Effect of Inotropic Stimulation on Mitochondrial Calcium in Cardiac Muscle*

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Ca\textsuperscript{2+}-dependent activation of citric acid cycle enzymes has been demonstrated in isolated cardiac mitochondria. These observations led to the hypothesis that Ca\textsuperscript{2+} is the signal coupling myofibrillar energy use to mitochondrial energy production in vivo. To test this hypothesis we have measured mitochondrial Ca\textsuperscript{2+} content during increased energy demand, using electron probe microanalysis. Mitochondrial Ca\textsuperscript{2+} was measured in hamster papillary muscles rapidly frozen at the peak rate of tension rise under control conditions and after stimulation with the \(\beta\)-adrenergic agonist isoproterenol (10\textsuperscript{-5} M). A third group of muscles was frozen after incubation in low (45.5 mM) Na\textsuperscript{+} solution to Ca\textsuperscript{2+} load the cells. Pyruvate dehydrogenase activity was measured in each of the muscles. Isoproterenol caused a 39% increase in force and a 43% increase in pyruvate dehydrogenase activity compared with control (0.54 ± 0.12). In contrast, low Na\textsuperscript{+} increased pyruvate dehydrogenase activity by 56% and also elevated mitochondrial Ca\textsuperscript{2+} to 1.28 ± 0.31 (\(p < 0.02\)). These results demonstrate that mitochondrial Ca\textsuperscript{2+} is not elevated after inotropic stimulation. Cardiac muscle by \(\beta\)-adrenergic agonists although pyruvate dehydrogenase is increased. We conclude that Ca\textsuperscript{2+} uptake by mitochondria is not a requirement for activation of mitochondrial respiration after increased energy demand.

Cardiac muscle depends almost exclusively upon mitochondrial oxidative phosphorylation to supply its energy needs for contraction (Kobayashi and Neely, 1979; Randle and Tubbs, 1979). A mechanism must therefore exist whereby mitochondria regulate their rate of ATP production in response to changes in energy demand, as occurs after inotropic stimulation. The means by which mitochondria regulate ATP synthesis have, for many years, been an area of intense investigation. After the classical demonstration of mitochondrial respiratory control by ADP in isolated mitochondria (Chance and Williams, 1955), much attention has focused on the roles of ATP, ADP, and P, as regulators of mitochondrial metabolism in vivo (Giesen and Kammermeier, 1980; Hassinen, 1986; McMillin and Pauly, 1988). However, a number of studies performed in isolated perfused hearts (Wikman-Coffelt et al., 1985; From et al., 1986) or in vivo by \(31P\) NMR (Katz et al., 1980) have not been able to demonstrate a consistent relationship between changes in the concentration of ATP and its metabolites and changes in cardiac performance. Thus, additional regulatory mechanisms have been sought to explain the tight coupling between cellular energy use and mitochondrial energy production.

One hypothesis that has recently gained support is the proposal that uptake of Ca\textsuperscript{2+} by mitochondria is a necessary step in the activation of mitochondrial metabolism. It has been proposed that the increase in cytoplasmic Ca\textsuperscript{2+} which occurs upon hormonal stimulation results in an uptake of Ca\textsuperscript{2+} into mitochondria (Hansford, 1985; McCormack and Denton, 1984). This increase in mitochondrial Ca\textsuperscript{2+} is believed to activate key Ca\textsuperscript{2+}-sensitive regulatory enzymes of the citric acid cycle such as pyruvate dehydrogenase (McCormack and Denton, 1984; Hansford, 1985) and \(\alpha\)-ketoglutarate dehydrogenase (Denton et al., 1972). The data supporting this hypothesis come from several studies performed in isolated mitochondria loaded with fura-2 (Lukacs et al., 1988; Davis et al., 1987; McCormack et al., 1988; Wan et al., 1989) or indo-1 (Moreno-Sanchez and Hansford, 1988). These studies have demonstrated that increases in mitochondrial matrix free Ca\textsuperscript{2+} concentration occur in concert with increases in pyruvate dehydrogenase or \(\alpha\)-ketoglutarate dehydrogenase activity over a physiological range of extramitochondrial Ca\textsuperscript{2+} concentrations (Moreno-Sanchez and Hansford, 1988; McCormack et al., 1989; Lukacs et al., 1988; Wan et al., 1989).

