The α -adrenergic-mediated activation of the cardiac mitochondrial Ca^{2+} uniporter and its role in the control of intramitochondrial Ca^{2+} *in vivo*

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1. Administration of methoxamine ($10\mu\text{M}$, 2 min) to perfused rat hearts increased the rate at which subsequently isolated mitochondria accumulated Ca^{2+} . Methoxamine did not change significantly the development of $\Delta\psi$ with time or the basal rates of Ca^{2+} flux on inhibition of the uniporter with Ruthenium Red. 2. With $200\mu\text{M-P}_i$, the rates of Ca^{2+} uptake at constant $\Delta\psi$ were unaffected by the small variations in endogenous $[\text{P}_i]$ between mitochondrial preparations, and were also unaffected by changes in internal Ca^{2+} over the approximate range 8–43 nmol of Ca^{2+} /mg. At low internal Ca^{2+} (about 8 nmol/mg of protein) the rates of Ca^{2+} uptake at constant $\Delta\psi$ were unaffected by addition of $200\mu\text{M-P}_i$. Under these conditions, the uniporter activity and the uniporter conductance were increased by 38–40% by methoxamine pretreatment. 3. The endogenous Ca^{2+} content of mitochondria from control heart was about 1.8 nmol of Ca^{2+} /mg of protein. Perfusion with agonist increased the Ca^{2+} content as follows: $10\mu\text{M}$ -methoxamine (2 min), 48%; $1\mu\text{M}$ -isoprenaline (2 min), 100%; $1\mu\text{M}$ -adrenaline (2 min), 140%. 4. The implications of the data for the adrenergic control of oxidative metabolism by intramitochondrial Ca^{2+} is discussed.

It is now generally accepted that the distribution of Ca^{2+} across the inner membrane of heart mitochondria is established by steady-state recycling via independent carriers for influx, i.e. the Ca^{2+} uniporter, and efflux, i.e. the Na^+ – Ca^{2+} carrier (Crompton *et al.*, 1976b, 1977, 1978b; Crompton & Heid, 1978; Denton *et al.*, 1980; Hansford, 1981; reviewed by Nicholls & Akerman, 1982). The possible importance of this cycle in controlling intramitochondrial Ca^{2+} has emerged in the light of evidence that Ca^{2+} activates several matrix dehydrogenases (reviewed by Denton & McCormack, 1980). Hiraoka *et al.* (1980) and McCormack & Denton (1981) proposed that increased cytoplasmic free Ca^{2+} in heart, occurring, for example, during β -adrenergic stimulation (Tsien, 1977; Allen & Kurihara, 1980; Fabiato, 1981), may be relayed by the cycle into an increase in matrix free Ca^{2+} with consequent activation of matrix dehydrogenases.

Abbreviations used: $\Delta\psi$, inner membrane potential; $\Delta[\text{Ca}^{2+}]$, concentration gradient of free Ca^{2+} across the inner membrane; $\Delta\tilde{\mu}_{\text{Ca}^{2+}}$, electrochemical gradient of Ca^{2+} across the inner membrane $\{=RT \ln([\text{Ca}^{2+}]_{\text{out}}/[\text{Ca}^{2+}]_{\text{in}}) + 2F\Delta\psi\}$; CCCP, carbonyl cyanide *m*-chlorophenylhydrazine; TPP⁺, tetraphenylphosphonium ion; HEDTA, *N*-(2-hydroxyethyl)ethylenediamine-*NN'*-triacetic acid.

The capacity of the cycle in heart to respond to changes in cytoplasmic Ca^{2+} may be limited by its slow kinetic behaviour relative to the rapidity of the beat-to-beat changes in cytoplasmic Ca^{2+} , and it is likely that the cycle responds to the time-average free Ca^{2+} in this case (Crompton, 1980; McCormack & Denton, 1981; Robertson *et al.*, 1982). The basic relay function of the cycle may be facilitated by regulation (inhibition) of the Na^+ – Ca^{2+} carrier by cytoplasmic Ca^{2+} (Hayat & Crompton, 1982).

