Journal of Bioenergetics and Biomembranes, Vol. 26, No. 5, 1994

Physiological Role of Mitochondrial Ca^{2+} Transport

Richard G. Hansford[1]

Received June 15, 1994

A model has been proposed in which mitochondrial Ca^{2+} ion transport serves to regulate mitochondrial matrix free Ca^{2+} ($[Ca^{2+}]_m$), with the advantage to the animal that this allows the regulation of pyruvate dehydrogenase and the tricarboxylate cycle in response to energy demand. This article examines recent evidence for dehydrogenase activation and for increases in $[Ca^{2+}]_m$ in response to increased tissue energy demands, especially in cardiac myocytes and in heart. It critiques recent results on beat-to-beat variation in $[Ca^{2+}]_m$ in cardiac muscle and also briefly surveys the impact of mitochondrial Ca^{2+} transport on transient changes in cytosolic free Ca^{2+} in excitable tissues. Finally, it proposes that a failure to elevate $[Ca^{2+}]_m$ sufficiently in response to work load may underlie some cardiomyopathies of metabolic origin.

KEY WORDS: Oxidative phosphorylation; ion transport; tricarboxylate cycle; cardiomyopathy.

INTRODUCTION

Mitochondria from mammalian tissues contain a Ca^{2+} transport system which carries out electrophoretic Ca^{2+} uptake, mediated by a carrier designated as a "uniporter," balanced by electroneutral Ca^{2+} release, mediated by the joint functioning of a $2Na^+/Ca^{2+}$ exchanger, or "antiporter," and a Na^+/H^+ exchanger. Thus, Ca^{2+} uptake is driven by the $\Delta\psi$ component of the overall proton electrochemical gradient ($\Delta\bar{\mu}H^+$) across the mitochondrial membrane, whereas Ca^{2+} release is driven by the ΔpH component. These relations are described in more detail in the review by T. E. Gunter in this issue of the Journal (and see Gunter and Pfeiffer, 1990). The presence of these carriers allows the net uptake of Ca^{2+} into the mitochondria, or the net release of Ca^{2+}, in response to changes in cytosolic free Ca^{2+} ($[Ca^{2+}]_c$). The biological advantage of such a system became more apparent with the discovery during the 1970's of Ca^{2+} sensitivity of three dehydrogenases residing within the permeability barrier of the inner mitochondrial membrane, viz. the pyruvate dehydrogenase system (Denton *et al.*, 1972), the NAD-isocitrate dehydrogenase (Denton *et al.*, 1978), and the 2-oxoglutarate dehydrogenase (McCormack and Denton, 1979). This raised the possibility that the increased $[Ca^{2+}]_c$, indicative of an increased energy demand upon the tissue, could serve to raise intramitochondrial free Ca^{2+} ($[Ca^{2+}]_m$) and thus act as a signal to activate substrate dehydrogenation and hence energy provision by the process of oxidative phosphorylation (Denton *et al.*, 1980; Denton and McCormack, 1985; Hansford, 1985). In the last 15 years, convincing evidence has been produced that these intramitochondrial dehydrogenase do respond to changes in extramitochondrial Ca^{2+} over the range 10^{-7}–10^{-6} M, in *in vitro* studies involving mitochondrial suspensions (reviewed by Hansford, 1985, 1991; McCormack *et al.*, 1990a). However, these findings do not translate readily into the *in vivo* situation, as the cytosolic concentration of effectors of mitochondrial Ca^{2+} transport, e.g., Mg^{2+} (Crompton *et al.*, 1976a); Fry *et al.*, 1984) and polyamines (Lenzen *et al.*, 1986), may not be known and indeed there may be other effectors, yet undescribed. In addition, it has become apparent that increases in $[Ca^{2+}]_c$ are normally transient (Berridge and Galione, 1988; Berridge *et al.*, 1988), such that mitochondria are exposed to oscillating $[Ca^{2+}]_c$, of a periodicity that varies with the tissue. The question arises as to how

[1] Gerontology Research Center, National Institute on Aging, Baltimore, Maryland 21224

0145-479X/94/1000-0495$07.00/0

the mitochondiral Ca^{2+} transport mechanisms respond to these oscillations in $[Ca^{2+}]_c$. This clearly determines the fidelity with which the message of an impending energy demand is passed from the cytosol to the machinery of oxidative phosphorylation.

This article will discuss evidence from studies of cells and tissues which bears upon this question, and aims to update a previous mini-review in this journal (Hansford, 1991). It is also apparent that the response of the mitochondrial Ca^{2+} transport cycle to changes in $[Ca^{2+}]_c$ will itself cause changes in $[Ca^{2+}]_c$. These will involve the damping of transients in $[Ca^{2+}]_c$ though probably not changes in steady-state $[Ca^{2+}]_c$, which in the last resort is set by the transport mechanisms of the plasma membrane. Recent results bearing on the extent to which the kinetics of changes in $[Ca^{2+}]_c$ are altered by the existence of the mitochondrial Ca^{2+} transport cycle will also be discussed briefly. Finally, there are now several studies suggesting altered homeostasis of $[Ca^{2+}]_m$ in various disease states, and this emerging area of research will be explored.

ACTIVATION OF ENZYMES BY $[Ca^{2+}]_m$

The availability of the fluorescent Ca^{2+} chelating agents fura-2 and indo-1 in membrane-permeable ester forms has allowed the loading of isolated mitochondria with these compounds and the continuous monitoring of $[Ca^{2+}]_m$ (Davis *et al.*, 1987; Lukács and Kapus, 1987; Gunter *et al.*, 1988; Moreno-Sánchez and Hansford, 1988; Reers *et al.*, 1989). Studies with heart mitochondria have given $K_{0.5}$ values for the activation of pyruvate dehydrogenase by $[Ca^{2+}]_m$ of 0.7 μM (Moreno-Sánchez and Hansford, 1988; Hansford, 1991) and 1 μM Ca^{2+} (Wan *et al.*, 1989). Values of $K_{0.5}$ for the activation of 2-oxoglutarate dehydrogenase have fallen within the range 120–800 nM (Lukács *et al.*, 1988; McCormack *et al.*, 1989; Wan *et al.*, 1989). These values are quite close to those originally described for the activation of pyruvate dehydrogenase phosphatase by Ca^{2+} (Denton *et al.*, 1972) and for the activation of 2-oxoglutarate dehydrogenase (McCormack and Denton, 1979) in experiments involving mitochondrial extracts or purified enzymes. A major theme of the next section of this article will be the question of whether these enzymes become activated when muscle contracts, neurons fire, or nonexcitable tissues are challenged by Ca^{2+}-mobilizing hormones, and to what extent this involves changes in $[Ca^{2+}]_m$ in this range (0.1–2 μM). In this context, some of the work involves measurement of total mitochondrial calcium, whether by atomic absorption spectroscopy or by electron probe microanalysis. Thus, it is useful to know that the range 0.1–2 μM for $[Ca^{2+}]_m$ corresponds to approximately 0.2–4 nmol of total Ca^{2+}/mg of mitochondrial protein ($[Ca^{2+}]_{m-total}$, see Coll *et al.*, 1982; Hansford and Castro, 1982). This was originally shown in null-point experiments involving addition of Ca^{2+}-ionophores to mitochondria and detection of net movements of Ca^{2+} with (extramitochondrial) metallochromic indicators. These basic findings, which indicate that only one part in 10^3 of total mitochondrial Ca^{2+} is free, have since been confirmed with the fluorescent chelating agent technology (see McCormack *et al.*, 1990a; Hansford, 1991).

Recently, some of these issues have been revisited in a report by Cox and Matlib (1993) which examines oxidative phosphorylation by heart mitochondria, with nonsaturating 2-oxoglutarate as substrate, as a function of $[Ca^{2+}]_m$, measured with fura-2. The authors show that the rates of NAD reduction and state 3 oxygen uptake are lower in the presence of Na^+, which potentiates Ca^{2+} efflux via Na^+/Ca^{2+} exchange (Crompton *et al.*, 1976b; Crompton and Heid, 1978), and can be raised by application of benzothiazepines which inhibit this carrier. This is a nice confirmation of the earlier literature (Hansford and Castro, 1981; Denton et al., 1980).

ACTIVATION OF DEHYDROGENASES IN HEART LINKED TO RAISED $[Ca^{2+}]_m$

The previous review (and see also McCormack *et al.*, 1990a) documented the evidence of activation of pyruvate dehydrogenase, i.e., increase in the fraction of the dephospho form of the enzyme (PDH_A), in response to increased work performance by the isolated perfused heart. As there are other effectors of pyruvate dehydrogenase interconversion, namely NAD^+/NADH and ATP/ADP ratios (see Hansford, 1976; Wieland, 1983; Reed *et al.*, 1985) which may change under these conditions, the question arises as to how much of this increase in PDH_A content can be attributed to increased $[Ca^{2+}]_m$. This has been approached using a number of different methodologies.

The first to be used involved the rapid isolation of mitochondria from the heart using media designed to minimize loss or gain of mitochondrial Ca^{2+} during isolation and thus preserve the activation status of

pyruvate dehydrogenase (Crompton *et al.*, 1983; McCormack and Denton, 1984). The $[Ca^{2+}]_{m-total}$ was found to be raised from 1.8 to 4.2 nmol/mg of mitochondrial protein by treatment of the isolated, perfused rat heart with epinephrine, with the majority of the response being β-adrenergic (Crompton *et al.*, 1983; McCormack and Denton, 1984). Similar experiments with guinea-pig hearts showed an increase in $[Ca^{2+}]_{m-total}$ from 2.73 to 4.10 nmol/mg of mitochondrial protein as a consequence of electrical stimulation at 2 Hz, relative to the arrested heart (Hansford *et al.*, 1990). Substantial increases in PDH_A content were recorded in both studies, together with an increased activity of oxidation of nonsaturating 2-oxoglutarate by mitochondria recovered from the epinephrine-stimulated hearts (McCormack and Denton, 1984).

