The effects of Mg^{2+} and adenine nucleotides on the sensitivity of the heart mitochondrial Na^+-Ca^{2+} carrier to extramitochondrial Ca^{2+}

A study using arsenazo III-loaded mitochondria

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The technique of reversible Ca^{2+} -induced permeabilization [Al Nasser & Crompton (1986) *Biochem. J.* **239**, 19–29, 31–40] has been applied to the preparation of heart mitochondria loaded with the Ca^{2+} indicator arsenazo III (2 nmol of arsenazo III/mg of mitochondrial protein). The loaded mitochondria ('mitosomes') were used to study the control of the Na^+-Ca^{2+} carrier by extramitochondrial Ca^{2+} mediated by putative regulatory sites. The V_{max} of the Na^+-Ca^{2+} carrier and the degree of regulatory-site-mediated inhibition were similar to normal heart mitochondria. Ca^{2+} occupation of the sites in mitosomes yields partial inhibition, which is half-maximal with 0.8 μM external free Ca^{2+} . The inhibition consists of a small decrease in V_{max} and a relatively large increase in apparent K_m for internal Ca^{2+} . Mg^{2+} also appears to interact with the sites, but this is largely abolished by ATP and ADP (but not AMP) under conditions in which the free $[Mg^{2+}]$ is maintained constant. The results indicate that the regulatory sites are effective in controlling the Na^+-Ca^{2+} carrier at physiological concentrations of adenine nucleotides, Mg^{2+} , intra- and extra-mitochondrial free Ca^{2+} .

INTRODUCTION

Heart mitochondrial Ca^{2+} is established kinetically by (quasi) steady-state cycling across the inner membrane, in which influx is mediated by the Ca^{2+} uniporter and efflux by the Na^+-Ca^{2+} carrier (Crompton *et al.*, 1976b; reviewed by Crompton, 1985). The kinetic properties of these two transport systems are responsible, together with the extramitochondrial $[Ca^{2+}]$, for setting intramitochondrial free $[Ca^{2+}]$ at values that are thought to control the activities of several Ca^{2+} -sensitive dehydrogenases of the matrix [around 1 μM : for reviews see Denton & McCormack (1980) and McCormack *et al.* (1986)]. In essence, the basis of this function of the cycle is the quasi-steady-state relation established between the free $[Ca^{2+}]$ in the mitochondrial matrix and the cytosol, and, although it is clear that increased cytosolic Ca^{2+} would produce increased mitochondrial Ca^{2+} , there are indications that the relation is complex (Crompton, 1985; Crompton *et al.*, 1986), since extramitochondrial Ca^{2+} inhibits the Na^+-Ca^{2+} carrier, in addition to being a substrate of the uniporter (Hayat & Crompton, 1982, 1985). The characteristics of Na^+-Ca^{2+} -carrier inhibition differ in several respects from those associated with the action of Ca^{2+} on externally presented sites involved in Ca^{2+} translocation (product-binding sites), and the presence of distinct Ca^{2+} -regulatory (inhibitory) sites was proposed (Hayat & Crompton, 1982).

In order to assess the potential importance of the regulatory sites, measurements need to be made at physiological values of intramitochondrial and extramitochondrial free $[Ca^{2+}]$, and there are obvious difficulties in doing this by conventional means (see the

Results and discussion section of this paper). However, a new technique has been developed whereby liver mitochondria may be loaded with Ca^{2+} -indicators via reversible Ca^{2+} -induced permeabilization (Al Nasser & Crompton, 1986a,b). Such 'permeabilized' and 'resealed' mitochondria, or 'mitosomes', exhibit near-normal capacities for respiration, $\Delta\psi$ generation and Ca^{2+} transport. As shown in the present paper, mitosomes prepared from heart mitochondria permit continuous rate measurements under the physiological constraints noted above. A related issue concerns the action of Mg^{2+} . Mg^{2+} inhibits the Na^+-Ca^{2+} carrier (Hansford & Castro, 1981; Crompton, 1985), and it has been suggested that Mg^{2+} interacts with the external regulatory sites (Lukacs & Fonyo, 1986). At face value, this implies that the sites may be largely occupied by Mg^{2+} *in vivo* and thereby redundant, unless their intrinsic Mg^{2+} -sensitivity is relieved by other factors. In the present paper, a procedure is described for the preparation of arsenazo III-loaded heart mitosomes, which are used to address the questions raised above.

