Intramitochondrial free calcium in cardiac myocytes in relation to dehydrogenase activation

Fabio Di Lisa, Giovanni Gambassi, Harold Spurgeon, and Richard G Hansford

Objective: The aim was to quantitate intramitochondrial free Ca\(^{2+}\) ([Ca\(^{2+}\)\(_{m}\)]) in cardiac myocytes under conditions of stimulation previously shown to cause activation of pyruvate dehydrogenase. Methods: [Ca\(^{2+}\)\(_{m}\)] was monitored in single, isolated rat cardiac myocytes using fluorescence microscopy following the loading of the cells with the fluorescent chelating agent indo-1, in its permeant acetoxy methylester form, and the selective quenching of cytosolic fluorescence with MnCl\(_2\). The extent of contraction upon electrical stimulation was also measured. Results: Electrical stimulation at 2 Hz and higher frequency raised [Ca\(^{2+}\)\(_{m}\)] significantly, and this was potentiated by exposure to isoprenaline. However, isoprenaline had no effect in quiescent cells, in which [Ca\(^{2+}\)\(_{m}\)] was raised above resting values by partial replacement of Na\(^+\) in the medium. The mitochondrial uncoupling agent carboxylic acid p-trifluoromethoxyphenylhydrazone (FCCP) raised [Ca\(^{2+}\)\(_{m}\)] in unstimulated cells, but lowered it in cells subjected to electrical stimulation at 2 Hz or more, to partial Na\(^+\) replacement, or to the alkaloid veratridine. Conclusions: The values of [Ca\(^{2+}\)\(_{m}\)] in quiescent myocytes (approximately 100 nmol.litre\(^{-1}\)) would be associated with very little activation by Ca\(^{2+}\) of pyruvate dehydrogenase phosphatase, based on determination of Ki, value of 650 nmol.litre\(^{-1}\) in work with mitochondrial suspensions. By contrast, the values of [Ca\(^{2+}\)\(_{m}\)] associated with electrical stimulation at 2 Hz or greater in the presence of β adrenergic activation (>500 nmol.litre\(^{-1}\)) would be associated with significant dehydrogenase activation. The effect of β adrenergic activation is only seen in the presence of electrical stimulation and probably involves enhancement of systolic transients in cytosol [Ca\(^{2+}\)]. Effects of uncoupling agents validate the conclusions on the direction and magnitude of the mitochondrial Ca\(^{2+}\) gradient in situ in living myocytes.

Cardiovascular Research 1993;27:1840-1844

A model has been advanced in which the activation of pyruvate, NAD isocitrate, and 2-oxoglutarate dehydrogenases by Ca\(^{2+}\) allows the signalling of increased work performance by the heart to the tricarboxylic cycle, resulting in more active substrate dehydrogenation and oxidative phosphorylation.\(^1\)\(^-\)\(^4\) As all three of these enzyme systems are present in the mitochondrial matrix, that is, within the permeability barrier of the inner mitochondrial membrane, increases in cytosolic free Ca\(^{2+}\) associated with increased contractility have to be transduced into increases in mitochondrial matrix free Ca\(^{2+}\) ([Ca\(^{2+}\)\(_{m}\)],) by the operation of the mitochondrial Ca\(^{2+}\) transport cycle.\(^4\) Until very recently, it has been impossible to confirm the control significance of [Ca\(^{2+}\)\(_{m}\)] in situ in living cells, because it has not been possible to measure this variable. Instead, one time destructive techniques have been applied to the measurement of total Ca\(^{2+}\) content of mitochondria isolated rapidly from perfused hearts and have shown it to increase with protocols producing a positive inotropic effect on the heart and in response to increased frequencies of electrical stimulation.\(^5\)\(^-\)\(^6\) The recent development of a technique allowing the measurement of [Ca\(^{2+}\)\(_{m}\)] in single, isolated cardiac myocytes has enabled us to examine further the role of [Ca\(^{2+}\)\(_{m}\)] as a signal for enhanced substrate oxidation.\(^7\) In the previous communication from this laboratory,\(^7\) it was shown that electrical stimulation of cardiac myocytes at 2, 3, and 4 Hz raised [Ca\(^{2+}\)\(_{m}\)] in the presence of β adrenergic stimulation. Here we isolate the effects of electrical stimulation from those of β adrenergic activation, showing the profound importance of the latter.

