methods section). Fig. 4 shows the time courses for the effects of RR (panel *b* versus panel *a*) on the response of all of these parameters to positive inotropic stimulation by isoprenaline.

In the absence of RR (Fig. 4*a*), the administration of isoprenaline caused a transient fall of about 50% in PCr concentration. However, this had returned to pre-stimulus values within about 1 min. A similar transient was previously noted in hearts which were freeze-clamped

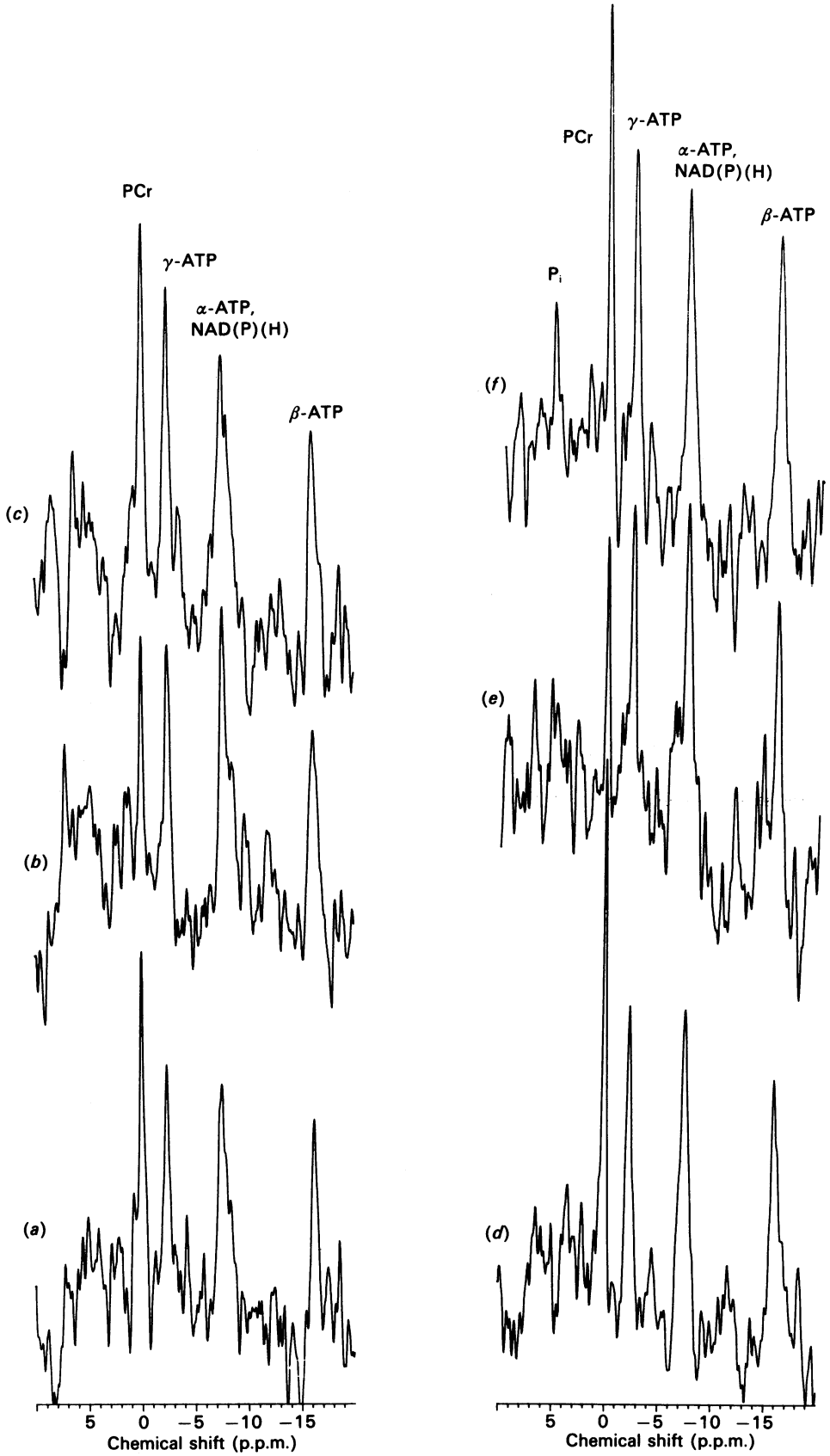

Fig. 3. Typical ^{31}P-n.m.r. spectra of perfused rat hearts in the absence and presence of Ruthenium Red and isoprenaline