METHODS

Preparation of arsenazo III-loaded mitosomes from rat heart mitochondria

Rat heart mitochondria were prepared as described by Hayat & Crompton (1982) with 0.05% (w/v) bovine serum albumin (fatty acid-free) in the initial homogenization medium. The protein content of mitochondria and mitosomes (below) was measured by a modified biuret procedure (Al Nasser & Crompton, 1986b).

Abbreviations used: $\Delta\psi$, mitochondrial inner-membrane potential; ΔpH , pH gradient.

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Arsenazo III-loaded mitochondria were prepared from the heart mitochondria by the procedure described previously for liver mitochondria (Al Nasser & Crompton, 1986b), except that (a) higher concentrations of Ca^{2+} and P_i were used (3- and 4-fold respectively), since mitochondria of heart are more resistant to permeabilization than those of liver (Palmer & Pfeiffer, 1981; Crompton, 1985) and (b) the resealing period from arsenazo III addition to the first wash was increased 5-fold. Heart mitochondria (10 mg of protein/ml) were first permeabilized by incubation for 10 min at 25 °C in medium (pH 7.0) comprising 120 mM-KCl, 10 mM-Hepes/Tris, 10 mM- KH_2PO_4 , 1 mM- CaCl_2 , 10 μg of rotenone/ml and 8 mM-succinate. The mitochondria were resealed by addition of 7 mM-arsenazo III, which decreased free $[\text{Ca}^{2+}]$ to $< 1 \mu\text{M}$. As discussed previously, indicator entry into permeabilized mitochondria is sufficiently rapid with respect to the rate of resealing to allow entrapment of the indicator in the matrix (Al Nasser & Crompton, 1986b). The mitochondria were sedimented 10 min later and washed three times by resuspension in 50 vol. of 210 mM-mannitol/70 mM-sucrose/10 mM-Hepes (pH 7.0), followed by sedimentation. The final mitochondrial pellet was deep purple, characteristic of the Ca^{2+} /arsenazo complex, with little or no colour visible in the supernatant. Intramitochondrial arsenazo III was determined as described by Al Nasser & Crompton (1986b). In eight such preparations, intramitochondrial arsenazo III amounted to 2.2 ± 0.2 nmol/mg of protein (mean $\pm$ S.E.M.).

Na^+ - Ca^{2+} carrier assay in arsenazo III-loaded mitochondria

Na^+ - Ca^{2+} carrier activity was determined from the rate of change in intramitochondrial arsenazo III absorbance at 675 minus 685 nm with a Perkin-Elmer model 356 dual-wavelength spectrophotometer. Arsenazo III-loaded mitochondria (3 mg of protein) were preincubated at 25 °C in 1.5 ml of standard reaction medium (pH 7.0) containing 120 mM-KCl, 10 mM-Hepes, 0.2 mM- KH_2PO_4 , 10 μM -oligomycin, 6 nmol of Ruthenium Red, 5 mM-succinate and 100 μM -EGTA. Reactions were started by Ca^{2+} buffer with or without 10 mM- NaCl as shown in Fig. 1, and Na^+ - Ca^{2+} carrier activity was obtained by difference ($\pm \text{Na}^+$). EGTA (4 mM) was used as buffer from 0 to 2.4 μM free Ca^{2+} and HEDTA (*N*-hydroxyethylethylenediaminetriacetic acid) for higher free Ca^{2+} concentrations. The free $[\text{Ca}^{2+}]$ established with each buffer was determined with a Ca^{2+} electrode and a Nernstian calibration curve extrapolated to the free $[\text{Ca}^{2+}]$ range of interest, as stated previously (Al Nasser & Crompton, 1986a). Assays with Mg^{2+} in the absence of adenine nucleotides were conducted by adding Ca^{2+} /EGTA (4 mM) buffers containing 2.6–3.0 mM- Mg^{2+} . In assays with adenine nucleotides the Mg^{2+} added was increased (1 mol of Mg^{2+} /mol of ATP; 0.58 mol of Mg^{2+} /mol of ADP; none with AMP) to take account of Mg^{2+} binding by the nucleotides. In all cases, the final free $[\text{Mg}^{2+}]$ was determined to be 2.5 mM by measurement with arsenazo III at the wavelength pair 604 minus 650 nm, which discriminates between the absorbances of the Mg^{2+} /arsenazo III and Ca^{2+} /arsenazo III complexes. Nucleotides were added simultaneously with the Ca^{2+} buffers. The extra-mitochondrial $[\text{ATP}]$ in assays with ADP was determined