Further, we report a series of experiments in which the addition of mitochondrial uncoupling agents to the myocytes allows the description of the direction of the mitochondrial Ca\(^{2+}\) concentration gradient, that is, whether [Ca\(^{2+}\)\(_{m}\)]/[Ca\(^{2+}\)\(_{cyt}\)] is greater than 1 or not, under conditions of both electrical excitation of the cells and graded chronic depolarisation of the sarcolemma. These results provide independent confirmation of estimates of [Ca\(^{2+}\)\(_{m}\)]/[Ca\(^{2+}\)\(_{cyt}\)] based upon measurement of the former with indo-1 acetoxy methyl-ester (indo-1:AM) loaded and Mn\(^{2+}\) quenched cells and the latter with indo-1: salt loaded cells. The introduction of the pentapotassium salt of indo-1 into the cells, by the process described by Sollott et al,\(^8\) allows measurement of fluorescence from the cytosol only, whereas use of the membrane permeant indo-1:AM generates fluorescence from both cytosol and mitochondrial compartments. Finally, we relate values of [Ca\(^{2+}\)\(_{m}\)] measured in this study of myocytes to the sensitivity of pyruvate dehydrogenase interconversion to [Ca\(^{2+}\)\(_{m}\)] as measured with suspensions of indo-1 loaded rat heart mitochondria.\(^9\) Methods

Single cardiac myocytes were isolated by perfusion of rat hearts with collagenase containing medium, as previously described.\(^10\) Cells were loaded with indo-1 by incubation at 23°C in a HEPES buffered medium (composition in mmol.litre\(^{-1}\): NaCl 154, KCl 5, MgSO\(_4\) 1.2, NaH\(_2\)PO\(_4\) 1.2, D-glucose 11, CaCl\(_2\) 1.5, and Na HEPES 10) containing 25 μmol.litre\(^{-1}\) indo-1:AM. After 30 min of exposure to indo-1:AM, cells were washed by sedimentation and resuspension in the same medium, lacking indo-1:AM, and allowed a further 45-60 min for de-esterification of the indo-1:AM prior to study.

Laboratory of Cardiovascular Science, Gerontology Research Center, National Institute on Aging, National Institutes of Health, 4940 Eastern Avenue, Baltimore, MD 21224, USA: F Di Lisa, G Gambassi, H Spurgeon, R G Hansford. Correspondence to Dr Hansford.
Single cells were studied in a custom designed fluorescence microscope described in detail in a previous publication. The cells were superfused in a bicarbonate buffer comprising (mmol. litre⁻¹): NaCl 137, KCl 5, MgSO₄ 1.2, NaH₂PO₄ 1.2, D-glucose 11, CaCl₂ 1.5, and NaHCO₃ 25, at pH 7.4. A gas phase of 95% O₂, 5% CO₂ was maintained. Fluorescence emission was collected at 391 to 434 nm, corresponding to the calcium bound form of indo-1, and at 477 to 507 nm, corresponding to the calcium-free form. These wavelengths bands are referred to hereafter as the 410 and 490 nm channels.