In a recent variant of these experiments, Wolska and Lewartowski (1991) showed that stimulation of guinea pig heart at 1 Hz increased the amount of mitochondrial Ca^{2+} which could subsequently be released by uncoupler (CCCP) addition to thin strips of muscle excised from the ventricles at low temperature. The increase with stimulation was from 1.58 to 3.86 nmol/mg of mitochondral protein, which is within the range of sensitivity of the dehydrogenases.

Experiments of this type have recently been broadened to include a measurement of $[Ca^{2+}]_m$, as distinct from the determination of $[Ca^{2+}]_{m-total}$. This has been accomplished by preperfusion of the heart with fura-2/AM, such that the mitochondria which were subsequently harvested contained not only their preisolation load of Ca^{2+} but also fura-2. Incubation with oxidizable substrate then allowed generation of fura-2 fluorescence reflecting $[Ca^{2+}]_m$, with minimal changes from the in situ value (Allen *et al.*, 1992). Increased contractile work raised $[Ca^{2+}]_m$ from a basal value of 172 nM, achieved with the heart beating at its intrinsic rhythm, to 916 nM: treatment with epinephrine gave a value of 727 nM. The corresponding values of PDH_A content were 1.81, 2.66, and 3.59 arbitrary units, respectively. Comparison of these values of $[Ca^{2+}]_m$ with the $K_{0.5}$ values for pyruvate dehydrogenase activation measured in mitochondrial suspensions loaded with indo-1 or fura-2 (Moreno-Sánchez and Hansford, 1988; Wan *et al.*, 1989) indicates that changes in $[Ca^{2+}]_m$ are indeed likely to be largely responsible for the changed activity status of pyruvate dehydrogenase in these heart perfusions.

To establish such a cause-and-effect relation, an attractive approach is to block changes in $[Ca^{2+}]_m$ and to determine whether enzyme activation is also blocked. This has been attempted several times using ruthenium red, a glycoprotein stain which is a very potent inhibitor of the mitochondrial Ca^{2+} uniport, in experiments with isolated mitochondria (Rossi *et al.*, 1973; Reed and Bygrave, 1974; Gupta *et al.*, 1989). Despite the severely limited permeability of this hexavalent cation into intact cells (Gupta *et al.*, 1988), perfusion with ruthenium red has been shown to block the activation of pyruvate dehydrogenase due to epinephrine or to raised perfusate Ca^{2+} (McCormack and England, 1983) and to allow the appearance of a drop in adenine nucleotide phosphate potenial in response to raised work load (Unitt *et al.*, 1989). Experiments with suspensions of isolated cardiac myocytes, in which $[Ca^{2+}]_c$ could be monitored with fluorescent probes, revealed that the action of ruthenium red is not at the level of sarcolemmal Ca^{2+} influx, but must involve the mitochondrial membrane (Hansford, 1987).

The model of partial control of oxidative phosphorylation by Ca^{2+} ions (Hansford, 1985; Heineman and Balaban, 1990; McCormack *et al.*, 1990a) states that increased dehydrogenase activation by Ca^{2+} results in increased mitochondrial $NADH/NAD^+$ ratios and an increased flux through oxidative phosphorylation. In the event that raised $[Ca^{2+}]_m$ activates processes which consume $\Delta\bar{\mu}H^+$, e.g., the F_1F_0 ATPase, as well as the dehydrogenases (see below), then it is possible for increased flux through oxidative phosphorylation to occur without an increase in the mitochondrial $NADH/NAD^+$ ratio. In any event, on this model, activation of intramitochondrial metabolism by Ca^{2+} is considered to be the reason why cytosolic ATP/ADP × Pi ratios fall to only a very minimal extent in response to raised work load in the heart (Balaban *et al.*, 1986; Clarke and Willis, 1987; From *et al.*, 1986; Katz *et al.*, 1987, 1988, 1989). The action of ruthenium red to render more prominent the transitory fall in ATP/ADP × Pi, on imposition of increased work load, is in keeping with this proposal. A very recent paper by Hak *et al.* (1993) extends these inquiries by examining the effect of ruthenium red on the lag in onset of increased O_2 uptake due to raised mechanical work of the isolated, perfused rabbit heart. At a concentration of 2.1 μg/ml, ruthenium red increased this response time from 12 to 21 s: however, a lower concentration of ruthenium red (0.9 μg/ml) had no such effect. The authors argue from these findings that $[Ca^{2+}]_m$ does activate oxidative phosphorylation but that Ca^{2+} uptake by the mitochondrial

uniporter has a low control strength in the control of the overall process of oxidative phosphorylation (see Kacser and Burns, 1973; Heinrich and Rapoport, 1974; Kacser and Porteous, 1987, for a discussion of metabolic control theory). The latter conclusion is perhaps premature in view of the limited cell permeability to ruthenium red alluded to above, which means that the intracellular concentrations of the compound may well not be directly proportional to the concentrations in the perfusion fluid. More incisive experiments than these await the development of cell penetrant, specific inhibitors of the mitochondrial transport cycle.

There is one notable exception to the general conclusion that positive inotropic stimulation of heart muscle results in increased values of $[Ca^{2+}]_m$. This involves work with electron probe x-ray microanalysis (EPMA) which has the ability to measure $[Ca^{2+}]_{m-total}$ in rapidly frozen muscles. An EPMA study by Moravec and Bond (1992) failed to show an increase in $[Ca^{2+}]_{m-total}$ due to β-adrenergic stimulation (10^{-6} M isoproterenol) of hamster papillary muscles, frozen at maximum $+dT/dt$. Values were 0.46 ± 0.19 (SEM) versus 0.54 ± 0.12 mmol of Ca^{2+}/kg^{-1} dry wt. for stimulated and control, respectively. Despite this, a modest increase in muscle PDH_A content occurred as a consequence of stimulation by isoproterenol. It is perhaps not surprising that little increase in $[Ca^{2+}]_{m-total}$ occurred under these conditions, as such low stimulation frequencies (0.2 Hz)—which are of course nonphysiological—gave rise to no appreciable increase in $[Ca^{2+}]_m$, even in the presence of isoproterenol, using the indo-1 loading and Mn^{2+}-quench technique described below (Miyata *et al.*, 1991). However, a very recent study reported in abstract form by Moravec *et al.* (1994) also failed to show an increase in $[Ca^{2+}]_{m-total}$ in isolated, perfused hamster hearts stimulated at 4 Hz in response to treatment with isoproterenol or exposure to raised perfusion pressure. Although it is tempting to mitigate the impact of these findings by pointing out that the parameter measured is $[Ca^{2+}]_{m-total}$ rather than $[Ca^{2+}]_m$, it is likely that the latter is related nearly linearly to the former, over this concentration range (Hansford and Castro, 1982). Thus, these negative results from the EPMA technique remain an enigma.

The development of a direct, continuous measurement technique for $[Ca^{2+}]_m$ *in situ* in cardiac myocytes by Miyata *et al.* (1991) has allowed the description of the relation between frequency of contraction and $[Ca^{2+}]_m$. In this technique, the fluorescent signal originating from the cytosol of indo-1-loaded myocytes is selectively quenched by exposure of the cells to low concentrations of Mn^{2+}. The stable signal remaining is attributed to intramitochondrial indo-1 on the grounds of its resistance to release by low concentrations of digitonin and its sensitivity to ruthenium red (Miyata *et al.*, 1991). This allows a continuous read-out of $[Ca^{2+}]_m$ in single cells. Most importantly, $[Ca^{2+}]_m$ was found to rise from values of approximately 100 nM in cells stimulated at low frequencies (0.2 Hz) to values of 450–650 nM in cells stimulated at 4 Hz in the presence of a β-adrenergic agonist (Miyata *et al.*, 1991). Much of this increase reflects the larger transients in $[Ca^{2+}]_c$ seen in the presence of β-adrenergic stimulation, and electrical excitation alone causes only very modest increases in $[Ca^{2+}]_m$, when imposed at frequencies of 2 Hz (Di Lisa *et al.*, 1993b). Unfortunately, higher rates of stimulation are not always possible in the absence of β-adrenergic activation, as the cells may not follow the pacing. Although it is not possible to study enzyme activation on a single myocyte at this time, it is clear by reference to the work with indo-1-loaded and fura-2-loaded suspensions of mitochondria (Moreno-Sánchez and Hansford, 1988) that the dynamic range of $[Ca^{2+}]_m$ identified in the studies by Miyata *et al.* (1991) and Di Lisa *et al.* (1993b) is very appropriate for regulating pyruvate and 2-oxoglutarate dehydrogenases. The missing ingredient in these studies is the performance of external work as the cells are not mechanically loaded. Conceivably they could be made to perform work by making them contract against a surrounding fibrin matrix (Sollott and Lakatta, 1994). Other drawbacks of this technique include possible direct effects of Mn^{2+} on the carriers of the mitochondrial Ca^{2+} cycle and limited applicability to other types of cell. In the case of the former, the free Mn^{2+} concentration in the cytoplasm is probably limitingly low, as it is used just to "titrate" the cytosolic indo-1, which binds Mn^{2+} with very high affinity (K_d for the indo-1/Mn^{2+} chelate of approximately 10 nM). There will presumably be competition for any Mn^{2+} in excess of the molarity of the cytosolic indo-1 among all of the high-affinity Ca^{2+}-binding sites of the cytosol and cellular membranes. Clearly, transport of Mn^{2+} into the mitochondria is very slow under these intracellular conditions, though Mn^{2+} is well established as a substrate for the Ca^{2+} uniport in studies with isolated mitochondria (Chappell *et al.*, 1963) and has been shown to be taken up by mitochondria in perfused heart (Hunter *et al.*, 1980), as the

signal remaining after quenching cytosolic Ca^{2+}: indo-1 transients is remarkably stable in rat myocytes (Miyata *et al.* 1991). The method is not so useful for cardiac myocytes from the guinea pig, which fail to show two such clearly separable phases of quenching (Janczewski and Lakatta, unpublished observations). It does, however, work very well with cells from hamster hearts, and results of a study with cardiomyopathic hamsters are discussed below.

