Ca²⁺ is a widely used hormonal second-messenger in the cytosol of mammalian cells [1]. The processes stimulated by increased cytosolic Ca²⁺ are often energy-requiring, e.g. contraction, secretion, yet rarely are decreases in cellular ATP/ADP ratios observed under such circumstances [2]. There is now good evidence that a compensatory increase in oxidative metabolism and hence ATP production is responsible for maintaining energy homeostasis under such conditions which involves a relay of the Ca²⁺ signal into the mitochondrial matrix [3]. Ca²⁺ is thus a key regulator of intramitochondrial oxidative metabolism in mammalian cells through its activation of the pyruvate (PDH), NAD⁺-isocitrate, and 2-oxoglutarate dehydrogenases [3] which are major sites of NADH production for the respiratory chain. Ca²⁺ activates PDH by increasing the amounts of active, non-phosphorylated enzyme (PDHₙ) through its stimulation of PDH phosphate phosphatase, whereas the other two enzymes are activated non-covalently through marked decreases in their Kₘ values for isocitrate and oxoglutarate respectively.

In extracts or after purification, the Ca²⁺ activatory ranges for the enzymes is approximately 0.1-3μM, although NAD⁺-isocitrate dehydrogenase may respond to a higher range 141. The fluorescent 
Ca²⁺ indicator fura 2 can be loaded into isolated rat heart mitochondria and can thus be used to measure matrix Ca²⁺ directly, and hence to correlate this with the activities of these Ca²⁺-dependent enzymes in intact, viable mitochondria [5,6]. There are also reports that some fura 2 localises to mitochondria in intact cells from heart and other mammalian tissues (e.g. [7,8]).

We have previously shown that the positive inotropic activation of the perfused rat heart (via raised cytosolic Ca²⁺) is associated with increases in the amounts of PDHₙ [9]. Moreover, these activations of PDH were found to persist through to isolated and then subsequently incubated (at 30°C in KCl-based buffer containing EGTA) mitochondria [10]. Parallel activations of 2-oxoglutarate dehydrogenase were also observed in mitochondria prepared from stimulated hearts, and others showed that these activations were accompanied by increases in total mitochondrial Ca content within the range associated with enzyme activation (0-5 nmol/mg protein) [11]. Furthermore these persistent activations could be diminished by adding Na⁺ ions to the incubations to promote Ca²⁺-egress from the mitochondria [10].

The present studies were therefore undertaken to see whether some fura 2 could be loaded into the mitochondrial matrix in intact perfused hearts, and whether this could be used to report on any persistent changes in matrix Ca²⁺ concentration (due to prior inotropic intervention) in subsequently isolated mitochondria.

Male rat (200-250g) hearts were perfused at 37°C with a gassed (O₂/CO₂; 19/1) Krebs-bicarbonate buffer containing 10mM glucose [9]. After an initial 5min flow-through period, the perfusions were switched to a (pumped) 50ml re-circulating system and the acetoxymethyl ester of fura 2 (fura 2/AM) added to a concentration of 10μM. Perfusions were then continued at 12ml/min for 5min, and then for a further 2min with no change (control), or for 2min at 24ml/min to produce a positive inotropic response ('high work'). A small portion of each heart (0.1-0.2g) was then quickly freeze-clamped for subsequent tissue PDH analysis (as in [9]), and the remainder rapidly homogenised, under conditions and in buffer designed to minimise any artefactual Ca²⁺ movements across the mitochondrial inner membrane [10]. After mitochondrial isolation [10], matrix entrapped fura 2 was evident (results not shown) and this was then used to measure matrix Ca²⁺ and was calibrated as described previously [6].

Table 1. Effects of positive inotropy on the amounts of PDHₙ in the perfused rat heart and in subsequently isolated mitochondria

<table>
<thead>
<tr>
<th>Sample</th>
<th>Control</th>
<th>High work</th>
</tr>
</thead>
<tbody>
<tr>
<td>freeze-clamped heart</td>
<td>0.56±0.22</td>
<td>0.92±0.19a</td>
</tr>
<tr>
<td>freshly-isolated mitochondria</td>
<td>20±9</td>
<td>40±11a</td>
</tr>
</tbody>
</table>

Table 1 shows that the high-work condition caused about a twofold increase in the amount of PDHₙ in the perfused rat heart, and that this increase caused by tissue pretreatment largely persisted through to the level of isolated mitochondria. This degree of activation is about 50% of the full Ca²⁺-dependent response (see [9]), and could also be maintained in mitochondria incubated for 5min at 30°C in KCl-based buffer containing respiratory substrates and EGTA (not shown, but as shown previously [10]).

Preliminary experiments (not shown) revealed that perfusion with 10μM fura 2/AM for 1h gave good loading of fura 2 into mitochondria. The mitochondria were then incubated (see [6]) in a fluorimeter at appropriate wavelength settings [6] with a stirring cuvette unit at 30°C and in KCl-based media containing 10mM-2-oxoglutarate, 0.5M-malonate and 0.5mM EGTA. After a 2min stabilisation period [6], the signal obtained was then used to obtain estimates of matrix Ca²⁺ as fully described previously [6]. The values obtained in mM (as means ± s.e.m. (n)) for the mitochondria prepared from control and 'high-work' hearts respectively were 187±40(10) and 682±149(6) (p<0.05, unpaired t-test). These values are entirely consistent with matrix Ca²⁺ being responsible for the degrees of PDH activation seen under the two conditions (Table 1, [6]). These differences in both Ca²⁺ and PDH due to 'high-work' pretreatment (versus controls) were diminished by adding Na⁺ (10mM) to incubations (not shown, see above, [10]).

The results obtained provide strong supporting evidence that the activation of PDH by positive inotropes is at least partly due to raised intramitochondrial Ca²⁺, and that such increases can be maintained through to isolated and suitably incubated mitochondria.

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