There is little direct evidence, however, that Ca\textsuperscript{2+} uptake by mitochondria is a physiological regulator of mitochondrial metabolism in vivo. Although pyruvate dehydrogenase has been shown to be activated after stimulation of cardiac muscle with \(\beta\)-adrenergic agonists (Hiraoka et al., 1980; McCormack and Denton, 1981, 1984), increases in mitochondrial Ca\textsuperscript{2+} after \(\beta\)-adrenergic stimulation have only been observed after mitochondrial isolation (Crompton et al., 1983; McCormack and Denton, 1984). An increase in mitochondrial Ca\textsuperscript{2+} upon inotropic stimulation of cardiac muscle has also been inferred from an increase in the size of the uncoupler-releasable Ca\textsuperscript{2+} pool (Wolska and Lewartowski, 1991) and from the observation that treatment of isolated myocytes or perfused hearts with ruthenium red prevents the increase in pyruvate dehydrogenase activity (Hansford, 1987; Unitt et al., 1989; McCormack and Engvall, 1983). Because of the possibility of Ca\textsuperscript{2+} redistribution occurring during mitochondrial isolation and/or the fact that a subpopulation of Ca\textsuperscript{2+}-loaded mitochondria may artificially elevate the mean Ca\textsuperscript{2+} value mea-
ured or inferred, extrapolation of the Ca\(^{2+}\) content measured in isolated mitochondria or determined from the size of an uncoupler-releasable pool to the intact cardiac muscle may not be valid. An additional complication is that ruthenium red is not a specific inhibitor of mitochondrial Ca\(^{2+}\) uptake but also blocks Ca\(^{2+}\) release from the sarcoplasmic reticulum (Chamberlain et al., 1984) and binds to negatively charged sites on the sarcolemma (Gupta et al., 1988). Therefore the site of action of ruthenium red in the experiments described above cannot be determined with certainty. It therefore remains to be determined, by direct measurement of mitochondrial Ca\(^{2+}\) content in cardiac muscle in situ, whether the increased energy required after inotropic stimulation is accompanied by, and dependent upon, an increase in mitochondrial Ca\(^{2+}\).

We have previously measured the Ca\(^{2+}\) content of cardiac mitochondria in situ by electron probe microanalysis (EPMA) during the peak of contraction and during relaxation. Our data demonstrate that mitochondrial Ca\(^{2+}\) is not elevated during a single cardiac muscle twitch (Moravec and Bond, 1991). In the current study we have extended these investigations to the question of whether mitochondrial Ca\(^{2+}\) uptake occurs during inotropic stimulation of contracting cardiac muscle. The experimental approach utilized in these studies, rapid freezing of contracting papillary muscles followed by EPMA on cyrosections of the frozen tissue, avoids the potential problems inherent in the measurement of Ca\(^{2+}\) after mitochondrial isolation. Our results reveal that although uptake of Ca\(^{2+}\) by mitochondria can occur in Ca\(^{2+}\)-overloaded cardiac muscle cells, inotropic stimulation of cardiac muscle by \(\beta\)-adrenergic agonists is not accompanied by an increase in mitochondrial Ca\(^{2+}\) even though pyruvate dehydrogenase, one of the key regulatory enzymes of the citric acid cycle, is activated.

**MATERIALS AND METHODS**

**Isolated Hamster Papillary Muscle Preparation**—Left ventricular papillary muscles were obtained from the hearts of male golden hamsters (Canadian Hybrid Farms, Centerville, Nova Scotia, Canada) of average age 137.0 ± 2.8 (S.E.) days and body weight of 138.0 ± 1.3 g. Isolation, incubation, and stimulation of the muscles were as described previously (Moravec et al., 1990; Moravec and Bond, 1991) with the exception that to minimize mounting time stainless steel spring clips were used to mount the muscle instead of Ethicon silk. Experiments were performed in a hyperosmotic muscle bath containing Krebs-Henseleit buffer (100 mM NaCl; 4.0 mM KCl, 1.5 mM MgSO\(_4\), 1.5 mM NaH\(_2\)PO\(_4\), 20 mM NaHCO\(_3\), 20 mM Na\(_2\)H\(_3\)PO\(_4\), 10 mM glucose, 1.5 mM CaCl\(_2\), pH 7.4) at 28–29 °C, continuously bubbled with 95% O\(_2\), 5% CO\(_2\). Stimulation of the muscle (0.2 Hz, 5-s duration, 30% above threshold) was achieved by means of two parallel platinum electrodes incorporated into the muscle holder. This arrangement permitted uninterrupted stimulation up to the moment that the muscle was frozen. All experiments were performed with the muscle contracting isometrically at \(I_{max}\), the length at which maximal developed tension (DT) is obtained. Parameters of isometric contraction (resting tension, RT, DT; rate of rise of developed tension, +d\(T/dt\); rate of fall of developed tension, −d\(T/dt\); and rate of fall of developed tension, −d\(T/dt\); rate of fall of developed tension, −d\(T/dt\)) were recorded continuously up to the time at which the muscle was frozen.

**Experimental Conditions**—To investigate the question of mitochondrial Ca\(^{2+}\) uptake as a function of altered cardiac energy demand, the energy demand of the contracting papillary muscle was increased by stimulation with a high dose (10 MV) of the \(\beta\)-adrenergic agonist isoproterenol and incubating in low Na\(^{+}\) solution (Krebs-Henseleit buffer with 93.5 mM Na\(^{+}\) substituted by a 93.5 mM concentration of the CI\(^{–}\) salt of the impermeant cation, N-methyl D-glucamine\(^{–}\)). N-Methyl D-glucamine\(^{–}\) has the advantage over other Na\(^{+}\) ion substitutes that both the Na\(^{+}\) ion and the ion of the substitute are impermeant to the control solution. The low Na\(^{+}\) protocol served as a positive control to increase the Ca\(^{2+}\) load on the cells by inhibiting Ca\(^{2+}\) efflux via the Na\(^{+}\)/Ca\(^{2+}\) exchanger (Chapman, 1986; Sheu and Fozzard, 1982).