One conclusion from these direct determinations of $[Ca^{2+}]_m$ *in situ* is that the gradient of Ca^{2+} across the mitochondrial membrane—$[Ca^{2+}]_m/[Ca^{2+}]_c$—may in fact be less than one (Miyata *et al.* 1991). This was found to be so at low, and nonphysiological, frequencies of electrical stimulation. Since this conclusion followed from comparison of different sets of cells, as measurements of $[Ca^{2+}]_c$ were made in cells loaded with the membrane-impermeant pentapotassium salt of indo-1 (Sollott *et al.*, 1992), it seemed wise to look for some independent verification. This was achieved by the addition of the uncoupling agent FCCP to cardiac myocytes (Di Lisa *et al.*, 1993b). By collapsing the proton electrochemical gradient, FCCP would be expected to also reduce the gradient $[Ca^{2+}]_m/[Ca^{2+}]_c$ to unity. Such studies showed that FCCP indeed caused a rise in $[Ca^{2+}]_m$ when added to unstimulated cells, while causing a decline when added to stimulated cells showing values of $[Ca^{2+}]_m$ above approximately 200 nM (Di Lisa *et al.*, 1993b). Thus, the conclusions above concerning the directionality of the mitochondrial Ca^{2+} concentration gradient were probably correct. It is noted that there is very good agreement between results of these studies and those involving suspensions of heart mitochondria (Hansford, 1991).

Notably, there was no evidence of beat-to-beat variation in $[Ca^{2+}]_m$ in the studies of Miyata *et al.* (1991) and Di Lisa *et al.* (1993b). This is in keeping with modelling predictions by Crompton (1985) and Robertson *et al.* (1982) which indicated that the carriers of the mitochondrial membrane were not sufficiently active for $[Ca^{2+}]_m$ to track $[Ca^{2+}]_c$ with fidelity. Clearly in the adjustment to a raised rate of cellular contraction, there must be an increment in $[Ca^{2+}]_m$ with each beat, reflecting a net gain of flux through the uniporter relative to the antiporter, but the rate of such an adjustment is quite slow ($T_{1/2}$ of approximately 20 s) and the gain in $[Ca^{2+}]_m$ with each beat is too small to measure (Miyata *et al.*, 1991).

Another technique which has been applied to the question of beat-to-beat variations in $[Ca^{2+}]_m$ is EPMA, and this has yielded disparate answers in the hands of different workers. Thus, application of EPMA to rapidly frozen cardiac myocytes isolated from guinea pig has been taken to show beat-to-beat variations in $[Ca^{2+}]_m$ (Isenberg *et al.*, 1993; Wendt-Gallitelli and Isenberg, 1991). Using a protocol of paired voltage clamp pulses which gave contractions of maximal amplitude, these authors showed a rise in $[Ca^{2+}]_{m-total}$ which began after a 20 ms lag and almost dissipated by 90 ms, at which point contraction was at a maximum. The maximum $[Ca^{2+}]_{m-total}$ achieved, 40 ms after the stimulation, was 3.2 ± 0.25 mmol kg^{-1} dry wt., up from an end-diastolic value of 1.34 ± 0.24, recorded during a train of paired stimuli. In unstimulated cells, $[Ca^{2+}]_{m-total}$ was found to be 0.51 ± 0.34 mmol kg^{-1} dry wt., which is near the limit of detection of the method. On the basis of the findings that $[Ca^{2+}]_m$ is approximately $[Ca^{2+}]_{m-total} \times 10^{-3}$ (Hansford and Castro, 1982), values of $[Ca^{2+}]_m$ were approximately 0.2, 1.0, and 0.4 μM for rest, potentiated systole, and potentiated disatole, respectively. Clearly, these values fall in the range where dehydrogenase regulation is achieved (Hansford, 1985; McCormack *et al.*, 1990a).

Other work using EPMA fails to show any beat-to-beat variation in $[Ca^{2+}]_m$ (Moravec and Bond, 1991). There are a number of differences in experimental protocol between this study and those of Wendt-Gallitelli and Isenberg (1991) and Isenberg *et al.* (1993), which may explain the disparate result. Firstly, Moravec and Bond (1991) used hamster papillary muscles, stimulated electrically at 0.2 Hz. Hamster cardiac myocytes resemble those of the rat in the studies of Di Lisa *et al.* (1993a), whereas guinea pig cells show some evidence of beat-to-beat variation in $[Ca^{2+}]_m$ in Mn^{2+} quench experiments but are hard to investigate using that technique, owing to instability of the mitochondrial indo-1 signal (Janczewski and Lakatta, unpublished). Secondly, Moravec and Bond (1991) froze the muscles at the time of maximum rate of rise of tension ($+dT/dt$), a time at which there is no statistically significant increase in $[Ca^{2+}]_{m-total}$ in the experiments of Isenberg *et al.* (1993). Thirdly, the studies by Wendt-Gallitelli and Isenberg (1991) and Isenberg *et al.* (1993) used a paired-stimulus protocol, involving a longer period of depolarization than found in a normal cardiac action potential, possibly allowing for more net transport into, and out of, the mitochondria.

Clearly, these different techniques have different pros and cons in the attempt to describe the dynamics

of $[Ca^{2+}]_m$. Thus, the EPMA technique, while attractive in its ability to pinpoint individual mitochondria *in situ* in tissue, is limited in sensitivity—a particular problem in resting muscle. In addition the rapid freezing protocol precludes the use of a physiological frequency of electrical stimulation, if different time points of the cardiac cycle are to be described. The indo-1/Mn^{2+}-quench paradigm, on the other hand, allows selection of myocytes and near-physiological rates of stimulation, but has the intrinsic danger of requiring titration with an ion which interacts with Ca^{2+}-transport processes.

A procedure which avoids this difficulty, yet has the potential to measure $[Ca^{2+}]_m$ directly in isolated cells, is laser-scanning confocal microscopy. A very recent study by Chacon *et al.* (1993) of cardiac myocytes loaded with both fluo-3 and tetramethylrhodamine methyl ester showed transients in $[Ca^{2+}]_m$ of the same size and shape as those of $[Ca^{2+}]_c$. The mitochondrial origin of fluo-3 fluorescence was attributed on the basis of the localization of the potential-dependent rhodamine compound. The lack of any damping of the transients in $[Ca^{2+}]_m$ makes this an implausible result. It seems likely that a fluo-3 signal from the cytosol was also being sampled: certainly, it would seem to be very difficult to exclude this in an actively contracting cell. Potentially this problem can be avoided by a Ca^{2+} probe which distributes very largely into the mitochondrial space, and this approach has been described very recently by Jou and Sheu (1994). They applied confocal microsocopy to the study of the Ca^{2+}-dependent fluorescence of the compound rhod-2 which is positively charged and therefore accumulated into mitochondria due to $\Delta\psi$. The found evidence for an increase in $[Ca^{2+}]_m$ during spontaneous contractions of cultured neonatal rat cardiac myocytes, with fluorescence rising to a value of 1.7-fold the resting value. As there was no indication of $[Ca^{2+}]_c$ in this study, it is not easy to assess to what extent the changes in $[Ca^{2+}]_m$ were damped, or lagged those of $[Ca^{2+}]_c$.

In summary, there is reasonably convincing evidence that $[Ca^{2+}]_m$ increases with inotropic activation and increased work performance of cardiac muscle. Further, these changes are in the zone where meaningful dehydrogenase activation will occur. Other evidence in favor of these conclusions is presented below in the section on the effect of mitochondrial Ca^{2+} transport on $[Ca^{2+}]_c$. By contrast, there is no agreement on the existence of beat-to-beat changes in $[Ca^{2+}]_m$, with different methodologies yielding different conclusions.

ACTIVATION OF ENZYMES LINKED TO RAISED $[Ca^{2+}]_m$ IN OTHER EXCITABLE TISSUES

Nerve is characterized in its functioning by the rapid depolarization and repolarization of the plasma membrane and the accompanying transient increases in $[Ca^{2+}]_c$ which are involved in the mechanism of transmitter substance release (Katz and Miledi, 1967; Blaustein, 1975; Raiteri and Levi, 1978). The repolarization of the neuron involves active pumping of Na^+ and K^+ by the Na^+/K^+ ATP-ase, and thus it may be anticipated that more frequent firing will place a substantially raised energy demand upon oxidative phosphorylation in this tissue. To what extent does the increase in time-average $[Ca^{2+}]_c$ during repetitive firing cause increased $[Ca^{2+}]_m$ and the activation of oxidative phosphorylation?

Early work with preparations of synaptosomes, viz pinched-off presynaptic nerve terminals, established that chronic depolarization protocols involving raised medium K^+ concentration, or opening of Na^+ channels with the alkaloid veratridine, resulted in activation of pyruvate dehydrogenase, as well as the long-term elevation of $[Ca^{2+}]_c$ (Schaffer and Olson, 1980; Hansford and Castro, 1985). A recent paper (Martínez-Serrano and Satrústegui, 1992) has extended these studies and shown that mitochondria *in situ* in synaptosomes carry out the net accumulation of Ca^{2+} from the cytosol when values of $[Ca^{2+}]_c$ are greater than 100 nM or so. This was demonstrated by monitoring the release of Ca^{2+} from the mitochondria in response to FCCP plus oligomycin, or to rotenone plus oligomycin, using fluo-3 as an indicator of $[Ca^{2+}]_c$. Values of $[Ca^{2+}]_c$ were manipulated by altering the Ca^{2+} concentration of the medium in which the synaptosomes were incubated or by graded depolarization of the plasma membrane with media of varying K^+ concentration.