Hearts were perfused in the n.m.r. probe as described in the Materials and methods section, and spectra shown were acquired over 20 s in either the absence (*a–c*) or the presence (*d–f*) of RR (2.5 μg/ml). Traces (*a*) and (*d*) represent control spectra and were acquired before addition of isoprenaline (0.1 μM); (*b*) and (*e*) were acquired over the first 20 s of the challenge, and (*c*) and (*f*) were acquired over 20 s, but 10 min after the addition of isoprenaline. Chemical shifts are shown in p.p.m. from the PCr signal; this signal and the positions of other major peaks are indicated.

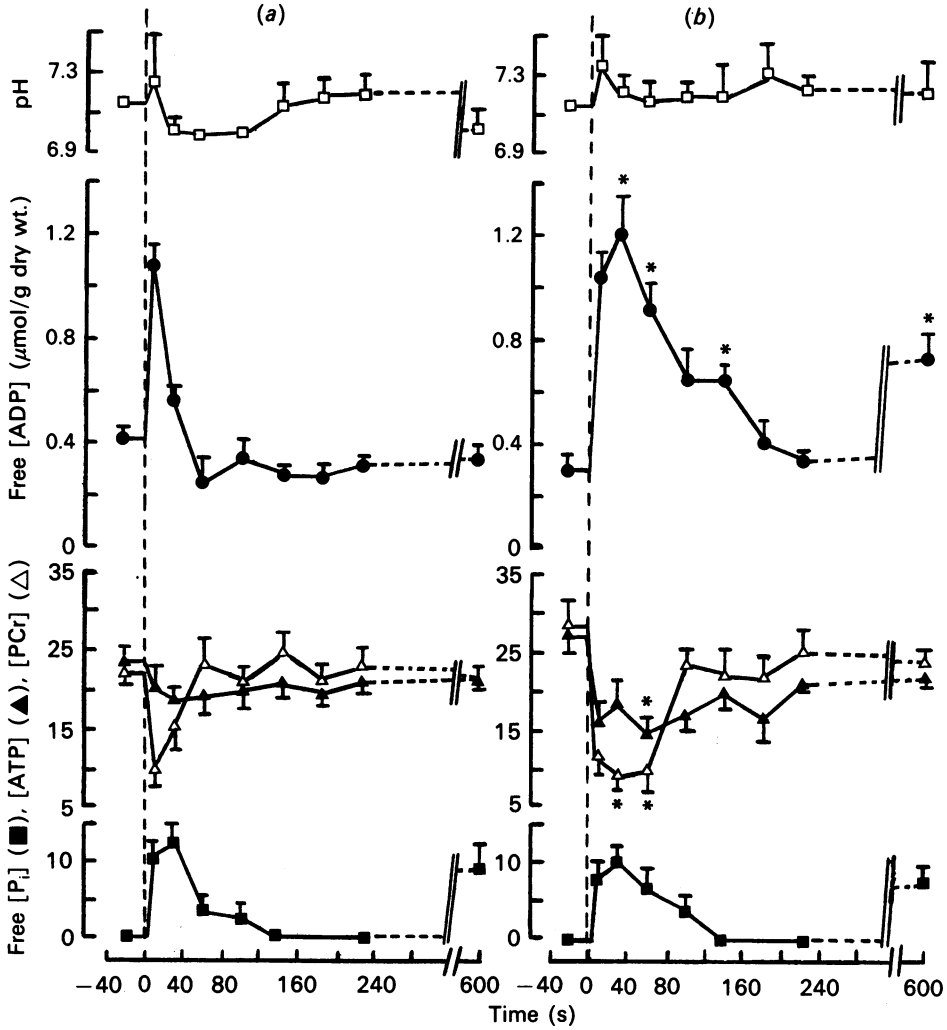

Fig. 4. Time courses of the effects of Ruthenium Red on the concentrations of free ATP, PCr, ADP and P_i, and on pH, in the rat heart perfused in the absence and presence of isoprenaline