The effect of adding 10 MV isoproterenol or incubating in low Na\(^{+}\) solution on the parameters of isometric contraction (RT, DT, +d\(T/dt\), −d\(T/dt\)) and −d\(T/dt\) was determined. The significance of the observed differences was assessed by a paired Student's t test which compared the value of each parameter after the intervention with its preintervention value.

The muscles frozen, cryosectioned, and analyzed by EPMA were then extracted and assayed for pyruvate dehydrogenase activity (see below). Six to seven additional muscles in each of the three experimental groups were also frozen using an identical procedure, extracted, and assayed for pyruvate dehydrogenase activity (see below).

**Rapid Freezing of the Muscle**—All muscles were rapidly frozen at peak +d\(T/dt\), which has been shown by others (Peterson et al., 1981; Yue, 1987) to correspond to the peak of the free cytoplasmic Ca\(^{2+}\) transient. When free cytoplasmic Ca\(^{2+}\) is maximal, the amount of Ca\(^{2+}\) bound to troponin C will also be maximal (the on-rate of Ca\(^{2+}\) for the regulatory Ca\(^{2+}\) binding sites on troponin C is very rapid), and thus myofibrillar ATP requirements are greatest. The time point of peak +d\(T/dt\) was separately determined in advance of the freeze for each muscle. The range of times from stimulus to +d\(T/dt\) for the muscles used in these experiments was 80–130 ms with a mean of 100.6 ± 3.0 ms. Timed rapid freezing was accomplished by a computerized rapid freezing fixture as described previously (Moravec and Bond, 1991). Immediately prior to the freeze, excess solution was blotted from around the muscle. The solenoid was then activated by computer to rapidly raise (3.5 m/s) a beaker of liquid N\(_2\)-cooled ethane at the predetermined time point. The frozen muscle was stored under liquid N\(_2\) until it was cryosectioned.

**Cryoultramicrotomy**—Cryoultramicrotomy and freeze-drying were performed as described previously (Bond et al., 1987; Moravec and Bond, 1991). Ultrathin cryosections (100–150 nm thick) were cut parallel to the long axis of the frozen muscle in a Reichert-Jung FC4D cryoultramicrotome (ambient temperature, −125 °C; knife and specimen temperature, −100 °C). Sections were cut from three different areas of the muscle to sample multiple faces of the tissue. Sections on carbon-coated copper grids (determined previously to be Ca\(^{2+}\) free by EPMA) were freeze-dried overnight in a liquid N\(_2\)-chilled brass block in a vacuum evaporator. The next day, the freeze-dried cryosections were carbon coated and stored in a desicator.

After cryoultramicrotomy, the muscles were returned directly to the cryostorage dewar. Since the temperature of the muscle did not exceed −100 °C at any time during sectioning, it was possible to extract these same muscles later and measure pyruvate dehydrogenase activity (described below).

**Electron Probe Microanalysis**—X-ray spectra were collected at 80 kV accelerating voltage in a Philips CM/12 transmission electron microscope equipped with an LaB\(_6\) gun and an Edax 30-mm\(^2\) Si(Li) energy dispersive x-ray detector and multichannel analyzer.

Grids were transferred into the microscope using a Gatan temperature-regulated specimen stage and the specimen cooled to −100 °C before turning on the beam. Spectra were collected from the A-band in the region of overlap of the thick and thin filaments, using 700 nm–1 μm diameter stationary probes and from mitochondria using an average probe size of 300-nm diameter.

For each of the three experimental conditions, one x-ray spectrum was collected from the A-band of one spectrum from the mitochondrion in each of 5–10 cells (average 9 cells) from each muscle. To sample from different areas, cells were generally analyzed from at least two (usually three) different sectioned areas of the frozen muscle.

X-ray spectra were analyzed on a 386/20 IBM-compatible personal computer by minimum least squares to stored reference spectra (Kitts et al., 1985; Shuman et al., 1986) according to the Hall thin film quantitation procedure (Hall, 1971).

The concentrations of all elements measured in the A-band and in mitochondria by EPMA (Na, Mg, P, S, Cl, K, and Ca) were analyzed by nested one-way analysis of variance (cell nested within animal and animal nested within treatment group) as described previously (Bond et al., 1989; Moravec and Bond, 1991). Standard errors reported under "Results" include contributions of between-animal and within-animal variability.
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**RESULTS**

**Base-line Contractile Response and Responses to Inotropic Interventions**—Base-line contractile parameters of the hamster papillary muscles were similar to those reported previously (Moravec et al., 1990; Moravec and Bond, 1991). Examples of the effect of $10^{-6}$ M isoproterenol and low Na$^+$ solution on $DT$, $dT/dt$, and $-dT/dt$ are shown in Fig. 1. On average, $10^{-6}$ M isoproterenol increased the amplitude of $DT$ by $43 \pm 8\%$ ($p < 0.0005$), increased peak $+dT/dt$ by $62 \pm 9\%$ ($p < 0.0001$), and increased peak $-dT/dt$ by $99 \pm 12\%$ ($p < 0.0005$).