Recent studies with isolated dorsal root ganglion neurons have also shown that $[Ca^{2+}]_c$ remains elevated for longer periods of time when mitochondrial Ca^{2+} uptake is precluded, either by the inhibition of respiration or by intracellular dialysis with solutions containing ruthenium red, in whole-cell patch-clamp experiments. The greater the degree of cell Ca^{2+} loading, the larger the fractional role of mitochondrial Ca^{2+} transport to the buffering of the cytosol (Thayer and Miller, 1990). The degree of cell Ca^{2+} loading was determined in these studies by the concentration of extracellular K^+ or by the duration of

voltage-clamp steps to 0 mV (Thayer and Miller, 1990). Important from the perspective of this section of this review article was the finding that the mitochondrial content of Ca^{2+} was very low in resting neurons, but was much increased for some time following a pulsed depolarization of the cell (Thayer and Miller, 1990). Mitochondrial Ca^{2+} content was estimated from the size of increase in $[Ca^{2+}]_c$ upon dumping the content of the mitochondria by exposure to the uncoupling agent CCCP. The raised mitochondrial Ca^{2+} content persisted during a "plateau" of elevated $[Ca^{2+}]_c$ (300–400 nM) which followed a rapid transient of $[Ca^{2+}]_c$ to higher values (500–800 nM), upon plasma membrane depolarization. The conclusion of the authors that mitochondria accumulate Ca^{2+} during the rapid, high transient in $[Ca^{2+}]_c$, and slowly release it during the subsequent "plateau" phase, seems quite tenable. The implications for activation of oxidative phosphorylation are clear.

From the viewpoint of activation of oxidative phosphorylation, the recent study of isolated dorsal root ganglion neurons by Duchen (1992) is particularly informative. Pulses of KCl of 100–500 ms duration, or voltage steps to 0 mV for 50 ms in the patch-clamp protocol, gave rise to increases in $[Ca^{2+}]_c$, which reversed in a biphasic manner, as described by Thayer and Miller (1990), and to a transient decrease in mitochondrial membrane potential, as indicated by rhodamine-1,2,3 fluorescence. This is the expected consequence of the net influx of Ca^{2+} through the electrophoretic Ca^{2+} uniporter. However, measurement of changes in cell autofluorescence, which reflect mainly changes in mitochondrial NADH content, reveals a more complex pattern, with an initial oxidation occurring synchronously with the large, rapid transient in $[Ca^{2+}]_c$, followed by increased reduction, which persists as long as the "plateau" phase of $[Ca^{2+}]_c$ persists. This is consistent with the increased activation of intramitochondrial dehydrogenases occurring as a consequence of net mitochondrial Ca^{2+} uptake. All metabolic changes were blocked by the intracellular application of ruthenium red, in the patch-clamp protocol, establishing the necessity for the mitochondrial uptake of Ca^{2+} for the changes in $\Delta\psi$ and NADH to occur. This work clearly shows that the activity status of the intramitochondrial dehydrogenases, as judged by the mitochondrial NADH content, is raised for extended periods following brief excursions of $[Ca^{2+}]_c$ into the near-micromolar range. Thus there is "memory" in the system. This is quite consistent with modelling predictions based upon the *in vitro* behavior of mitochondria (Crompton 1985; Gunter and Pfeiffer, 1990) and at odds with claims (in another tissue) of syncronous changes in $[Ca^{2+}]_c$ and $[Ca^{2+}]_m$ (Chacon *et al.*, 1993).

It should be pointed out that measurements of steady-state mitochondrial $NADH/NAD^+$ ratio of this type (Duchen, 1992) may underestimate the degree of dehydrogenase activation which has occurred. The reason is that there is evidence, at least in heart, of an activation of the F_1-F_0 ATP-ase by increases in $[Ca^{2+}]_m$. The evidence derives from experiments with isolated cardiac myocytes in which electrical stimulation led to stable increases in F_1F_0 ATP-ase activity, in a process which was sensitive to ruthenium red (Das and Harris, 1990a,b; Harris and Das, 1991). The kinetics of activation and inactivation, upon cessation of electrical stimulation, were remarkably similar to the kinetics of changes in $[Ca^{2+}]_m$ seen in stimulated myocytes by Miyata *et al.* (1991). Thus, increased flux through oxidative phosphorylation, due to activation by Ca^{2+}, could occur with increases or decreases in $NADH/NAD^+$ ratio. The quantitative contribution of the activation of the F_1F_0 ATP-ase by Ca^{2+} to the control of the overall process of oxidative phosphorylation remains to be seen, as the range of activities in the studies by Harris and Das (1991) was only of the order of 3-fold. Conceivably, the enzyme activation was not fully stable during the extraction necessary before measurement. Whether an activation of the F_1F_0 ATP-ase by increased $[Ca^{2+}]_m$ occurs in nuerons, or in other excitable tissues, remains an open and important question.

There is also evidence of activation of pyruvate dehydrogenase in pancreatic islet cells, in response to increasing the glucose concentration or to raising the bathing Ca^{2+} from 1.2 to 4.2 mM (McCormack *et al.*, 1990b). Presumably this involves net influx of Ca^{2+} into the mitochondria in response to the "spikes" of $[Ca^{2+}]_c$ which are known to accompany challenge by glucose (Longo *et al.*, 1991). A recent direct measurement of $[Ca^{2+}]_m$ in a pancreatic β-cell line (INS-1) using a novel technique of targeting recombinant aequorin to the mitochondria (described more fully in the next section) has shown that there are indeed large increases in $[Ca^{2+}]_m$ in response to ATP or depolarization by K^+ (Rutter *et al.*, 1993). Values of more than 4 μM were measured for $[Ca^{2+}]_m$, in response to increases in $[Ca^{2+}]_c$ to less than 600 nM, but lasting many seconds. Unfortunately, Ca^{2+}-mobilization by

glucose could not be studied in these experiments with aequorin targeted to the mitochondria, as cells respond nonsynchronously to stimulation by glucose, and the sensitivity of the aequorin technique requires that light be collected from many cells. It is known that increased glucose concentration leads to increased adenine nucleotide phosphate potentials in islet cells, and this has been linked to the closure of K_{ATP} channels, and consequent plasma membrane depolarization, entry of Ca^{2+} on voltage-dependent-channels, and increased secretion of insulin (see Ashcroft and Rorsman, 1990). Although it might have been attractive to speculate that the rise in $[Ca^{2+}]_c$ was the cause of the increased ATP/ADP ratio, rather than the effect, this possibility has now been laid to rest in a study by Duchen *et al.* (1993), which establishes that the rise in $[Ca^{2+}]_c$ due to glucose addition, as measured with fluo-3, is a late event (latency 129 s), whereas fluorescence of mitochondrial NADH and $\Delta\Psi$ rise earlier (latency 28 and 49 s, respectively). Thus, in this unique tissue, the rise in $[Ca^{2+}]_m$ likely reinforces a condition of enhanced oxidative activity which originated in enhanced glycolysis and substrate provision to the mitochondria.

ACTIVATION OF ENZYMES LINKED TO RAISED $[Ca^{2+}]_m$ IN NONEXCITABLE TISSUES

Previous reviews have clearly documented the activation of pyruvate and 2-oxoglutarate dehydrogenases which occurs in the perfused liver and in isolated hepatocytes upon exposure to the Ca^{2+}-mobilizing hormones vasopressin, glucagon, and α_1-adrenergic agonists (McCormack *et al.* 1990a; Hansford, 1991). At the same time, mitochondrial NADH content increases and so does the rate of O_2 uptake, despite an increase in the cytosolic adenine nucleotide phosphate potential. Concomitant with these changes is an increased content of $[Ca^{2+}]_{m-total}$, as determined by the rapid isolation of mitochondria in conditions minimizing redistribution of the ion (Assimacopoulos-Jeannet *et al.*, 1986). These results will not be reiterated here and the reader is referred to the previous reviews (McCormack *et al.*, 1990a; Hansford, 1991) for more details. An important recent addition to this field is the analysis by Brown *et al.* (1990) of the flux control coefficients of the various processes producing and consuming $\Delta\psi$ in the control of the overall process of oxidative phosphorylation in hepatocytes. These authors conclude that the control coefficient for the processes which produce mitochondrial NADH (which, of course, includes the Ca^{2+}-sensitive dehydrogenases) is 0.15–0.3, in cells from fed rats. For the processes of ATP synthesis, transport, and consumption by activities in the cytosol the coefficient is 0.49. These figures are consistent with a significant role of control of dehydrogenases by Ca^{2+}, and with a substantial potential role for control of F_1F_0 ATP-ase by Ca^{2+}, in the control of the overall process of oxidative phosphorylation. It remains to be seen whether there is a stable Ca^{2+}-dependent activation of the F_1F_0 ATPase in hepatocytes, as there is in cardiac myocytes (Harris and Das, 1991).