in neutralized HClO_4 -treated samples with hexokinase and glucose-6-phosphate dehydrogenase.

Calibrations of intramitochondrial arsenazo III absorbance versus intramitochondrial free Ca^{2+} ($[\text{Ca}^{2+}]_{\text{free}}$) were made with mitochondria in standard reaction medium (above) plus Ca^{2+} /HEDTA buffer, and 0.5 nmol each of Ca^{2+} ionophore A23187 and nigericin to set the values of $[\text{Ca}^{2+}]_{\text{free}}$ and internal pH equal to those of the medium. Control experiments showed that under these and under normal assay conditions there was negligible ΔpH (< 0.1 unit; measured as described by Goldstone *et al.*, 1983). Values of $[\text{Ca}^{2+}]_{\text{total}}$ were obtained using the relation between free $[\text{Ca}^{2+}]$ and total $[\text{Ca}^{2+}]$; this relation was obtained with a Ca^{2+} electrode in incubations containing arsenazo III at a concentration equal to intramitochondrial [arsenazo III]. Absolute rates of Ca^{2+} efflux were then calculated from $[\text{Ca}^{2+}]_{\text{total}}$ versus time.

RESULTS AND DISCUSSION

Utility of mitochondria for Na^+ - Ca^{2+} carrier assay

There are limitations in conventional Na^+ - Ca^{2+} carrier assays, in which changes in external free $[\text{Ca}^{2+}]$ are measured with external electrodes or indicators, when applied to physiological conditions of internal and external free $[\text{Ca}^{2+}]$. Firstly, inhibition of the carrier by increase in external $[\text{Ca}^{2+}]$ makes accurate rate measurements difficult by conventional means when external free $[\text{Ca}^{2+}]$ increases with time. Secondly, conventional assays give no indication of internal free $[\text{Ca}^{2+}]$. Arsenazo III-loaded mitochondria offer a means of dealing with these two problems: thus rates may be determined from changes in internal free $[\text{Ca}^{2+}]$ with external free $[\text{Ca}^{2+}]$ clamped at constant predetermined values with Ca^{2+} buffers. Also, internal free $[\text{Ca}^{2+}]$ may be obtained from internal indicator absorbance.

It was unnecessary to preload the mitochondria with Ca^{2+} for efflux measurements, since they contained Ca^{2+} when prepared [4.2 ± 0.3 nmol/mg protein (mean $\pm$ S.E.M.) in eight preparations]. Fig. 1 reports typical traces of mitochondrial Ca^{2+} efflux as monitored by the changes in internal arsenazo III absorbance. The rate of Ca^{2+} efflux was stimulated about 10-fold by 10 mM- Na^+ in both the presence (traces b and d) and absence (traces a and c) of external free Ca^{2+} . Thus the corrections for Na^+ -independent Ca^{2+} efflux that needed to be applied in calculating Na^+ - Ca^{2+} carrier activity (see the Methods section) were small, and similar to those with normal mitochondria (Hayat & Crompton, 1982).