In principle, a new steady-state distribution may also be brought about by modification of the kinetic properties of the component carriers of the cycle (Nicholls & Crompton, 1980). Three groups of investigators have observed that administration of adrenaline or the α -adrenergic agonists, phenylephrine and methoxamine, to heart and liver stimulates the rate of respiration-dependent accumulation of Ca^{2+} by mitochondria subsequently isolated (Friedman *et al.*, 1980; Taylor *et al.*, 1980; Kessar & Crompton, 1981). In contrast with the other studies, the stimulation in heart mitochondria by methoxamine was not accompanied by changes in the rate of respiration or $\Delta\psi$, and a possible α -adrenergic-mediated activation of the uniporter was indicated (Kessar & Crompton, 1981). If this interpretation is correct, the activation would be

evident at constant driving force for Ca^{2+} influx ($\Delta\tilde{\mu}_{\text{Ca}^{2+}}$) and would yield an increase in uniporter conductance. Moreover, such comparisons should allow the degree of uniporter activation to be evaluated precisely. In the present study, these predictions were studied under conditions designed to exclude possible complications caused by changes in internal Ca^{2+} and other critical factors. In addition, the basic question was investigated of whether matrix Ca^{2+} in heart is indeed changed by β -adrenergic and α -adrenergic mechanisms in a manner competent for regulation of oxidative metabolism.

Methods

Preparation of mitochondria from perfused hearts

Hearts from female albino Sprague–Dawley rats were removed and washed in ice-cold perfusion medium containing 120 mM-NaCl, 4.8 mM-KCl, 1.2 mM- KH_2PO_4 , 0.6 mM- MgSO_4 , 2.3 mM- CaCl_2 and 25.3 mM- NaHCO_3 . Each heart was perfused under hydrostatic pressure (85 cm of H_2O) by the Langendorff technique without recirculation with the above medium pre-gassed at 37°C (O_2/CO_2 , 19:1). After 15 min perfusion, 10 μM -methoxamine was introduced into the perfusion medium and, 2 min later, the heart was removed and homogenized immediately with a Polytron homogenizer (Kinematica G.m.b.H., Luzern, Switzerland) as described previously (Crompton *et al.*, 1976b), except that bovine serum albumin (fatty acid-free) was introduced into the homogenization medium [210 mM-mannitol, 70 mM-sucrose, 10 mM-Tris/HCl, pH 7.4, 0.1 mM-EDTA, and 0.05% (w/v) bovine serum albumin]. The inclusion of bovine serum albumin was advantageous, since it yielded mitochondria with extremely low basal rates of Ca^{2+} efflux in the presence of Ruthenium Red (defined below). The mitochondria were isolated from the homogenate exactly as described previously (Crompton *et al.*, 1976b). Each heart was perfused in parallel with a control heart, in which case methoxamine was omitted.

Measurement of mitochondrial protein and P_i

Mitochondrial protein was determined by a modified biuret procedure (Kröger & Klingenberg, 1966) with bovine plasma albumin as standard (Sigma Chemical Co., St. Louis, Mo, U.S.A.). In practice, it was found that the day-to-day reproducibility of the specific rates of Ca^{2+} uptake was improved by referring these to the cytochrome oxidase content, which was determined as described by Williams (1968). The cytochrome oxidase contents were converted into mg of mitochondrial protein by reference to a standard stock preparation of mitochondria used throughout this study. The

stock preparation contained 0.93 nmol of cytochromes ($a + a_3$)/mg of protein, which is close to the content in rat heart mitochondria reported by Williams (1968; 0.98 nmol/mg). P_i was determined as described by Stewart (1974).

Determination of intramitochondrial Ca^{2+} contents in vivo

Hearts were perfused and homogenized as described above, except that the agonists were 10 μM -methoxamine, 1 μM -isoprenaline or 1 μM -adrenaline (all for 2 min), and the [EDTA] of the homogenization medium was increased to 0.5 mM. The homogenate was centrifuged for 3 min at 2°C and 1130 g (r_{av} , 8.3 cm), and the supernatant containing the mitochondria was retained on ice. At intervals, 1 ml samples of the supernatant were centrifuged for 30 s in an Eppendorf bench centrifuge (model 5412). The mitochondrial pellets were suspended in 50 μl of water, and then extracted with 0.5 M-HCl containing 0.5% (w/v) LaCl_3 . The Ca^{2+} contents of the extracts were measured with a Perkin–Elmer atomic-absorption spectrophotometer (model 305). Mitochondrial protein was determined from the content of cytochrome oxidase (as above). Negligible amounts of unbroken cells were sedimented with the mitochondria, as judged by the fact that mitochondria isolated as described and suspended in 0.24 M-sucrose/10 mM-Tris/HCl (pH 7.3) contained less than 3% of the total lactate dehydrogenase activity of the homogenate. Other experiments indicated that microsomal Ca^{2+} was excluded from the mitochondrial fraction. Microsomal fractions were isolated and preloaded with $^{45}\text{Ca}^{2+}$ as described by Harigaya & Schwartz (1969), and introduced into the homogenate immediately before centrifugation. Less than 2% of the $^{45}\text{Ca}^{2+}$ was sedimented.