Progressive exposure of the cells to MnCl₂, then selectively quenched the fluorescence signal from the cytosol. Proof that causes no rise in $[\text{Ca}^{2+}]_{i}$, when applied to a cell stimulated-the remaining Mn⁺⁺ resistant fluorescence originated from mitochondria. Exposure of cardiac myocytes to indo-lactate dehydrogenase. Figure 1 presents the representative results from experiments of this type are compiled and analysed statistically in the table and confirm the relatively minor response of $[\text{Ca}^{2+}]_{i}$, to electrical stimulation and the very significant potentiation of this effect by β adrenergic stimulation. Although the most complete series of experiments was carried out at 25°C, some studies were conducted at 37°C to allow the cells to follow high rates of pacing (4 Hz) without the need for β adrenergic stimulation. It is clear from these studies at 37°C that stimulation at 4 Hz itself is not sufficient to generate substantial values of $[\text{Ca}^{2+}]_{i}$, These results extend previous findings from this laboratory, by allowing this separation of the effects of electrical stimulation and β adrenergic stimulation upon $[\text{Ca}^{2+}]_{i}$.

The effect of isoprenaline in increasing $[\text{Ca}^{2+}]_{i}$, could be due to either an enhancement of the systolic transients in $[\text{Ca}^{2+}]_{i}$, concentration³ or a stable change at the level of the mitochondrial membrane, resetting the balance of $\text{Ca}^{2+}$ uniport and antiport activities (for review see Gunter and Pfeiffer). Although the latter seemed less likely in view of the failure of isoprenaline to raise $[\text{Ca}^{2+}]_{i}$, when a cell was stimulated at 0.2 Hz (fig 2), we re-examined this issue with cells in which both $[\text{Ca}^{2+}]_{i}$, and $[\text{Ca}^{2+}]_{m}$, had been raised in chronic depolarization protocols. When Na⁺ ions in the medium were largely replaced with K⁺, it was found that addition of 1 × 10⁻⁶ M isoprenaline had no effect in raising $[\text{Ca}^{2+}]_{i}$, in cells not subjected to electrical stimulation (result not shown). We therefore favour the view that β adrenergic...
stimulation increases \([Ca^{2+}]_{in}\) through an enhancement of the amplitude of systolic transients in \([Ca^{2+}]_{e}\).

One of the remarkable things to emerge from the previous communication from this laboratory\(^7\) is that, at low pacing rates (ie, <2Hz), \([Ca^{2+}]_{in}\) is actually lower than \([Ca^{2+}]_{e}\) — that is, the \(Ca^{2+}\) concentration gradient across the mitochondrial membrane (\([Ca^{2+}]_{in}/[Ca^{2+}]_{e}\)) is less than unity. Although consistent with findings from suspensions of heart mitochondria loaded with indo-1 :AM and exposed to known values of extramitochondrial \([Ca^{2+}]_{e}\)\(^9\)\(^13\), this conclusion runs counter to most published reports, which assume that mitochondria are “Ca\(^{2+}\) stores”. In the work by Miyata \textit{et al}’\(^1\) our conclusion was based upon a comparison of values of \([Ca^{2+}]_{in}\), measured as described here, with values of \([Ca^{2+}]_{e}\), measured in different cells loaded with indo-1 :salt.

To confirm these conclusions without using different cells, we added the mitochondrial uncoupling agent carbonyl-cyanide p-trifluoromethoxyphenylhydrazone (FCCP) to cardiac myocytes exposed to different stimuli. The rationale was that, by collapsing the proton electrochemical gradient across the mitochondrial membrane which energises both processes of the \(Ca^{2+}\) transport cycle\(^4\), FCCP would be expected to equilibrate \([Ca^{2+}]_{in}\) and \([Ca^{2+}]_{e}\). It is seen in Fig 3 that addition of FCCP leads to a small increase in \([Ca^{2+}]_{in}\) in a cell stimulated at 0.2 Hz, whereas it leads to a decrease in a cell stimulated at 4 Hz in the presence of \(1 \times 10^{-8}\) M isoprenaline. This is consistent with a gradient \([Ca^{2+}]_{in}/[Ca^{2+}]_{e}\) of less than 1 in the former instance and more than 1 in the latter, prior to the moment of addition of the uncoupling agent.