The initial Na^+ - Ca^{2+} carrier activity from Fig. 1 was markedly inhibited by 2 μM external free Ca^{2+} , i.e. from 0.0156 to 0.0066 A/min. In six such experiments (Table 1) the mean inhibition by 2 μM external free Ca^{2+} was 58%. This value is the same as that determined conventionally with normal heart mitochondria.

The relation between the inhibition by 2 μM external free Ca^{2+} and that with higher external free $[\text{Ca}^{2+}]$ is shown in Fig. 2. The curve contains essentially two domains. The domain of strong inhibition ($< 2 \mu\text{M}$ free Ca^{2+}) may be attributed to the interaction of Ca^{2+} with regulatory sites on the carrier. The weak inhibition ($> 2 \mu\text{M}$ free Ca^{2+}) presumably reflects Ca^{2+} interaction with translocation sites and carrier reversal, since the rate of net Ca^{2+} efflux decreased to zero when the internal and external concentrations of free Ca^{2+} were about equal (40 μM). Other similar experiments confirmed the lack of

Fig. 1. Ca^{2+} efflux from mitochondria in the presence and absence of Na^+

Arsenazo III-loaded mitochondria were preincubated as described in the Methods section without Mg^{2+} and adenine nucleotides. The following additions were made at the arrow: (a) 10 mM- Na^+ plus EGTA; (b) 10 mM- Na^+ plus Ca^{2+} /EGTA (2 μM free Ca^{2+}); (c) EGTA; (d) Ca^{2+} /EGTA (2 μM free Ca^{2+}).

net Ca^{2+} flux when the free $[\text{Ca}^{2+}]$ gradient was zero over the range 5–40 μM free Ca^{2+} . As stated in the Methods section, there was no detectable pH gradient (ΔpH) across the inner membrane in these experiments. Since the Na^+ gradient is established by ΔpH (Crompton & Heid, 1978), a zero Na^+ gradient could be predicted. These conditions for zero net Ca^{2+} flux are therefore consistent with an electroneutral mechanism of $\text{Na}^+/\text{Ca}^{2+}$ exchange proposed by Brand (1985).

Although efflux in the absence of Na^+ was also inhibited by external Ca^{2+} (Fig. 1, traces c and d), this cannot be taken as evidence that a Na^+ -independent Ca^{2+} transport system is sensitive to external Ca^{2+} , since some efflux may have occurred via re-permeabilization. In liver mitochondria, Na^+ -independent efflux comprises both carrier-mediated and re-permeabilization-dependent mechanisms (Al Nasser & Crompton, 1986b).

Influence of Mg^{2+} and adenine nucleotides on $\text{Na}^+/\text{Ca}^{2+}$ carrier activity

Table 1 reports the effect of 2.5 mM external free Mg^{2+} on $\text{Na}^+/\text{Ca}^{2+}$ carrier activity with zero external free Ca^{2+} and with 2 μM external free Ca^{2+} , which is sufficient to saturate the Ca^{2+} -regulatory sites (see below). This concentration of free Mg^{2+} is probably reasonably close to the cytosolic free $[\text{Mg}^{2+}]$, although this is not known with certainty; Hess *et al.* (1982) obtained a value of 3 mM cytosolic free Mg^{2+} in ventricular cells with

Table 1. Effects of Mg^{2+} and adenine nucleotides on $\text{Na}^+/\text{Ca}^{2+}$ carrier activity in mitochondria

Assays were performed as in Fig. 1 in the presence or absence of 2 μM external free Ca^{2+} , 2.5 mM free Mg^{2+} and 3 mM adenine nucleotides as indicated. All rates are given as a percentage of the rates in the absence of external Ca^{2+} , Mg^{2+} and nucleotides with the same mitochondria preparations (means $\pm$ S.E.M., six mitochondrial preparations).