The oscillatory transients of $[Ca^{2+}]_c$ found in single hepatocytes in response to Ca^{2+}-mobilizing hormones usually persists for many seconds (Kawanishi *et al.*, 1989; Rink and Hallam, 1989; Woods *et al.*, 1986), and indeed more prolonged elevations of cytosolic Ca^{2+} may be a feature which distinguishes nonexcitable from excitable tissues. Clearly, mitochondrial Ca^{2+} transport would be expected to respond more fully to these longer transients in $[Ca^{2+}]_c$. This is what was seen in a remarkable recent study of cellular Ca^{2+} homeostasis in endothelial cells transfected with a chimeric gene which allows the synthesis of the luminescent jellyfish protein aequorin and its insertion into the mitochondria of these mammalian cells (Rizzuto *et al.*, 1992). Challenge with extracellular ATP caused a rapid rise in $[Ca^{2+}]_c$, as indicated with fura-2, and a large, rapid rise in $[Ca^{2+}]_m$, as indicated by the luminescence of aequorin. The rise in $[Ca^{2+}]_c$ was maintained at approximately 600 μM for at least 30 s, and so the large rise in $[Ca^{2+}]_m$ is perhaps not surprising. Certainly, the mitochondria have much more opportunity for net uptake of Ca^{2+} in this situation than they do during the much shorter action potential of an electrically stimulated cardiac myocyte—a paradigm in which no beat-to-beat variation in $[Ca^{2+}]_m$ is observed (Miyata *et al.*, 1991). What is surprising in the study by Rizzuto *et al.* (1992) is that $[Ca^{2+}]_m$ apparently decays more rapidly than $[Ca^{2+}]_c$. This would be contrary to what has been seen in other cell types (see, e.g., Thayer and Miller, 1990) and contrary to modelling predictions. Conceivably the result is an artefact of the nonlinear dependence of aequorin light on Ca^{2+}—though that is a speculation. The amplitude of the rise in $[Ca^{2+}]_m$ in this study is also surprising, reaching, it is estimated, a maximum of approximately 5 μM, while $[Ca^{2+}]_c$ only reaches 0.6 μM. This indicates a gradient

$[Ca^{2+}]_m/[Ca^{2+}]_c$ which is substantially larger than that found in several studies of mitochondria *in vitro* at comparable values of extramitochondrial $[Ca^{2+}]$ (Hansford, 1991; McCormack *et al.*, 1989). Though this might have been explained by the presence of factors in the cytosol affecting the net balance of uniport and antiport, another, quite intriguing, explanation has been offered (Rizzuto *et al.*, 1993). Thus, it appears that mitochondria, or at least some mitochondria, are exposed to high local concentrations of Ca^{2+} when ER stores are released by IP_3. In a study of a HeLa cell clone expressing mitochondrial aequorin, mitochondria were found to be much more sensitive to Ca^{2+} released from the ER due to exposure of the cells to histamine, than to Ca^{2+} entering the cell through the plasma membrane (Rizzuto *et al.*, 1993). Thus, comparison of global measures of $[Ca^{2+}]_c$, as achieved with fura-2 fluorescence, with values of $[Ca^{2+}]_m$ may not be appropriate. These results from this new and creative technique raise intriguing questions. If different populations of mitochondria within a cell are exposed to different values of $[Ca^{2+}]_c$ on cell activation, do they respond by generating different values of $\Delta\bar{\mu}H^+$? If so, are there gradients of adenine nucleotide phosphate potential within a cell, especially in cells lacking the creatine kinase shuttle? The question of the magnitude of $\Delta\bar{\mu}H^+$, or at least $\Delta\psi$, of individual mitochondria in a cell is now being approached (Loew *et al.*, 1993).

EFFECT OF MITOCHONDRIAL Ca^{2+} TRANSPORT UPON $[Ca^{2+}]_c$

For many years it was considered that mitochondria play a primary role in setting, or "buffering," $[Ca^{2+}]_c$ (see, e.g., Nicholls, 1978), a view that was supported by the powerful ability of mammalian mitochondria to accumulate or release Ca^{2+} so as to achieve a steady-state value of $[Ca^{2+}]_0$, referred to as the "set-point" (Åkerman and Nicholls, 1983). The key to the observation of this behavior was that the mitochondria had to be loaded with Ca^{2+} to the point that their efflux pathway was saturated with $[Ca^{2+}]_m$. Under these conditions, net accumulation of Ca^{2+} upon adding a pulse of $CaCl_2$ to the medium resulted in no enhancement in efflux rate and therefore proceeded to the point that the influx rate fell back to that seen prior to the pulse of $CaCl_2$: this was achieved when $[Ca^{2+}]_0$ also returned to prepulse levels. The first paper to challenge the operation of this system under physiological conditions was that by Hansford and Castro (1982), which showed that heart mitochondria could either "buffer" values of $[Ca^{2+}]_0$, if their $[Ca^{2+}]_{m-total}$ was above 10 nmol/mg of mitochondrial protein—in which case their matrix dehydrogenases were saturated with Ca^{2+} and not controlled—or they could regulate the activity of the dehydrogenases, if their $[Ca^{2+}]_{m-total}$ was 0 to 5 nmol/mg of protein—in which case they acted as less than perfect buffers of $[Ca^{2+}]_0$. Clearly, the value of $[Ca^{2+}]_{m-total}$ *in situ* in tissues became of paramount importance. Improvement of rapid-freezing and EPMA techniques over the last decade has generally supported the notion that $[Ca^{2+}]_{m-total}$ is in the range 0–5 nmol/mg protein—certainly in resting tissues (Somlyo *et al.*, 1985; Bond *et al*, 1987)—consistent with mitochondrial functioning in the "dehydrogenase-regulation" mode.

The case has been made quite eloquently that Ca^{2+}-sensitive intramitochondrial dehydrogenases and high-affinity mitochondrial Ca^{2+} transport systems have evolved together in the animal kingdom, to allow the regulation of the operation of the Krebs tricarboxylate cycle (Denton and McCormack, 1980; McCormack and Denton, 1981). Clearly, the sampling of $[Ca^{2+}]_c$ by the mitochondria must affect $[Ca^{2+}]_c$, and this section will deal with some recent experiments on the degree of this impact, emphasizing muscle and nerve cells.

The blockade of net mitochondrial Ca^{2+} uptake by uncoupling agents results in larger transients in $[Ca^{2+}]_c$ and larger contractions in isolated cardiac myocytes, until ATP/ADP × Pi ratios decline to the point where contraction fails (Wolska and Lewartowski, 1991; Chapman, 1986; Isenberg *et al.*, 1993). The difference can be quite large, with Isenberg *et al.* (1993) reporting transients in $[Ca^{2+}]_c$ reaching approximately 1 μM in the presence of the uncoupler dinitrophenol, compared to approximately 0.4 μM in the absence of uncoupler. Comparison of the records of $[Ca^{2+}]_c$, using indo-1, revealed net uptake into the mitochondria for 30 ms or so, followed by net release over 150 ms, in response to their paired stimulus protocol. These results are consistent with, and the mirror image of, the results on $[Ca^{2+}]_{m-total}$ obtained by these authors by EPMA. Clearly they are subject to the same questions involving the physiological significance of these protocols involving lengthy depolarizations.

Mitochondrial Ca^{2+} uptake serves to damp increases in $[Ca^{2+}]_c$ in nerve preparations, especially at the higher Ca^{2+} loads achieved with more lengthy

depolarizations (Thayer and Miller, 1990; Duchen *et al.*, 1990) or by Na^+-replacement protocols (Baker and Umbach, 1987). Blockade of the mitochondrial respiratory chain (Duchen *et al.*, 1990), uncoupling of oxidative phosphorylation (Duchen *et al.*, 1990; Martínez-Serrano and Satrústegui, 1992), or introduction of ruthenium red into neurons (Baker and Umbach, 1987; Thayer and Miller, 1990) all increase the duration and size of transients in $[Ca^{2+}]_c$ occurring in response to activation of plasma membrane voltage-dependent Ca^{2+} channels. Interference with mitochondrial functioning during hypoxia of the central nervous system is likely to act synergistically with stimulation of neurons by excitotoxic neurotransmitters to flood the cytosol with Ca^{2+}, to disastrous effect.

ACTIVATION OF MITOCHONDRIAL ENZYMES BY Ca^{2+} IN PATHOLOGICAL STATES OF THE HEART

Response to Anoxia and Reoxygenation

There is no question that mitochondria can accumulate much larger amounts of Ca^{2+} than discussed so far in this article, under pathophysiological conditions. Thus, the reperfusion of ischemic heart or brain leads to a large Ca^{2+} uptake, much of which is localized to the mitochondria, and this may play a role in cell death under these conditions.

How much mitochondrial Ca^{2+} accumulation occurs? Two recent papers have directly measured $[Ca^{2+}]_m$ of cardiac myocytes under reoxygenation protocols, with somewhat different results. Application of the technique of Miyata *et al.* (1991), described above, to rat myocytes which were made truly anoxic and then reoxygenated revealed that no increase occurs in $[Ca^{2+}]_m$ or $[Ca^{2+}]_c$ during anoxia until the cells undergo a rigor contraction (Miyata *et al.*, 1992). Similar results have been obtained previously using techniques which measure $[Ca^{2+}]_c$ solely (Allshire *et al.*, 1987) or predominantly (Li *et al.*, 1988). During continued anoxia, $[Ca^{2+}]_m$ and $[Ca^{2+}]_c$ rise slowly and in parallel. Upon reoxygenation, $[Ca^{2+}]_m$ rises, while $[Ca^{2+}]_c$ falls—suggesting a net accumulation of Ca^{2+} by the mitochondria once O_2 availability allows generation of $\Delta\psi$. The subsequent fate of the cell depends upon how long it spent in anoxia following rigor, and to what level $[Ca^{2+}]_m$ has risen. Cells with lower values of $[Ca^{2+}]_m$ "survive" reoxygenation in that they lengthen slightly and begin to beat again, while cells with the higher values of $[Ca^{2+}]_m$ during early reoxygenation undergo hypercontracture, become rounded and die. The value of $[Ca^{2+}]_m$ which separates the sheep from the goats was found to lie between 200 and 500 nM (Miyata *et al.* 1992). This is surprisingly low, being very much in the range described above as potentiating dehydrogenase activation. Conceivably the autofluorescence controls in this work, though careful, were not correct. Other work, measuring only $[Ca^{2+}]_c$ and not $[Ca^{2+}]_m$, has indicated higher thresholds for cell survival (Allshire *et al.*, 1987; Li *et al.*, 1988).