Chronic depolarisation of the plasma membrane by the alkaloid veratridine, which favours the open state of Na\(^+\) channels\(^14\), was found to give prompt and massive increases in \([Ca^{2+}]_{in}\) (Fig 4). That the mitochondrial \(Ca^{2+}\) gradient, \([Ca^{2+}]_{in}/[Ca^{2+}]_{e}\), is greater than 1 under these conditions is illustrated by the rapid decrease in \([Ca^{2+}]_{in}\) on adding FCCP. When data are compiled from all cells which were treated with FCCP, it becomes apparent that the direction of change of \([Ca^{2+}]_{in}\) upon uncoupling depends solely on the pre-existing value of \([Ca^{2+}]_{in}\) regardless of whether the cell was stimulated electrically or subjected to chronic depolarisation (Fig 5).

**Discussion**

The fraction of pyruvate dehydrogenase existing in the active, dephospho form (PDH\(_a\)) is increased with increasing \([Ca^{2+}]_{in}\), owing to an effect of \(Ca^{2+}\) on the binding of the phosphatase to the transacetylase core of the multisubunit enzyme complex.\(^15\)\(^16\) At the same time, the PDH\(_a\) content of isolated perfused hearts is increased by \(\beta\) adrenergic stimulation,\(^17\)\(^18\) the increase of perfusion fluid \(Ca^{2+}\) or the increase of pacing frequency or of afterload.\(^19\) Rapid isolation of mitochondria from perfused hearts using a technique\(^5\) which minimises loss or gain of mitochondrial \(Ca^{2+}\) has shown that total mitochondrial \(Ca^{2+}\) content is increased in response to raised perfusion fluid \(Ca^{2+}\); electrical stimulation,\(^6\) or \(\beta\) adrenergic stimulation.\(^2\) As there is an approximately linear relationship between \([Ca^{2+}]_{in}\) and
total mitochondrial Ca\(^{2+}\) in this concentration range,\(^{20}\) these results can be taken to indicate that the raised content of PDH\(_{m}\) in the stimulated heart is associated with an increase in the modulator [Ca\(^{2+}\)\(_{m}\)]. However, pyruvate dehydrogenase interconversion is also sensitive to other effectors\(^{21}\) and a raised PDH\(_{m}\) content could also reflect decreases in mitochondrial ATP/ADP or NADH/NAD\(^{+}\) ratio. With this in mind, we have sought to relate enzyme activation, as studied in suspensions of mitochondria, to values of [Ca\(^{2+}\)\(_{m}\)] measured in single cardiac myocytes in response to various imposed workloads.

Work with suspensions of rat heart mitochondria, loaded with indo-1 and allowed to respire in the presence of Ca\(^{2+}\) buffered at 10\(^{-7}\) to 10\(^{-6}\) M in the medium, has established that the K\(_{0.5}\) for pyruvate dehydrogenase activation is 0.65 \(\mu\)M [Ca\(^{2+}\)\(_{m}\)]\(^{13}\) a figure remarkably close to that determined for the activation of the phosphatase in extracts and in toluene permeabilised mitochondria.\(^{2,3}\) How do values of [Ca\(^{2+}\)\(_{m}\)] measured in situ in surviving cardiac myocytes compare with this number? It is clear that values of [Ca\(^{2+}\)\(_{m}\)] recorded in cells stimulated at 2 Hz or less (table) would have little effect in activating pyruvate dehydrogenase phosphate. By contrast, stimulation at 4 Hz in the presence of \(\beta\) adrenergic stimulation would give significant activation (table). Although PDH\(_{m}\) content would be expected to be only slightly more than 50% of maximum under these conditions, it is noted that in the living animal the cells would be subjected to frequencies in excess of 4 Hz and would be subject to mechanical loading not preserved in experiments with isolated myocytes.