Additions	External free $[\text{Ca}^{2+}]$ (μM)	Rate of Na^+ -induced Ca^{2+} efflux (%)	Inhibition of Ca^{2+} efflux by external Ca^{2+} (%)
None	0	100	—
	2	42 $\pm$ 10	58
Mg^{2+}	0	53 $\pm$ 5	—
	2	42 $\pm$ 7	20
Mg^{2+} , ATP	0	88 $\pm$ 5	—
	2	42 $\pm$ 8	52
Mg^{2+} , ADP	0	64 $\pm$ 9	—
	2	32 $\pm$ 10	50
Mg^{2+} , AMP	0	53 $\pm$ 4	—
	2	45 $\pm$ 4	16

micro-electrodes. ^{31}P -n.m.r. measurements of frog skeletal muscle yielded values of 0.6–4 mM (Gupta & Moore, 1980; Cohen & Burt, 1977). The influence of external free $[\text{Mg}^{2+}]$ on the direction of net Mg^{2+} flux in rat heart mitochondria is consistent with 2 mM free Mg^{2+} in the cytosol if it is assumed that little Mg^{2+} is lost during mitochondrial preparation (Crompton *et al.*, 1976a).

It is evident that, whereas Mg^{2+} inhibited the $\text{Na}^+/\text{Ca}^{2+}$ carrier by about 50% in the absence of external Ca^{2+} , it caused no inhibition in the presence of

Fig. 2. Effect of external free $[\text{Ca}^{2+}]$ on mitochondrial $\text{Na}^+/\text{Ca}^{2+}$ carrier activity

Assays were performed as described in the Methods section in the absence of Mg^{2+} and adenine nucleotides. External free $[\text{Ca}^{2+}]$ was set at the values indicated with Ca^{2+} buffers. Intramitochondrial free $[\text{Ca}^{2+}]$ was 38–41 μM .

2 μM external Ca^{2+} . This behaviour suggests that Mg^{2+} may substitute for Ca^{2+} in interacting with the regulatory sites, such that 2.5 mM- Mg^{2+} confers near-maximal inhibition. This would explain the lack of further inhibition when the sites are fully occupied by Ca^{2+} . This agrees also with the conclusion of Lukacs & Fonyo (1986), who observed non-competitive inhibition by Mg^{2+} with respect to Na^+ ; since previous studies (Hayat & Crompton, 1982) revealed that Ca^{2+} occupation of the regulatory sites is non-competitive with Na^+ , whereas Ca^{2+} occupation of externally presented sites involved in Ca^{2+} translocation inhibits competitively with Na^+ , Mg^{2+} interaction with the regulatory sites was indicated.

As shown in Table 1, however, this effect of Mg^{2+} was very largely abolished in the presence of ATP with external free $[\text{Mg}^{2+}]$ maintained constant. The restoration of Ca^{2+} -sensitivity by ATP was wholly due to stimulation of the rate of Ca^{2+} efflux in the absence of external Ca^{2+} . The $[\text{ATP}]$ -dependence of this stimulation is reported in Fig. 3; maximal stimulation was attained with about 1.5 mM-ATP.

Ca^{2+} -sensitivity in the presence of Mg^{2+} was also restored by ADP (Table 1). Although ADP inhibited slightly, the percentage inhibition by 2 μM external free Ca^{2+} in the presence of Mg^{2+} /ADP (50%) was similar to that with Mg^{2+} /ATP (52%) and in the absence of Mg^{2+} (58%). In these experiments with ADP, the $[\text{ATP}]$ measured in the incubation medium after sedimentation of the mitochondria was < 0.1 mM, which is insufficient to restore Ca^{2+} -sensitivity detectably (Fig. 3). Unlike the other nucleotides, AMP did not restore Ca^{2+} -sensitivity (Table 1).

The effectiveness of ATP is reported more fully in Fig. 4. With ATP present, the form of the inhibition by external Ca^{2+} was clearly partial, yielding a maximal inhibition of about 60% under these experimental conditions and half-maximal inhibition at 0.8 μM external free Ca^{2+} . The effectiveness of both ADP and ATP appears to rule out phosphorylation as the cause of the restoration of Ca^{2+} -sensitivity. Nevertheless, the presence of nucleotide-binding sites is clearly indicated. Con-

Fig. 3. Activation of the Na^+ - Ca^{2+} carrier by ATP in the presence of Mg^{2+} .