Incubation in low Na$^+$ solution resulted in a significant (p < 0.01) increase in resting tension of $39 \pm 6\%$. On average, after an initial peak in $DT$, there was a consistent decrease of $15\%$, but this was not statistically significant. Peak $+dT/dt$ and peak $-dT/dt$ also decreased in the low Na$^+$ group by $24 \pm 6\%$ ($p < 0.02$) and $23 \pm 6\%$ ($p < 0.05$), respectively.

**Elemental Concentrations of Myofibrils and Mitochondria**—The average elemental concentrations measured over myofibrils in the region of the A-band for each of the experimental conditions are summarized in Table I. Stimulation with $10^{-6}$ M isoproterenol resulted in no significant changes in the concentrations of any of the elements in the A-band as compared with controls. Incubation in low Na$^+$ solution caused a significant decrease in A-band Na$^+$, which was accompanied by significant decreases in A-band K$^+$, Mg$^{2+}$, and P.

The elemental concentrations of mitochondria in muscles frozen under each of the experimental conditions generally reflected the concentrations measured in the A-band (Table II). In the group of muscles stimulated with isoproterenol there was no difference in mitochondrial Ca$^{2+}$ (0.46 ± 0.19

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| TABLE II
| Elemental content of mitochondria |
|----------------|-----------------|----------------|
|                 | Control (n=34)  | Isoproterenol (n=47) | Low Na⁺ (n=46) |
| Na             | 61.1 ± 4.2    | 56.2 ± 6.8          | 40.3 ± 1.3*    |
| Mg             | 38.9 ± 3.8    | 39.7 ± 2.3          | 41.6 ± 1.6    |
| P              | 504.1 ± 38.5  | 490.3 ± 23.9        | 472.4 ± 16.1  |
| S              | 346.1 ± 14.0  | 374.5 ± 11.8        | 320.0 ± 6.5   |
| Cl             | 40.0 ± 6.1    | 42.4 ± 6.1          | 33.7 ± 6.5   |
| K              | 322.5 ± 24.1  | 312.9 ± 14.6        | 258.9 ± 7.6*  |
| Ca             | 0.54 ± 0.12   | 0.46 ± 0.19         | 1.28 ± 0.31*  |

**mmol/kg dry weight ± S.E.** Total number of cells (with one spectrum/cell) in each experimental group is indicated under each column. A significant increase in mitochondrial Ca²⁺ was measured in the low Na⁺ group; p < 0.02 versus control.

mmol/kg, dry weight) compared with controls (0.54 ± 0.12 mmol/kg, dry weight). As in the A-band, incubation in low Na⁺ caused significant decreases in mitochondrial Na⁺ and K⁺ content. In addition, a significant increase in mitochondrial Ca²⁺ was measured, with Ca²⁺ increasing to 1.28 ± 0.31 mmol of Ca²⁺/kg, dry weight, in the low Na⁺ group (p < 0.02). The average mitochondrial Ca²⁺ concentrations for each of the three groups are presented in Fig. 2.

**Pyruvate Dehydrogenase Activity**—The average pyruvate dehydrogenase activity measured in 11 control muscles frozen at peak +dT/dt was 27 ± 4 nmol/min/mg of muscle protein. The effect of inotropic stimulation on pyruvate dehydrogenase activity is shown in Fig. 3. In a total of 11 muscles stimulated with 10⁻⁶ M isoproterenol, pyruvate dehydrogenase activity increased by 42%, as compared with controls, to 38 ± 5 nmol/min/mg of protein (p < 0.05). Incubation of a group of 11 muscles in low Na⁺ caused a 56% increase in pyruvate dehydrogenase activity, compared with the control group (p < 0.01), to 42 ± 3 nmol/min/mg of protein.

**DISCUSSION**

In this study we have investigated the question of whether the mitochondrial Ca²⁺ content changes after an alteration of the inotropic state of cardiac muscle. To achieve this goal we have utilized methods developed recently in our laboratory to rapidly freeze contracting papillary muscles at precise time points of the cardiac cycle under defined inotropic conditions

**FIG. 2.** Total mitochondrial Ca²⁺ content measured by EPMA in cryosections of hamster papillary muscles frozen under each of the three experimental conditions, control (CONT), isoproterenol (ISO), and low Na⁺ (LOWNA). Mitochondrial Ca²⁺ is expressed as mmol/kg, dry weight, of mitochondrion ± S.E. Total number of cells (with one spectrum/cell) in each experimental group is indicated under each column. A significant increase in mitochondrial Ca²⁺ was measured in the low Na⁺ group; p < 0.02 versus control.