Hearts were perfused in the magnet chamber as described in the Materials and methods section for a total of approx. 40 min. After stabilization (approx. 20 min; see the Materials and methods section) in control medium, perfusions were continued in either (*a*) the absence or (*b*) the presence of RR (2.5 μg/ml). At 10 min after this, 0.1 μM-L-isoprenaline was added; this is indicated as zero time in the Figures (dashed vertical lines). Results are expressed as means ± S.E.M. for at least four hearts in each case, and for free concentrations of ATP (▲), PCr (△), ADP (●) and P_i (■) and for pH (□). The data for ATP, PCr and P_i are given in the same arbitrary units; however, these allocated values approximate very well to μmol/g dry wt. (see From *et al.*, 1986). Also, 1 μmol/g dry wt. approximates to a concentration of about 400 μM, based on the assumptions given in From *et al.* (1986), i.e. wet-wt./dry-wt. ratio approx. 5.7 (From *et al.*, 1986), and a cytosolic volume of 0.44 ml/g wet wt. (Morgan *et al.*, 1964). *Indicates a significant difference of P at least $\leqslant 0.05$ for the effects of RR. The points shown correspond to the middle of the collection times.

and the PCr was measured enzymically (England & Shahid, 1987). There was a slight (< 20 %) but not significant depression in ATP concentration; however, there was a large (about 2.5-fold) increase in derived ADP concentration, although this was again very transient in nature and had returned to pre-stimulus values after about 1 min. There was also a transient increase in P_i; however, pH was largely unaffected (Fig. 4*a*). An important conclusion from the results shown in Fig. 4(*a*) is that immediately after the administration of isoprenaline there is a very transient decrease in phosphorylation potential, which may coincide with the peak of the stimulated work output (Fig. 2, Table 2). A second conclusion is that, after this brief transient period of about 1 min, there is a rapid return almost exactly to pre-stimulus values, while increases in oxidative metabolism are still clearly evident (Fig. 1*c*).

In contrast, in hearts perfused in the presence of RR (Fig. 4*b* versus 4*a*), before stimulation the concentrations of ATP and PCr were marginally, though not significantly, higher than in those hearts perfused in the absence of RR, and the derived concentration of ADP was slightly lower. This may reflect the decreased work under such circumstances (Fig. 2). After stimulation with isoprenaline in the presence of RR there was a decline in PCr concentration, of slightly larger magnitude than in

Table 2. Effects of Ruthenium Red on the heart cytoplasmic phosphorylation potential and ATP/ADP ratio before and after stimulation with isoprenaline

The values shown were calculated from the data shown in Fig. 4 and are means ± S.E.M. for four hearts in each case. Significant effects of RR are indicated by $*P \leqslant 0.05$ or $**P \leqslant 0.01$ respectively.

Time after isoprenaline (s)	$10^3 \times$ Phosphorylation potential (M^{-1})		ATP/ADP ratio	
	Without RR	With RR	Without RR	With RR
−10	> 20†	> 20†	47.8 ± 3.5	84.3 ± 5.8**
10	2.16 ± 0.57	2.24 ± 0.18	19.1 ± 2.4	13.2 ± 1.3
30	3.56 ± 0.66	1.65 ± 0.34*	29.2 ± 5.7	14.5 ± 1.9
60	17.3 ± 4.22	1.45 ± 0.39*	95.9 ± 10.6	17.9 ± 0.8**
600	15.0 ± 2.80	3.38 ± 0.47*	63.0 ± 5.4	30.4 ± 6.1**