Assays were conducted with mitochondria in the presence of EGTA and 2.5 mM free Mg^{2+} (see the Methods section). Mg^{2+} /ATP (1 mol/mol) was added as indicated.

Fig. 4. Influence of ATP on the external free- $[\text{Ca}^{2+}]$ -dependence of Na^+ - Ca^{2+} carrier activity in the presence of Mg^{2+} .

The Na^+ - Ca^{2+} carrier of mitochondria was assayed as described in the Methods section with 2.5 mM external free Mg^{2+} and Ca^{2+} buffers yielding the free $[\text{Ca}^{2+}]$ stated in the presence (●) and absence (○) of 3 mM-ATP.

ceivably, nucleotide binding induces a change to a conformation with lower Mg^{2+} affinity. Moreover, the data provide firm indications that *in vivo*, in the presence of Mg^{2+} and adenine nucleotides, control by changes in cytosolic free $[\text{Ca}^{2+}]$ in the physiological range would be essentially fully expressed.

Influence of external free $[\text{Ca}^{2+}]$ on kinetic parameters of the Na^+ - Ca^{2+} carrier

Absolute values of mitochondrial Na^+ - Ca^{2+} carrier activity with varied internal and external free $[\text{Ca}^{2+}]$ are reported in Fig. 5. Since, as discussed in connection with Fig. 2, significant carrier reversal may occur when the gradient of free $[\text{Ca}^{2+}]$ across the inner membrane approaches zero, the ratio internal free $[\text{Ca}^{2+}]$ /external free $[\text{Ca}^{2+}]$ was always maintained high, and never less than 19.

The data yield a V_{max} value of 26 nmol of Ca^{2+} /min per mg of protein in the absence of external free Ca^{2+} . A similar value (27 nmol of Ca^{2+} /min per mg) was obtained isotopically with normal heart mitochondria in the presence of external EGTA (Hayat & Crompton, 1982), which indicates the absence of damage to the Na^+ - Ca^{2+} carrier during mitochondria preparations. The V_{max} value was decreased by external Ca^{2+} (Fig. 5b), i.e. by 9% (at 0.25 μM - Ca^{2+}) and by 27% (at 0.8 μM - Ca^{2+}). A more pronounced change was observed in apparent K_m (internal Ca^{2+}), which was increased from 5.0 μM (at zero external Ca^{2+}) to 9.4 μM (at 0.25 μM external Ca^{2+}) and 19.0 μM (at 0.8 μM external Ca^{2+}).

Conclusions

Although arsenazo III-loaded mitochondria offer clear potential advantages in Ca^{2+} -flux assays, their utility will depend ultimately on the degree to which native carrier properties are preserved during mitochondria preparation.

Fig. 5. Dependence of Na^+ - Ca^{2+} carrier activity on intramitochondrial free $[\text{Ca}^{2+}]$ with varied external free $[\text{Ca}^{2+}]$

Assays were carried out as in Fig. 1 with Ca^{2+} /EGTA buffers yielding the following external free $[\text{Ca}^{2+}]$: ●, zero; △, 0.25 μM ; ○, 0.8 μM . Intramitochondrial free $[\text{Ca}^{2+}]$ and the absolute rates of Ca^{2+} efflux (v) were calculated as described in the Methods section.

In fact, the properties of the Na^+ - Ca^{2+} carrier in mitosomes and normal mitochondria are essentially the same in two basic respects, namely V_{max} and the maximal inhibition conferred by the regulatory sites. This corroborates previous studies of liver mitosomes in which several basic bioenergetic features were unchanged (see the Introduction). Thus mitosome preparation does not appear to impair the properties of inner-membrane constituents. Indeed, previous work revealed the lack of protein loss during mitosome preparation (Al Nasser & Crompton, 1986a), and if, as proposed, the process merely involves the opening and closure of a Ca^{2+} -activated inner-membrane pore, then absence of structural damage would be expected. Mitosomes may therefore provide a means of investigating mitochondrial transport systems that preserves their structural integrity within a native membrane environment and that is extremely flexible in allowing manipulation of the low- M_r solute composition on both sides of the inner membrane.