**FIG. 3.** The average activity of pyruvate dehydrogenase in each experimental group. Data are expressed as nmol/min/mg of muscle protein ± S.E. In addition to the muscles in each group which were cryosectioned and analyzed by EPMA, an additional seven muscles from the control group, six from the isoproterenol-treated group, and six from the low Na⁺ group were rapidly frozen for analysis of the dehydrogenase activity later. Control and isoproterenol muscles showed an increase in pyruvate dehydrogenase activity compared with control. Significantly elevated after treatment with isoproterenol and incubation in low Na⁺. *p < 0.05 versus control; **p < 0.01 versus control.

(Moravec and Bond, 1981). Using this approach we have been able to measure by EPMA the total Ca²⁺ content of subcellular organelles as well as changes in the subcellular Ca²⁺ distribution during the cardiac contractile cycle (Moravec and Bond, 1991). In the current study we utilized EPMA to directly measure mitochondrial Ca²⁺ content in situ in the rapidly frozen muscles under different inotropic conditions. This approach has the advantage of allowing us to measure the mitochondrial Ca²⁺ concentration without homogenization of the tissue and mitochondrial fractionation. The spatial resolution using EPMA on ultrathin cryosections of rapidly frozen tissue is on the order of 30 nm, which is sufficient to quantify changes in the Ca²⁺ content of junctional sarcoplasmic reticulum (Moravec and Bond, 1991). This approach thus allows us to readily measure the Ca²⁺ content of individual mitochondria, which have a diameter on the order of 300-500 μm.

We are also able, in the same muscle preparation, both to assess the inotropic response of the muscle to experimental interventions and to measure the activity of pyruvate dehydrogenase, which has been proposed (Hansford, 1985; McCormack and Denton, 1984) to be Ca²⁺ regulated in vivo and to increase its activity after β-adrenergic stimulation (Crompton et al., 1983; Wolska and Lewartowski, 1991).

β-Adrenergic stimulation was the principal inotropic stimulus used in this study. Stimulation of cardiac muscle by β-adrenergic agonists is known to cause an increase in cytoplasmic Ca²⁺ (Endoh and Blinks, 1988) and an increase in the rate of conduction and an increase in the rate of contraction. Evidence suggests that the inotropic effect of β-adrenergic agonists is caused by an increase in the Ca²⁺ influx into the cell via voltage-sensitive Ca²⁺ channels and by an increase in the amount of Ca²⁺ released upon stimulation from the sarcoplasmic reticulum (Callewaert et al., 1988). Consistent with the expected positive inotropic effect of β-adrenergic stimulation on cardiac muscle, we observed significant increases in DT peak +dT/dt, and peak -dT/dt after isoproterenol stimulation of the isometrically contracting papillary muscles.

The increase in DT and the acceleration of the rate of tension development and rate of relaxation which occur upon
\(\beta\)-adrenergic stimulation all require a greater availability of ATP than is needed in the absence of an inotropic stimulus. As cardiac muscle depends almost exclusively upon oxidative phosphorylation to supply its energy needs (Kobayashi and Neely, 1979), the increased ATP must be provided by the activation of mitochondrial metabolism. It has been proposed (Hansford, 1985; McCormack and Denton, 1984) that this may occur by acceleration of the citric acid cycle by means of \(\beta\)-adrenergic activation of these enzymes such as pyruvate dehydrogenase and \(\alpha\)-ketoglutarate dehydrogenase. However, for \(\text{Ca}^{2+}\) activation of these enzymes to occur \textit{in vivo}, it would be necessary for the mitochondrial \(\text{Ca}^{2+}\) concentration to increase. We tested this hypothesis by comparing the average \(\text{Ca}^{2+}\) content of mitochondria in papillary muscles rapidly frozen at peak \(d\text{Ca}^{2+}/dt\) after isoproterenol stimulation, with the average mitochondrial \(\text{Ca}^{2+}\) content in muscles frozen at peak \(d\text{Ca}^{2+}/dt\) in the absence of inotropic stimulation. Mitochondrial \(\text{Ca}^{2+}\) in the isoproterenol-stimulated muscles (0.46 ± 0.19 mmol/kg, dry weight) was not increased compared with control muscles (0.54 ± 0.12 mmol/kg, dry weight). This lack of change in mitochondrial \(\text{Ca}^{2+}\) content is in distinct contrast to results obtained in mitochondria isolated from catecholamine-stimulated hearts (Crompton, 1983) or to the size of the uncoupler-releasable \(\text{Ca}^{2+}\) pool of stimulated guinea pig hearts (Wolska and Lewartowski, 1991). In these studies, total mitochondrial \(\text{Ca}^{2+}\) increased by as much as 2.4-fold, reaching 3.5-3.9 mmol/kg of protein. However, with EPMA of ultrathin cryosections, much smaller changes in magnitude can readily be detected. In our hands, EPMA can detect changes in mitochondrial \(\text{Ca}^{2+}\) content on the order of 0.3 mmol/kg, dry weight (LeFurgey et al., 1988). We can therefore conclude from the results of this study that the increase in free cytoplasmic \(\text{Ca}^{2+}\) that occurs during \(\beta\)-adrenergic stimulation does not activate mitochondrial \(\text{Ca}^{2+}\) uptake.