† These values are estimated, as P_i was not detectable before the addition of isoprenaline (see Fig. 4).

the hearts without RR (Fig. 4*b*). However, the most notable features are that the transient period of the decline is much longer in the presence of RR (2–3 min as opposed to less than 1 min), and that after the transient period the concentration of PCr does not quite return to pre-stimulus values. Even more dramatic is that in the presence of RR there was a large fall in ATP concentration and that this did not return at all to pre-stimulus values. This is in direct contrast with the perfusions carried out in the absence of RR. These differences caused by RR are clearly illustrated by comparing its effects (Fig. 4*b* versus 4*a*) on calculated ADP concentration after the positive inotropic intervention. In contrast with the brief increase in ADP in the absence of RR, in its presence there was a larger and much more sustained increase. The increase in P_i after isoprenaline in the presence of RR was similar to that in its absence, except that the increase was slightly more sustained (Fig. 4). However, RR appeared to have little effect on the intracellular pH (Fig. 4), except perhaps to prevent the small transient acidosis which followed the even briefer alkalosis which was seen in the hearts perfused in its absence, after the stimulus.

The data in Fig. 4, and especially that in Fig. 4(*b*) in the presence of RR, would also indicate that after stimulation the decline in ATP content of the hearts cannot be completely accounted for by ADP formation (see Fig. 4 legend). It is plausible that this may be due to the further formation of IMP or adenosine or other such compounds (see, e.g., Sparks & Bardenheuer, 1986). Adenosine may act as a vasodilator and may also have some negative inotropic effects (e.g. Sparks & Bardenheuer, 1986), and this may have some bearing on the results obtained here (e.g. Fig. 2), although under the conditions used such compounds should be quickly washed out in the perfusate.

General discussion

The effects of RR on the calculated phosphorylation potential and ATP/ADP ratio before and during the positive inotropic intervention are shown in Table 2. It is clear that in the absence of RR there is a very transient fall in these parameters, followed by a rapid return to pre-stimulus values, under conditions where the hearts are still stimulated and oxidative metabolism is clearly elevated (Figs. 2 and 1 respectively). In contrast with this, it can be seen (Table 2) that in the presence of RR, firstly, the declines in phosphorylation potential and ATP/ADP ratio are more pronounced than in its absence, and secondly that these decreases are much more sustained. The latter point is similar in many respects to the data reported by Katz *et al.* (1988*a*) who also demonstrated a decline in ATP and PCr in the presence of RR, but none in its absence, in hearts which were subjected to a work-jump. However, in that study the spectra for the phosphorylated metabolites were gathered over a much longer time period (30–40 min) than in the present study. The advantage of the shorter period of data acquisition in the present study has also allowed the examination of the effects of RR (and of isoprenaline) during the initial phase of the stimulus as well as at longer times, and has thus permitted the demonstration of both transient and sustained effects.

Interpretation of the data reported in the present paper is entirely consistent with the proposal that a rise in the intramitochondrial concentration of Ca^{2+} as a result of the 'time-averaged' increases in the cytosolic concentration of Ca^{2+} in stimulated hearts (McCormack & Denton, 1986*b*) is an important part of the response of oxidative metabolism to the increased demands for ATP production under such circumstances. This interpretation of the data also offers a plausible mechanism for the means whereby the stimulated mammalian heart is able to maintain high ATP/ADP ratios (see references in the Introduction). This feature is clearly of advantage to the heart (and other) cells, and the proposed role of intramitochondrial Ca^{2+} would allow for increased respiratory rates to occur without the diminution of this key ratio. Clearly, when this mechanism is not available, as in the hearts perfused with RR, then control of the stimulation of the mitochondrial respiratory rate reverts principally to the phosphorylation potential or some component of this. It is likely that in unstimulated heart cells there will be very little Ca^{2+} in the mitochondrial matrix (McCormack *et al.*, 1989), and presumably the bulk of the control of respiration is exerted by the phosphorylation potential. However, there has been a report of changes in PDH_a within the contractile cycle of unstimulated dog hearts (Krause & Beyerdorfer, 1988) from about 10% of total PDH to 15%, and back again. The response of O_2 uptake (Fig. 1) suggests also that the mechanism involving intramitochondrial Ca^{2+} is required

for the maximal response of oxidative metabolism to occur.