The other recent approach to this problem involved the rapid isolation of mitochondria which had been loaded with fura-2 from hearts made hypoxic and then reoxygenated (Allen *et al.*, 1993). Results of subsequent $[Ca^{2+}]_m$ determinations, as described by Allen *et al.* (1992) and discussed above, agreed with the findings of Miyata *et al.* (1992) in that $[Ca^{2+}]_m$ remained steady during hypoxia (near-anoxia) and rose sharply upon reoxygenation, to a level that depended upon the time spent in hypoxia. Thus, 20 min of reoxygenation after 50 min of hypoxia gave a modest increase in $[Ca^{2+}]_m$—to 500 nM—whereas reoxygenation after 80 min of hypoxia gave values of $[Ca^{2+}]_m$ which saturated the fura-2 (taken by the authors to be more than 5 μM). Notably, this overload of the mitochondria was largely prevented by perfusion of the heart with 2.5 μM ruthenium red and not with 1 μM (Allen *et al*, 1993).

Is mitochondrial Ca^{2+} uptake during reoxygenation protective of cell function or deleterious? The evidence in heart suggests that mitochondrial Ca^{2+} uptake underlies the loss of contractile efficiency seen in the reperfused heart (Benzi and Lerch, 1992). Thus, 30 min reperfusion with 6 μM ruthenium red normalized the ratio of contractile performance (the product of heart rate and left-ventricular pressure development) to myocardial oxygen consumption, whereas reperfusion of ischemic heart in the absence of ruthenium red resulted in a contractile performance/oxygen-uptake ratio of only 7% of control values. Ruthenium red also reduces cell damage, as measured by creatine kinase release, both in perfused heart (Stone *et al.*, 1989; Benzi and Lerch, 1992) and in suspensions of isolated cardiac myocytes (Stone *et al.* 1989).

How does excessive mitochondrial Ca^{2+} uptake lead to decreased contractile performance and, ultimately, to loss of cell membrane permeability proper-

ties? Two mechanisms come to mind. First, the energy dissipation of high fluxes of Ca^{2+} through the uniporter and antiporter may lower $\Delta\bar{\mu}H^+$ to the point where cytosolic adenine nucleotide phosphate potential cannot support adequate SR Ca^{2+} accumulation (Kammermeier *et al.*, 1982; Griese *et al.*, 1988), or the plasma membrane becomes inexcitable, owing to the voltage-clamping effect of a fraction of open K_{ATP} channels (Stern *et al.*, 1988). Contraction diminishes and eventually fails.

Secondly, a megachannel in the inner mitochondrial membrane, which is known as the "permeability transition pore" and which is reviewed by Gunter and Pfeiffer (1990) and is discussed extensively elsewhere in this volume, may open and allow the collapse of $\Delta\bar{\mu}H^+$ and the mixing of low-molecular-weight (< 1500 Da) components of the cytosol and matrix. The opening of this megachannel is completely dependent upon the generation of high concentrations of $[Ca^{2+}]_m$, such that 25 μM $[Ca^{2+}]_m$ gives substantial, but far from complete, opening (Al Nasser and Crompton, 1986). Thus, it is unlikely to be involved if the work of Miyata *et al.* (1992) gives credible values for $[Ca^{2+}]_m$ upon reoxygenation, but could be involved if $[Ca^{2+}]_m$ rises to levels which saturate fura-2 (Allen *et al*, 1993). The latter is quite likely to be the case. Opening of the megachannel is also potentiated by high inorganic phosphate concentrations (Crompton and Costi, 1990), likely to occur owing to some hydrolysis of creatine phosphate, and by oxidative stress, which is known to accompany reoxygenation of heart (McCord, 1987).

The biological significance of this mechanism can be tested by use of the immunosuppressant cyclosporin A, which is known to prevent the opening of the channel in experiments with suspensions of mitochondria and with excised patches of mitochondrial membranes (Crompton *et al.*, 1988; Szabo and Zoratti, 1991). Exposure of isolated cardiac myocytes to cyclosporin A, over a narrow concentration range, has been reported to retard the hypercontracture seen on reoxygenation (Nazareth *et al.*, 1991). Beneficial effects have also been reported in experiments exposing hepatocytes to high Ca^{2+} and oxidative stress (Broekemeier *et al.*, 1992). There is also a recent report suggesting that cyclosporin A increases net Ca^{2+} uptake into suspensions of cardiac myocytes under normal, physiological conditions, and decreases net Ca^{2+} release, in a process which is sensitive to ruthenium red (12 μM) (Altschuld *et al.*, 1992). This suggests that there is a significant frequency of opening of the megachannel under physiological conditions. This conclusion has to be treated with caution, owing to the inherent problems of heterogeneity in work with suspensions of cardiac myocytes. It is extremely difficult to rule out a contribution due to a fraction of Ca^{2+}- and Na^+-overloaded cells in this sort of study. It is of course possible that there is a finite, low rate of opening and resealing of the megachannel under normal physiological conditions, and that it may in fact allow the movement of molecules for which there are no transport mechanisms into, and out of, the mitochondria (see Gunter and Pfeiffer, 1990). In an intriguing and casual reference Loew *et al.* (1993) mention rare but large oscillations of $\Delta\psi$ in a study of individual mitochondria in a nerve cell line. Conceivably, this is opening of the megachannel.

Clearly, if a large enough fraction of the population of mitochondria in a cell is undergoing opening of the megachannel at the same time, ATP production will be limited and contractility will be comprised by the same mechanisms alluded to briefly above. Eventually, elevation of cell Ca^{2+} and diminution of phosphate potential lead to loss of plasma membrane permeability and cell death. The primacy of ATP depletion versus $[Ca^{2+}]_c$ elevation in the generation of this state is contentious (see Herman *et al.*, 1990) and beyond the scope of this review. Central nervous tissue is acutely sensitive to damage from ischemia and reperfusion and the issues discussed here for the heart are all germane to the brain. There appears to be less work in this area.

PERTURBATION OF $[Ca^{2+}]_m$ IN CARDIOMYOPATHY

Myopathies of cardiac muscle have long been associated with cellular Ca^{2+} loading (Dhalla *et al.*, 1974; Markiewicz *et al.*, 1986), and this has often been assumed to be the mechanism of deterioration of cell function. However, it is clear that the disturbance of cellular energetics for whatever reason will lead to Ca^{2+} loading, as the chemical gradient alone for Ca^{2+} ions across the plasma membrane is approximately 10^4 and thus it is hard to establish cause and effect. In the cardiomyopathic Syrian hamster, a well-known genetic model of cardiomyopathy, bulk assay of cardiac muscle from affected hamsters shows an increased total Ca^{2+}, as do mitochondrial prepara-

tions from these hearts (Proschek and Jasmin, 1982; Wrogemann and Nylen, 1978). However, cell-by-cell examination using electron probe microanalysis reveals that the large majority of myocytes do not have elevated cytosol total Ca^{2+} or $[Ca^{2+}]_{m-total}$, but that the extra Ca^{2+} of bulk preparations derives from necrotic areas and from a few cells on the periphery of these areas, which are presumably mechanically stressed (Bond *et al.*, 1989).

Indeed, a recent paper from the author's lab (Di Lisa *et al.*, 1993a) makes the case that $[Ca^{2+}]_m$ is actually lower in myocytes from the myopathic animal and that this compromises mitochondrial substrate oxidation. This was demonstrated by examining single isolated cardiac myocytes from cardiomyopathic (strain B10 14.6) and control (F1B) animals, and investigating the response of $[Ca^{2+}]_m$ to raised rates of electrical pacing. It was found that $[Ca^{2+}]_m$ only rose from 248 ± 15 nM at rest to 348 ± 44 nM with 4 Hz stimulation in the B10 14.6 cells, compared with a rise from 241 ± 35 to 830 ± 124 nM in cells from healthy F1B animals. Developed pressure from isolated, perfused hearts of the B10 14.6 animals was lower, as had been shown previously (Wikman-Coffelt *et al.*, 1986, 1991) and, most interestingly, the fraction of pyruvate dehydrogenase present as PDH_A was lower in glucose-perfused hearts from the myopathic animals (Di Lisa *et al.*, 1993a). The inability to increase PDH_A in response to work load of the heart is consistent with the inability to increase $[Ca^{2+}]_m$ in response to electrical stimulation of myocytes from the B10 14.6 animals. The basic lesion does not seem to be at the level of the mitochondrial membrane, but rather in the excitation-contraction coupling process. Thus, electrical stimulation of cardiac myocytes from B10 14.6 animals resulted in smaller transients in $[Ca^{2+}]_c$, as sensed with the salt form of indo-1, than those seen in control cells. One would expect mitochondria to respond to a weighted average of $[Ca^{2+}]_c$, with more weight given to the peak of the transients, owing to the sigmoidal nature of the v versus s plot for the Ca^{2+} uniporter of heart mitochondria (Crompton, 1985) and the fact that the V_{max} of the uniporter far exceeds that of the Na^+/Ca^{2+} exchanger. However, a recent study examining changes in $[Ca^{2+}]_m$ of heart mitochondria in vitro exposed to mechanically generated oscillations in buffer $[Ca^{2+}]$ concluded that $[Ca^{2+}]_m$ rose with the simple arithmetic mean of the external $[Ca^{2+}]$ (Leisey *et al.*, 1993). Whichever is true, smaller transients in $[Ca^{2+}]_c$ generate lower values of $[Ca^{2+}]_m$, if the frequency is unchanged, and this is what is found in the myopathic hamster cells.