The increase in free cytoplasmic \(\text{Ca}^{2+}\) reported to occur upon \(\beta\)-adrenergic stimulation was not reflected in a significant increase in total A-band \(\text{Ca}^{2+}\) measured by EPMA. This is not unexpected because free cytoplasmic \(\text{Ca}^{2+}\) constitutes an extremely small proportion of the total \(\text{Ca}^{2+}\) measured by EPMA in the A-band (most of the \(\text{Ca}^{2+}\) being bound to myofibrillar binding sites) (Fabiato, 1983). Because most of the \(\text{Ca}^{2+}\) associated with the myofibrils at physiological free \(\text{Ca}^{2+}\) and \(\text{Mg}^{2+}\) concentrations is bound to the two high affinity (\(\text{Ca}^{2+}/\text{Mg}^{2+}\)) sites and one low affinity (\(\text{Ca}^{2+}\)) specific site on troponin C (Fabiato, 1983; Pan and Solaro, 1987), an increase in free cytoplasmic \(\text{Ca}^{2+}\) should result primarily in a greater degree of saturation of the \(\text{Ca}^{2+}\)-specific site on troponin C. Because only a single \(\text{Ca}^{2+}\) binding site is involved, the resultant changes in total A-band \(\text{Ca}^{2+}\) would be relatively small. Consistent with this idea, the values that we have measured for total A-band \(\text{Ca}^{2+}\) content in the control muscles and in the isoproterenol-treated muscles (1.84 ± 0.34 and 2.25 ± 0.19 mmol/kg, dry weight, respectively) agree well with the value of 1.8 mmol/mg of myofibrillar protein reported by Pan and Solaro (1987) for the combined density of the two classes of \(\text{Ca}^{2+}\) binding sites on troponin C.

From the total mitochondrial \(\text{Ca}^{2+}\) content measured in papillary muscles frozen with or without \(\beta\)-adrenergic stimulation, we can obtain an estimate of the mitochondrial matrix free \(\text{Ca}^{2+}\). An average value of 0.5 mmol/kg, dry weight, is approximately equivalent to 0.6 mmol/mg of mitochondrial protein (assuming \(0.8\) mg protein/mg, dry weight, in the mitochondria). From correlative measurements of free and total mitochondrial \(\text{Ca}^{2+}\) in isolated mitochondria (Davis et al., 1987), a value of approximately 200 nM for matrix free \(\text{Ca}^{2+}\) can be obtained in both the presence and absence of \(\beta\)-adrenergic stimulation. This is comparable to values obtained by Reers and co-workers (1989) in fura-2-loaded mitochondria at extramitochondrial \(\text{Ca}^{2+}\) concentrations below 1 \(\mu\)M. According to the \(K_{0.5}\) obtained for \(\text{Ca}^{2+}\)-dependent activation of each of the regulatory citric acid cycle enzymes (Wan et al., 1989), 200 nM matrix free \(\text{Ca}^{2+}\) may be sufficient for activation of \(\alpha\)-ketoglutarate dehydrogenase (\(K_{0.5}\) of 0.15 \(\mu\)M) but only for partial activation of pyruvate dehydrogenase, which has a lower \(\text{Ca}^{2+}\) affinity (\(K_{0.5}\) \(\approx\) 1 \(\mu\)M) (Wan et al., 1989). Nevertheless, because our results imply no increase in mitochondrial matrix free \(\text{Ca}^{2+}\) upon inotropic stimulation, the data do not support a role for changes in mitochondrial matrix free \(\text{Ca}^{2+}\) in enzyme regulation.

Our results are consistent with studies which show that in the presence of physiological concentrations of \(\text{Na}^{+}\) and \(\text{Mg}^{2+}\), the \(K_{0.5}\) for mitochondrial \(\text{Ca}^{2+}\) uptake is on the order of 30-90 \(\mu\)M (Scarpa and Graziotti, 1973; Crompton et al., 1976) whereas the free cytoplasmic \(\text{Ca}^{2+}\) at the peak of a contraction in the presence of isoproterenol is only approximately 350 nM (O’Rourke et al., 1990). Taking into account the sigmoidal shape of the mitochondrial \(\text{Ca}^{2+}\) uptake curve (Scarpa and Graziotti, 1973), the amount of \(\text{Ca}^{2+}\) that would be taken up during a twitch after \(\beta\)-adrenergic stimulation should be negligible. Indeed, Robertson and co-workers (1982) calculated that the increase in mitochondrial \(\text{Ca}^{2+}\) in the presence of an inotropic stimulus is no more than 1.2 \(\mu\)mol/kg of mitochondrial protein (approximately 1.0 \(\mu\)mol/kg, dry weight, of mitochondria). When mechanisms for release of mitochondrial \(\text{Ca}^{2+}\) (e.g. \(\text{Na}^{+}/\text{Ca}^{2+}\) exchange) are taken into account (Robertson et al., 1982), net mitochondrial uptake would be even less than this estimate.