Clearly, Ca\(^{2+}\)\(_{m}\) is very sensitive to \(\beta\) adrenergic activation in contracting cells. The mechanism is inferred to be the generation of larger systolic transients in Ca\(^{2+}\)\(_{m}\). This has been shown in studies with myocytes loaded with indo-1: salt, which allows the monitoring of [Ca\(^{2+}\)],.\(^{11}\) As the heart mitochondrial Ca\(^{2+}\) uniporter shows sigmoidal kinetics\(^{4}\) towards extramitochondrial Ca\(^{2+}\), an increase in [Ca\(^{2+}\)], due to \(\beta\) adrenergic activation would be expected to give a more than proportionate increase in the activity of Ca\(^{2+}\) uptake. Other studies with suspensions of cardiac myocytes, in which it is possible to make measurements of enzyme activity, have shown that chronic depolarisation by Na\(^{+}\) replacement with K\(^{+}\) or by exposure to the alkaloid veratridine results in increases in the fractional content of PDH\(_{m}\).\(^{22}\) Here we confirm the results of Miyata et al.\(^{2}\) to the effect that Na\(^{+}\) replacement increases [Ca\(^{2+}\)\(_{m}\)], and extend these findings to veratridine treated cells (fig 4). This is a paradigm which we have used in experiments with suspensions of cardiac myocytes not only to activate pyruvate dehydrogenase but also to stimulate O\(_{2}\) uptake in the presence of glucose as substrate.\(^{23}\) The latter finding implies that ATP/ADP ratios fell, presumably as a function not only of increased Na\(^{+}/K^{+}\)-ATPase activity but also of myofibrillar Ca\(^{2+}\)-ATPase. For this reason, we hesitate to draw quantitative conclusions on the activation of pyruvate dehydrogenase by [Ca\(^{2+}\)\(_{m}\)] in chronically depolarised myocytes, as there may be changes in effectors other than [Ca\(^{2+}\)\(_{m}\)].

The experiments involving FCCP addition are informative, as they show that [Ca\(^{2+}\)\(_{m}\)/Ca\(^{2+}\)], can be less than unity. This suggests some caution in interpretation of many published reports involving a variety of cell types, in which addition of an uncoupling agent is assumed to release mitochondrial Ca\(^{2+}\). However, it is also true that the gradient [Ca\(^{2+}\)\(_{m}\)/Ca\(^{2+}\)] is likely to be greater than one when the myocyte is stimulated at in vivo frequencies. Similarly, this gradient may well be less than 1 in resting cells from non-excitatory tissues, but become positive in response to hormonal activation of the cell. This seems to be the case in very recent work on [Ca\(^{2+}\)\(_{m}\)] in endothelial cells transfected with the gene for the luminescent protein aequorin and challenged by the agonist ATP.\(^{24}\)

Finally, although increases in [Ca\(^{2+}\)\(_{m}\)] are discussed in terms of activation of pyruvate dehydrogenase in this paper, such increases will also activate NAD-isocitrate dehydrogenase\(^{25}\) and 2-oxoglutarate dehydrogenase\(^{26}\) and thus the operation of the tricarboxylate cycle. PDH\(_{m}\) content is used as an indicator of the degree of dehydrogenase activation which is occurring, as the state is stable to tissue extraction. By contrast, activation of the other two dehydrogenases by Ca\(^{2+}\) involves an allosteric mechanism which is less easily measured following tissue extraction. The sensitivity of 2-oxoglutarate dehydrogenase to Ca\(^{2+}\) is similar to that of pyruvate dehydrogenase phosphate, whereas NAD-isocitrate dehydrogenase is somewhat less sensitive (see McCormack et al for review).

The activity of the tricarboxylate cycle is clearly of profound importance for the energetics of the heart, which prefers fatty acid as an oxidisable substrate.

Key terms: oxidative phosphorylation, regulation of; pyruvate dehydrogenase; indo-1.

Received 23 December 1992; accepted 21 May 1993. Time for primary review 42 days.

5 McCormack JG, Denton RM. Role of Ca\(^{2+}\) ions in the regulation of intramitochondrial calcium in rat heart. Evidence from studies with isolated mitochondria that adrenaline activates the pyruvate dehydrogenase and 2-oxoglutarate dehydrogenase complexes by increasing the intramitochondrial concentration of Ca\(^{2+}\). Biochem J 1984;218:335-47.