The relative extent to which control is exerted by intramitochondrial Ca^{2+} in comparison with the phosphorylation potential is an important question (Brand & Murphy, 1987). It is clear, for instance, that even in the absence of RR (Fig. 4*a* and Table 2) there is an initial, albeit transient, fall in phosphorylation potential and PCr concentration on the introduction of isoprenaline, suggesting that this contributes to the regulation of the promotion of the initial response to the stimulus. In the presence of RR these changes are larger and more sustained. The full activation of PDH_a, which will determine rates of carbohydrate utilization, requires about 30–40 s from the initiation of the stimulus (McCormack & Denton, 1981). Therefore, during this initial phase it also may be necessary to decrease the phosphorylation potential. However, it should be noted that the isocitrate, citrate and 2-oxoglurate contents decrease within this time (Williamson, 1965; Neely *et al.*, 1972, 1976; Williamson *et al.*, 1976), which may reflect a more rapid activation of the non-covalently regulated NAD^+–isocitrate dehydrogenase and 2-oxoglutarate dehydrogenase. It may also take some time, on a beat-to-beat basis, for full transmission of the Ca^{2+} signal into the matrix (see Crompton, 1985; Hansford, 1985). However, in the later stages after stimulation, when the phosphorylated metabolites have returned to pre-stimulus values (Fig. 4*a*) (in the absence of RR), it is likely that intramitochondrial Ca^{2+} contributes a considerable proportion of the regulation of the stimulated respiratory rate. The lack of activation of PDH and carbohydrate utilization in the presence of RR may also affect the Respiratory Quotient (see Fig. 1).

In the previous work by McCormack & England (1983) it was reported that RR did not appear to affect contractile behaviour in the presence of positive inotropic stimuli. In the present work, in fact, slight increases in cardiac work were observed in the presence of RR and isoprenaline when compared with isoprenaline alone (Fig. 2). This may indeed contribute to increased ATP utilization and hence the decline in phosphorylation potential under such conditions. However, it is also worth suggesting that the reason for this behaviour is that in the presence of RR Ca^{2+} cannot enter the mitochondria, and therefore the proportion of the released Ca^{2+} pool which normally enters the mitochondria remains in the cytosol and further stimulates contraction when compared with controls. This offers evidence that a proportion of the Ca^{2+} released into the cytoplasm for contraction is actually normally designated for entry into the mitochondria. It is of interest therefore that cardiac muscle is thought to never quite reach saturation of the its pCa–tension curve (Fabiato, 1981). Hansford (1987) reported some similar findings in measuring cytoplasmic Ca^{2+} in the absence and presence of RR in quin-2-loaded myocytes. Also in this context, it has been noted previously that, after perfusion with high doses of positive inotropes, there is some deterioration of contractile performance after a few minutes (e.g. England & Shahid, 1987; see Fig. 2). The reason for this is unclear, but cannot be completely ascribed to receptor down-regulation (England & Shahid, 1987). It may be that some Ca^{2+} overload occurs, causing a slow and gradual deterioration, and that this may be protected against by RR.

RR has been shown to have potential cardio-protective properties under certain pathophysiological circumstances, such as ischaemia–reperfusion (see, e.g., Smith, 1980; Peng *et al.*, 1980; Ferrari *et al.*, 1982), and some of the observations reported here may be relevant to this. However, it is probable that these cardio-protective properties are due largely to the prevention of mitochondrial Ca^{2+} overload and their resultant damage (see McCormack, 1985), as under such circumstances there is thought to be abnormal Ca^{2+} influx across the sarcolemma (see the references above).

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