This may be more than just an isolated case. Das and Harris (1990a) reported less of a range of response of F_1F_0-ATP-ase activity to electrical stimulation of cardiac myocytes from a spontaneously hypertensive (SHR) rat, when compared to cells from normotensive controls. This adjustment to electrical stimulation requires net mitochondrial Ca^{2+} fluxes, as evidenced by sensitivity to ruthenium red (as discussed above). Das and Harris (1990a) describe elevated ATP-ase activity in unstimulated SHR cells, relative to controls, but a lesser activation of ATP-ase in response to 10 Hz, in the SHR cells. This would be consistent with increased $[Ca^{2+}]_m$ at rest, but a failure to raise $[Ca^{2+}]_m$ with electrical stimulation, as in the cardiomyopathic hamster. However, other explanations are possible, and it is notable that the Ca^{2+}-channel blocker verapamil lowers ATP-ase in unstimulated SHR cells (Das and Harris, 1990a). This makes it possible that heterogeneity of the population of SHR cells is a factor, with some cells spontaneously depolarizing and increasing $[Ca^{2+}]_c$. Clearly, it would be interesting to repeat the single-cell protocol of Di Lisa *et al.* (1993a) on cells from the SHR rat.

The case has been made that heart failure reflects an unbalancing of the cellular economy, with energy demand outstripping supply (Katz, 1991). Increases in $[Ca^{2+}]_c$ stimulate energy demands, whereas increases in $[Ca^{2+}]_m$, within the physiological range, stimulate supply. It seems that drugs to activate the mitochondrial Ca^{2+} uniporter, or to cause a fractional inhibition of the Na^+/Ca^{2+} exchanger, might be effective in reestablishing a balance of energy supply and demand. Potential candidates in this regard would be more specific forms of the benzothiazepine inhibitors of the Na^+/Ca^{2+} exchange described by Cox and Matlib (1993). In the meantime, it would be informative to find out whether decreased transients in $[Ca^{2+}]_c$, and responses of $[Ca^{2+}]_m$, characterize other forms of cardiomyopathy considered to be "metabolic" in origin, e.g., that associated with type-1 diabetes (Rodrigues and McNeill, 1992; Chatham and Forder, 1993).

REFERENCES

Åkerman, K. E. O., and Nicholls, D. G. (1983). *Rev. Physiol. Biochem. Pharmacol.* **95,** 149–201.

Allen, S. P., Stone, D., and McCormack, J. G. (1992). *J. Mol. Cell. Cardiol.* **24,** 765–774.

Allen, S. P., Darley-Usmar, V. M. McCormack, J. G., and Stone, D. (1993). *J. Mol. Cell. Cardiol.* **25,** 949–958.

Allshire, A., Piper, H. M., Cuthbertstone, K. S. R., and Cobbold, P. H. (1987). *Biochem. J.* **244,** 381–385.

Al Nasser, I., and Crompton, M. (1986). *Biochem. J.* **239,** 19–29.

Altschuld, R. A., Hohl, C. M., Castillo, L. C., Garleb, A. A., Starling, R. C., and Brierley, G. P. (1992). *Am. J. Physiol.* **262,** H1699–H1704.

Ashcroft, F. M., and Rorsman, P. (1990). *Prog. Mol. Biol. Biophys.* **54,** 87–143.

Assimacopoulos-Jeannet, F., McCormack, J. G., and Jeanrenaud, B. (1986). *J. Biol. Chem.* **261,** 8799–8804.

Baker, P. F., and Umbach, J. A. (1987). *J. Physiol.* **383,** 369–394.

Balaban, R. S., Kantor, H. L., Katz, L. A., and Briggs, R. W. (1986). *Science* **232,** 1121–1123.

Benzi, R. H., and Lerch, R. (1992). *Circ. Res.* **71,** 567–576.

Berridge, M. J., and Galione, A. (1988). *FASEB J.* **2,** 3074–3082.

Berridge, M. J., Cobbold, P. H., and Cuthbertson, K. S. R. (1988). *Philos. Trans. R. Soc. London B* **320,** 325–343.

Blaustein, M. P., (1975). *J. Physiol. (London)* **247,** 617–655.

Bond, M., Jaraki, A.-R., Disch, C. H., and Healy, B. P. (1989). *Circ. Res.* **64,** 1001–102.

Bond, M., Vadasz, G., Somlyo, A. V., and Somlyo, A. P. (1987). *J. Biol. Chem.* **262,** 15630–15636.

Broekemeier, K. M., Carpenter Deyo, L., Reed, D. J., and Pfeiffer, D. R. (1992). *FEBS Lett.* **304,** 192–194.

Brown, G. C., Lakin-Thomas, P. L., and Brand, M. D. (1990). *Eur. J. Biochem.* **192,** 355–362.

Chacon, E., Harper, I. S., Reece, J. M., Herman, B., and Lemasters, J. J. (1993). *Biophys. J.* **64,** A106.

Chapman, R. A. (1986). *J. Physiol.* **373,** 163–179.

Chappell, J. B., Cohn, M., and Greville, G. D. (1963). In *Energy-Linked Functions of Mitochondria*, Academic Press, New York, pp. 219–231.

Chatham, J. C., and Forder, J. R. (1993). *J. Mol. Cell. Cardiol.* **25,** 1203–1213.

Clarke, K., and Willis, R. J. (1987). *J. Mol. Cell. Cardiol.* **19,** 1153–1160.

Coll, K. E., Joseph, S. K., Corkey, B. E., and Williamson, J. R. (1982). *J. Biol. Chem.* **257,** 8696–8704.

Cox, D. A., and Matlib, M. A. (1993). *J. Biol. Chem.* **268,** 938–947.

Crompton, M. (1985) *Curr. Top. Membr. Transp.* **25,** 231–276.

Crompton, M., and Costi, A. (1990). *Eur. J. Biochem.* **178,** 489–501.

Crompton, M., and Heid, I. (1978). *Eur. J. Biochem.* **91,** 599–608.

Crompton, M., Sigel, E., Salzmann, M., and Carafoli, E. (1976a). *Eur. J. Biochem.* **69,** 429–434.

Crompton, M., Capano, M., and Carafoli, E. (1976b). *Eur. J. Biochem.* **69,** 453–462.

Crompton, M., Kessar, P., and Al-Nasser, I. (1983). *Biochem. J.* **216,** 333–342.

Crompton, M., Ellinger, H., and Costi, A. (1988). *Biochem. J.* **255,** 357–360.

Das, A. M., and Harris, D. A. (1990a). *Am. J. Physiol.* **259,** H1264–H1269.

Das, A. M., and Harris, D. A. (1990b). *Cardiovasc. Res.* **24,** 411–417.

Davis, M. H., Altschuld, R. A., Jung, D. W., and Brierley, G. P. (1987). *Biochem. Biophys. Res. Commun.* **149,** 40–45.

Denton, R. M., and McCormack, J. G. (1980). *FEBS Lett.* **119,** 1–8.

Denton, R. M., and McCormack, J. G. (1985). *Am. J. Physiol.* **249** *(Endocrinol. Metab. 12)*, E543–E554.

Denton, R. M., Randle, P. J., and Martin, B. R. (1972). *Biochem. J.* **128,** 161–163.

Denton, R. M., Richards, D. A., and Chen, J. G. (1978). *Biochem. J.* **176,** 899–906.

Denton, R. M., McCormack, J. G., and Edgell, N. J. (1980). *Biochem. J.* **190,** 107–117.

Dhalla, H. S., Sulakhe, P. V., Fedelson, M., and Yates, J. C. (1974). *Adv. Cardiol.* **13,** 282–300.

Di Lisa, F., Fan, C.-Z., Gambassi, G., Hogue, B. A., Kudryashova, I., and Hansford, R. G. (1993a). *Am. J. Physiol.* **264,** H2188–2197.

Di Lisa, F., Gambassi, G., Spurgeon, H., and Hansford, R. G. (1993b). *Cardiovasc. Res.* **27,** 1840–1844.

Duchen, M. R. (1992). *Biochem. J.* **283,** 41–50.

Duchen, M. R., Valdeolmillos, M., O'Neill, S. C., and Eisner, D. A. (1990). *J. Physiol.* **424,** 411–426.

Duchen, M. R., Smith. P. A., and Ashcroft, F. M. (1993). *Biochem. J.* **294,** 35–42.

From, A. H. L., Petein, M. A., Michurski, S. P., Zimmer, S. D., and Ugurbil, K. (1986). *FEBS Lett.* **206,** 257–261.

Fry, C. H., Powell, T., Twist, V. W., and Ward, J. P. T. (1984). *Proc. R. Soc. London B* **223,** 239–254.

Griese, M., Perlitz, V., Jüngling, E., and Kammermeier, H. (1988). *J. Mol. Cell. Cardiol.* **20,**

Gunter, T. E., and Pfeiffer, D. R. (1990). *Am. J. Physiol.* **258** *(Cell Physiol. 27)*, C755–C786.

Gunter, T. E., Restrepo, D., and Gunter, K. K. (1988). *Am. J. Physiol.* **255,** C304–C310.

Gupta, M. P., Innes, I. R., and Dhalla, N. S. (1988). *Am. J. Physiol.* **255,** H1413–H1420.

Gupta, M. P., Dixon, I. M. C., Zhao, D., and Dhalla, N. S. (1989). *Can. J. Cardiol.* **5,** 55–63.

Hak, J. B., Van Beek, J. H. G. M., Eijgelshoven, M. H. J., and Westerhof, N. (1993). *Am. J. Physiol.* **264** *(Heart Circ. Physiol. 33)*, H448–H453.

Hansford, R. G. (1976). *J. Biol. Chem.* **251,** 5483–5489.

Hansford, R. G. (1985). *Rev. Physiol. Biochem. Pharmacol.* **102,** 1–72.

Hansford, R. G. (1987). *Biochem. J.* **241,** 145–151.

Hansford, R. G. (1991). *J. Bioenerg. Biomembr.* **23,** 823–854.

Hansford, R. G., and Castro, F. (1981). *Biochem. J.* **198,** 525–533.

Hansford, R. G., and Castro, F. (1982). *J. Bioenerg. Biomembr.* **14,** 361–376.