The use of mitosomes in this study has revealed novel features of the interaction of external Ca^{2+} with the regulatory sites of the Na^+ - Ca^{2+} carrier. Thus, although Mg^{2+} interacts with these sites, this interaction is minimized by adenine nucleotides. The interaction of Ca^{2+} with these sites gives rise to mixed inhibition of the Na^+ - Ca^{2+} carrier, the predominant effect being a decreased affinity of the system for the binding of internal Ca^{2+} as substrate. Moreover, the values of apparent K_m for internal Ca^{2+} ($> 5 \mu\text{M}$) indicate that the carrier would be far from saturated by concentrations of internal free Ca^{2+} that regulate the Ca^{2+} -sensitive dehydrogenases of the matrix, i.e. 0.1–5 μM (McCormack *et al.*, 1986). Thus, if the transport cycle does set matrix free $[\text{Ca}^{2+}]$ in heart within this range, and various lines of evidence indicate that it does (Crompton *et al.*, 1983; McCormack & England, 1984; Crompton, 1985), then regulatory-site-mediated control of the Na^+ - Ca^{2+} carrier is competent at least to induce changes in matrix free $[\text{Ca}^{2+}]$ within the operative range of dehydrogenase control.

It is evident from computer simulations that the Ca^{2+} -transport cycle in heart mitochondria relays the large-scale (i.e. > 20 -fold) beat-to-beat changes in

cytosolic free $[\text{Ca}^{2+}]$ into a largely damped-out ripple of intramitochondrial free $[\text{Ca}^{2+}]$, which changes by about 2% only from beat to beat (Crompton, 1985; Crompton *et al.*, 1986). An important question concerns the relation between the quasi-steady-state matrix free $[\text{Ca}^{2+}]$ and peak cytosolic free $[\text{Ca}^{2+}]$, since this relation is the basis by which the cycle is thought to co-ordinate mitochondrial Ca^{2+} -dependent dehydrogenase activity with contractility. It is generally appreciated that, if the mean matrix free $[\text{Ca}^{2+}]$ is sufficiently high such that the Na^+ - Ca^{2+} carrier approaches saturation, then an increase in peak cytosolic free $[\text{Ca}^{2+}]$ yields a disproportionately large increase in matrix free $[\text{Ca}^{2+}]$. This property of the cycle whereby changes in cytosolic free $[\text{Ca}^{2+}]$ produce relatively large increases in matrix free $[\text{Ca}^{2+}]$ has been termed 'relay amplification' (Crompton, 1985). It is clear, however, from present considerations, that the matrix free $[\text{Ca}^{2+}]$ required to approach Na^+ - Ca^{2+} carrier saturation would greatly exceed the upper limit of the $[\text{Ca}^{2+}]$ range of dehydrogenase control; in other words, that relay amplification arising from Na^+ - Ca^{2+} carrier saturation can have little relevance to dehydrogenase control.

The essential importance of the present data is that they introduce amplification into relay behaviour at matrix $[\text{Ca}^{2+}]$ values that are insufficient to saturate the Na^+ - Ca^{2+} carrier, but that are within the effective range for dehydrogenase control. Thus, with $< 5 \mu\text{M}$ matrix free Ca^{2+} , an increase in the size, frequency or duration of the cytosolic Ca^{2+} transients would not only increase the mean rate of Ca^{2+} influx (uniporter) but also decrease the mean rate of Ca^{2+} efflux (Na^+ - Ca^{2+} carrier), producing a relatively large increase in mitochondrial Ca^{2+} in the new quasi-steady state. This relay amplification, arising from a dual action of cytosolic Ca^{2+} , may permit a sensitive response of oxidative metabolism to changes in contractility.

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