In previous studies we have demonstrated that in the absence of inotropic stimulation, no significant mitochondrial \(\text{Ca}^{2+}\) uptake takes place during a single cardiac muscle twitch (Moravec and Bond, 1991). During \(\beta\)-adrenergic stimulation the peak free cytoplasmic \(\text{Ca}^{2+}\) approximately doubles (Endoh and Blinks, 1988; O’Rourke et al., 1990; Spurgeon et al., 1990). Therefore, in conjunction with our earlier results we conclude that over the range of cytoplasmic \(\text{Ca}^{2+}\) concentrations occurring in the cardiac muscle cell under physiological conditions (from relaxation to inotropic stimulation), there is no significant \(\text{Ca}^{2+}\) uptake by mitochondria. It should be noted, however, that a 3-fold increase in mitochondrial \(\text{Ca}^{2+}\) content has been measured by EPMA after paired pulse stimulation of isolated myocytes rapidly frozen during voltage clamp (Wendt-Gallitelli and Isenberg, 1991). It is possible that the differences between these results and our results are caused by alterations in the intracellular milieu of the patched cells as a result of equilibration with the solution in the patch electrode. Alternatively, the differences observed could be related to the different methods of inotropic stimulation used. Further studies will be needed to resolve this question.

To confirm activation of mitochondrial metabolism by \(\beta\)-adrenergic stimulation we measured pyruvate dehydrogenase activity in each of the rapidly frozen muscles. As illustrated in Fig. 3, there was a significant (42%) increase in pyruvate dehydrogenase activity in the muscles stimulated by isoproterenol compared with the control group. This increase is similar to the 34% increase in pyruvate dehydrogenase activity measured by Hiroaka and co-workers (Hiroaka et al., 1980) after isoproterenol stimulation in isolated perfused hearts. These results, coupled with the lack of increase in mitochondrial \(\text{Ca}^{2+}\), therefore suggest that pyruvate dehydrogenase activation is independent of changes in mitochondrial \(\text{Ca}^{2+}\). Similar conclusions were reached by Burger and Perametter...
(1984) who demonstrated a lack of correlation between per-
fuse Ca\(^{2+}\) and pyruvate dehydrogenase activity in perfused
hearts in which the work performed and O\(_2\) consumption were
kept constant.

As we found no evidence for an increase in mitochondrial Ca\(^{2+}\) after stimulation by isoproterenol, we then investigated the question of whether mitochondria participate in Ca\(^{2+}\) regulation under conditions in which the Ca\(^{2+}\) load of the cell is increased. The protocol used to Ca\(^{2+}\) load the cells was incubation in a low Na\(^{+}\)-containing solution. This treatment has been shown to inhibit Ca\(^{2+}\) efflux from the cell via inhibition of the Na\(^{+}\)/Ca\(^{2+}\) exchanger (Allen et al., 1983).

When papillary muscles were incubated in low Na\(^{+}\) solution, we observed an increase in RT accompanied by decreases in both peak +dT/dt and peak −dT/dt. The increase in RT is likely to be a direct consequence of the slow rise in cytoplasmic Ca\(^{2+}\) measured previously by other investigators using aequorin-loaded papillary muscles incubated in low Na\(^{+}\) solu-

tions (Allen et al., 1983) or by Ca\(^{2+}\)-selective microelectrode measurements in ventricular trabeculae (Chapman et al., 1986; Sheu and Fozzard, 1982). The decrease in peak −dT/dt observed in this study is consistent with the demonstration by Bers and co-workers (1980) that approximately 20% of the decrease in cytoplasmic Ca\(^{2+}\) which occurs during relaxation is a result of the activity of the Na\(^{+}\)/Ca\(^{2+}\) exchanger and that removal of extracellular Na\(^{+}\) causes a significant slowing in the rate of decline of free cytoplasmic Ca\(^{2+}\) during relaxation.

In the papillary muscles incubated in low (46.5 mM) Na\(^{+}\), we observed a significant decrease in A-band (as well as mitochondrial) Na\(^{+}\) and K\(^{+}\) content. These results are comparable with previous observations obtained with Na\(^{+}\)-selective microelectrodes after incubation of ventricular preparations with low Na\(^{+}\) solution (Chapman, 1986; Sheu and Fozzard, 1982) or obtained by measurement of intracellular Na\(^{+}\) and K\(^{+}\) by atomic absorption spectrophotometry in isolated hearts perfused with low Na\(^{+}\) (Hoerter et al., 1986). The decrease in A-band K\(^{+}\) accompanying the decrease in A-band Na\(^{+}\) may be a result of decreased availability of ATP (Hoerter et al., 1988) for activation of the Na\(^{+}/K^{+}\) ATPase.