Hansford, R. G., and Castro, F. (1985). *Biochem. J.* **227,** 129–136.

Hansford, R. G., Hogue, B., Prokopczuk, A., Wasilewska-Dziubińska, E., and Lewartowski, B. (1990). *Biochim. Biophys. Acta* **1018,** 282–286.

Harris, D. A., and Das, A. M. (1991). *Biochem. J.* **280,** 561–573.

Heineman, F. W., and Balaban, R. S. (1990). *Annu. Rev. Physiol.* **52,** 523–542.

Heinrich, R., and Rapoport, T. A. (1974). *Eur. J. Biochem.* **42,** 89–95.

Herman, B., Gores, G. J., Nieminen, A. L., Kawanishi, T., Harman, A., and Lemasters, J. J. (1990). *Crit. Rev. Toxicol.* **21,** 127–148.

Hunter, D. R., Komai, H., Haworth, R. A., Jackson, M. D., and Berkoff, H. A. (1980). *Circ. Res.* **47,** 721–727.

Isenberg, G., Han, S., Schiefer, A., and Wendt-Gallitelli, M.-F. (1993). *Cardiovasc. Res.* **27,** 1800–1809.

Jou, M.-J., and Sheu, S.-S. (1994). *Biophys. J.* **66,** A94.

Kacser, H., and Burns, J. A. (1973). *Symp. Soc. Exp. Biol.* **27,** 65–104.

Kacser, H., and Porteous, J. W. (1987). *Trends Biochem. Sci.* **12,** 5–14.

Kammermeier, H., Schmidt, P., and Jungling, E. (1982). *J. Mol. Cell. Cardiol.* **14,** 267–277.

Katz, A. M. (1991). *Hosp. Pract.*, Aug. 15, 78–90.

Katz, B., and Miledi, R. (1967). *Proc. R. Soc. London Ser. B.* **167,** 23–38.

Katz, L. A., Koretsky, A. P., and Balaban, R. S. (1987). *FEBS Lett.* **221,** 270–276.

Katz, L. A., Koretsky, A. P., and Balaban, R. S. (1988). *Am. J. Physiol.* **255** *(Heart Circ. Physiol 24)*, H185–H188.

Katz, L. A., Swain, J. A., Portman, M. A., and Balaban, R. S.

(1989). *Am. J. Physiol.* **256,** *(Heart Circ. Physiol. 25)*, H265–H274.
Kawanishi, T., Blank, L. M., Harootunion, A. T., Smith, M. T., and Tsien, R. Y. (1989). *J. Biol. Chem.* **264,** 12859–12866.
Leisey, J. R., Grotyohann, L. W., Scott, D. A., and Scaduto, R. C., Jr. (1993). *Am. J. Physiol.* **265,** H1203–H1208.
Lenzen, S., Hickethier, R., and Panten, U. (1986). *J. Biol. Chem.* **261,** 16478–16483.
Li, Q., Altschuld, R. A., and Stokes, B. T. (1988). *Am. J. Physiol.* **255,** C162–C168.
Loew, L. M., Tuft, R. A., Carrington, W., and Fay, F. S. (1993). *Biophys. J.* **65,** 2396–2407.
Longo, E. A., Tornheim, K., Deeney, J. T., Varnum, B. A., Tillotson, D., Prentki, M., and Corkey, B. E. (1991). *J. Biol. Chem.* **266,** 9314–9319.
Lukács, G. L., and Kapus, A. (1987). *Biochem. J.* **248,** 609–613.
Lukács, G. L., Kapus, A., and Fonyo, A. (1988) FEBS Lett. **229,** 219–223.
Markiewicz, W., Wu, S. S., Parmley, W. W., Higgins, C. B., Sievers, R., James, T. L. Wikman-Coffelt, J., and James, G. (1986). *Circ. Res.* **59,** 597–604.
Martínez-Serrano, A., and Satrústegui, J. (1992). *Mol. Biol. Cell* **3,** 235–248.
McCord, J. M. (1987). *Fed. Proc.* **46,** 2402–2406.
McCormack, J. G., and Denton, R. M. (1979). *Biochem. J.* **180,** 533–544.
McCormack, J. G., and Denton, R. M. (1981). *Biochem. J.* **196,** 619–624.
McCormack, J. G., and Denton, R. M. (1984). *Biochem. J.* **218,** 235–247.
McCormack, J. G., and England, P. J. (1983). *Biochem. J.* **214,** 581–585.
McCormack, J. G., Browne, H. M., and Dawes, N. J. (1989). *Biochim. Biophys. Acta* **973,** 420–427.
McCormack, J. G., Halestrap, A. P., and Denton, R. M. (1990a). *Physiol. Rev.* **70,** 391–425.
McCormack, J. G., Longo, E. A., and Corkey, B. E. (1990b). *Biochem. J.* **267,** 527–530.
Miyata, H., Silverman, H. S., Sollott, S. J., Lakatta, E. G., Stern, M. D., and Hansford, R. G. (1991). Am. J. Physiol. **261** *(Heart Circ. Physiol. 30)*, H1123–H1134.
Miyata, H., Lakatta, E. G., Stern, M. D., and Silverman, H. S. (1992). *Circ. Res.* **71,** 605–613.
Moravec, C. S., and Bond, M. (1991). *Am. J. Physiol.* **260,** H989–H997.
Moravec, C. S., and Bond, M. (1992). *J. Biol. Chem.* **267,** 5310–5316.
Moravec, C. S., Desnoyer, R. W., Milovanovic, M., and Bond, M. (1994). *Biophys. J.* **66,** A92.
Moreno-Sánchez, R., and Hansford, R. G. (1988). *Biochem. J.* **256,** 403–412.
Nazareth, W., Yafei, N., and Crompton, M. (1991). *J. Mol. Cell. Cardiol.* **23,** 1351–1354.
Nicholls, D. G. (1978). *Biochem. J.* **176,** 463–474.
Proschek, L., and Jasmin, G. (1982). *Muscle Nerve* **5,** 26–32.
Raiteri, M., and Levi, G. (1978). *Rev. Neurosci.* **3,** 77–111.
Reed, K. C., and Bygrave, F. L. (1974). *Biochem. J.* **140,** 143–155.
Reed, L. J., Damuni, Z., and Merryfield, M. L. (1985). *Curr. Top. Cell. Regul.* **27,** 41–49.
Reers, M., Kelly, R. A., and Smith, T. W. (1989). *Biochem. J.* **257,** 131–142.
Rink, T. J., and Hallam, T. J. (1989). *Cell. Calcium* **10,** 385–395.
Rizzuto, R., Simpson, A. W. M., Brini, M., and Pozzan, T. (1992). *Nature (London)* **358,** 325–327.
Rizzuto, R., Brini, M., Murgia, M., and Pozzan, T. (1993). *Science* **262,** 744–747.
Robertson, S. P., Potter, J. P., and Rouslin, W. (1982). *J. Biol. Chem.* **257,** 1743–1748.
Rodrigues, B., and McNeill, J. H. (1992). *Cardiovasc. Res.* **26,** 913–922.
Rossi, C. S., Vasington, F. D., and Carafoli, E. (1973). *Biochem. Biophys. Res. Commun.* **50,** 846–852.
Rutter, G. A., Theler, J.-M., Murgia, M., Wollheim, C. B., Pozzan, T., and Rizzuto, R. (1993). *J. Biol. Chem.* **268,** 22385–22390.
Schaffer, W. T., and Olson, M. S. (1980). *Biochem. J.* **192,** 741–751.
Sollott, S. J., and Lakatta, E. G. (1994). *Am. J. Physiol.*, in press.
Sollott, S. J., Ziman, B. D., and Lakatta, E. G. (1992). *Am. J. Physiol.* **262,** H1941–H1949.
Somlyo, A. P., Bond, M., and Somlyo, A. V. (1985). *Nature (London)* **314,** 622–625.
Stern, M. D., Silverman, H. S., Houser, S. R., Josephson, R. A., Capogrossi, M. C., Nichols, C. G., Lederer, W. J., and Lakatta, E. G. (1988). *Proc. Natl. Acad. Sci. USA* **85,** 6954–6958.
Stone, D., Darley-Usmar, V., Smith, D. R., and O'Leary, V. (1989). *J. Mol. Cell. Cardiol,*. **21,** 963–973.
Szabo, I., and Zoratti, M. (1991). *J. Biol. Chem.* **266,** 3376–3379.
Thayer, S. A., and Miller, R. J. (1990). *J. Physiol.* **425,** 85–115.
Unitt, J. F., McCormack, J. G., Reid, D., Maclachlan, L. K., and England, P. J. (1989). *Biochem. J.* **262,** 293–301.
Wan, B., La Noue, K. F., Cheung, J. Y., and Scaduto, R. C., Jr. (1989). *J. Biol. Chem.* **264,** 13430–13439.
Wendt-Gallitelli, M.-F., and Isenberg, G. (1991). *J. Physiol. (London)* **435,** 349–372.
Wieland, O. H. (1983). *Rev. Physiol. Biochem. Pharmacol.* **96,** 123–170.
Wikman-Coffelt, J., Sievers, R., Parmley, W. W., and Jasmin, G. (1986). *Cardiovas. Res.* **20,** 471–481.
Wikman-Coffelt, J., Stefenelli, T., Wu, S. T., Parmley, W. W., and Jasmin, G. (1991). *Circ. Res.* **68,** 45–51.
Wolska, B. M., and Lewartowski, B. (1991). *J. Mol. Cell Cardiol.* **23,** 217–226.
Woods, N. M., Cuthbertson, K. S. R., and Cobbold, P. H. (1986). *Nature (London)* **319,** 600–602.
Wrogemann, K., and Nylen, E. G. (1978). *J. Mol. Cell. Cardiol.* **10,** 185–195.