Although an increase in free cytoplasmic Ca\(^{2+}\) has been reported to occur in response to incubation of cardiac muscle in low Na\(^{+}\) solution, we observed no increase in total A-band Ca\(^{2+}\) content under these conditions. As discussed previously, this is most likely because the changes in free cytoplasmic Ca\(^{2+}\) are not detectable by EPMA. In addition, we can infer that within the limits of sensitivity of the method, incubation in low Na\(^{+}\) solution causes no increase in bound Ca\(^{2+}\) in the A-band. Because the Ca\(^{2+}\)-specific binding site on troponin C constitutes a very limited buffer for large increases in cyto-

plasmic Ca\(^{2+}\), once this Ca\(^{2+}\) binding site is occupied the mitochondrial Ca\(^{2+}\) uptake system would likely be activated by supraphysiological increases in free cytoplasmic Ca\(^{2+}\). Al-

though mitochondria have been shown to have a low affinity for Ca\(^{2+}\), the rate of Ca\(^{2+}\) uptake is a sigmoidal function of extramitochondrial Ca\(^{2+}\) (Scarpa and Grazioti, 1973) and increases markedly as extramitochondrial Ca\(^{2+}\) increases above physiological levels.

Our observation of mitochondrial Ca\(^{2+}\) uptake in muscles incubated in low Na\(^{+}\) solution is consistent with results ob-
tained by others using different techniques. For example, the elevation of cytoplasmic Ca\(^{2+}\) and the increase in RT in low Na\(^{+}\) solution were greater in the presence of mitochondrial inhibitors than in their absence (Chapman, 1986; Allen et al., 1983), indicating that in low Na\(^{+}\) solution mitochondria play a role in buffering the increase in cytoplasmic Ca\(^{2+}\). In ferret ventricular trabeculae incubated in low Na\(^{+}\), Ca\(^{2+}\) could be released into the cytoplasm by the mitochondrial uncoupler carbonyl cyanide p-chlorophenylhydrazone whereas Ca\(^{2+}\) re-

lease was not observed after incubation in normal Tyrode's solution (Chapman, 1986).

In the muscles incubated in low Na\(^{+}\) solution in this study we observed not only an increase in mitochondrial Ca\(^{2+}\) but also an increase in pyruvate dehydrogenase activity. A similar increase in pyruvate dehydrogenase activity was observed by Hansford and co-workers (1990) in isolated myocytes exposed to low Na\(^{+}\). Because mitochondrial matrix -free Ca\(^{2+}\) has been shown to increase in proportion to an increase in total mito-

chondrial Ca\(^{2+}\) (Hansford and Castro, 1982; Davis et al., 1987), the increases in mitochondrial Ca\(^{2+}\) and pyruvate dehydrogen-

ase activity reported here in the muscles incubated in low Na\(^{+}\) are similar to results obtained by others in isolated mitochondria loaded with the Ca\(^{2+}\)-selective indicators fura-2 or indo-1 (Moreno-Sanchez and Hansford, 1988; McCormack et al., 1989; Lukacs et al., 1988; Wan et al., 1989). However, because it has been demonstrated (Hoerter et al., 1986) that there is a significant decrease in the cytosolic phosphorylation potential after incubation of cardiac muscle in low Na\(^{+}\) solution, this does not necessarily imply that there is a causal relationship between the observed elevation in pyruvate de-
hydrogenase activity and the increase in mitochondrial Ca\(^{2+}\).

In the low Na\(^{+}\) group, we also observed a decrease in A-

band phosphorus and Mg\(^{2+}\), which was not accompanied by decreases in mitochondrial Mg\(^{2+}\) or phosphorus. It is possible that these changes are caused by a decrease in the amount of Mg\(^{2+}\)ATP and/or Mg\(^{2+}\)ADP bound to myofibrillar proteins. However, because the phosphorus measured by EPMA does not distinguish among the different forms of phosphorus (i.e. bound to a protein as P, or in a high energy phosphate compound such as ATP, ADP, or creatine phosphate), these data alone do not permit us to state with certainty the reason for the decrease in A-band phosphorus.

In summary, although a relationship between increased mitochondrial Ca\(^{2+}\) and increased pyruvate dehydrogenase activity was observed in Ca\(^{2+}\)-loaded muscles, no change in mitochondrial Ca\(^{2+}\) was measured in response to β-adrenergic stimulation, despite marked increases in the inotropic state of the muscle and an increase in pyruvate dehydrogenase activity. From these results we conclude that an increase in mitochondrial Ca\(^{2+}\) is not a requirement for the increase in mitochondrial respiration (indicated by activation of pyruvate dehydrogenase) which accompanies increased cardiac performance. We propose that other regulatory mechanisms such as an alteration in the ATP/ADP ratio or NADH/NAD\(^{+}\) ratio may play a more important role in the coupling of mitochon-

drial energy production to energy demand by the cardiac muscle cell.

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