Measurement of Ca^{2+} fluxes and $\Delta\psi$

The techniques used are exemplified by the representative curves of Fig. 1. Ca^{2+} fluxes were measured at 25°C with the Ca^{2+} indicator arsenazo III and a Perkin–Elmer model 356 double-beam spectrophotometer operating at 675–685 nm (Scarpa *et al.*, 1978). The standard reaction medium (3 ml) contained 120 mM-KCl, 5 mM-4-(2-hydroxyethyl)-1-piperazine-ethanesulphonate (Tris salt, pH 7.0), 2 mg of mitochondrial protein, 2 μg of rotenone, 100 μM -arsenazo III, 50 μM -TPP $^+$, 200 μM - KH_2PO_4 (except where indicated) and 0–90 pmol of CCCP (as indicated). Although the electrode gave a Nernstian response with TPP $^+$ concentrations as low as 1 μM (Kamo *et al.*, 1979), a relatively high [TPP $^+$] was used, since this minimized electrode drift between experiments. The suspension was preincubated for 8–10 min, after which the efflux of endogenous Ca^{2+} was essentially complete. CaCl_2

Fig. 1. Uptake of Ca^{2+} and TPP^{+} by mitochondria from hearts perfused with and without methoxamine

The time courses of Ca^{2+} uptake (curves *a* and *b*) and TPP^{+} uptake (curves *c* and *d*) were measured in mitochondria from control (curves *a* and *c*) and methoxamine-treated (curves *b* and *d*) hearts as described in the Methods section with 0.67 mg of mitochondrial protein/ml and 200 μM - KH_2PO_4 in final volumes of 3 ml (Ca^{2+} measurements) and 1 ml (TPP^{+} measurements). The uptakes were started with 5 mM-succinate plus 20 μM -ATP as indicated. In separate experiments (curves *e* and *f*), 1 nmol of Ruthenium Red (RR)/mg of protein was added when the extramitochondrial free Ca^{2+} was approx. 1.5 μM (determined with Ca^{2+} /HEDTA buffers).

was then introduced to give an approximate total content of either 24 nmol or 85 nmol of Ca^{2+} as indicated in the Figures. Ca^{2+} uptake was started by addition of 5 mM-succinate plus 20 μM -ATP (K^{+} salts). When uptake was essentially complete, 5 mM-EGTA was added to give the point on the trace corresponding to zero free Ca^{2+} . The concentrations of free Ca^{2+} were determined by calibrating with Ca^{2+} /HEDTA buffers as described previously (Crompton *et al.*, 1976b). The calibration of Δ (absorbance) versus Δ [total Ca^{2+}] was performed when Ca^{2+} uptake was complete by adding Ruthenium Red (2 nmol) to prevent further uptake of Ca^{2+} on addition of standard amounts of CaCl_2 .

As shown in Fig. 1, the rates of Ca^{2+} uptake decreased as Ca^{2+} uptake progressed. The rates were determined at an average external free $[\text{Ca}^{2+}]$ of 1.5 μM (unless otherwise stated), when $\Delta\psi$ changed little with time. The rates were calculated from the times taken to decrease the total extramitochondrial Ca^{2+} from 1 nmol above to 1 nmol below the 1.5 μM point.

The uptake of Ca^{2+} monitored in this way is the resultant of uptake via the Ca^{2+} uniporter and slow net efflux (basal efflux) via a Ruthenium Red-independent route. True determinations of uniporter activity require that the basal rates of Ca^{2+} efflux be accounted for. This was done by blocking the uniporter with Ruthenium Red (1 nmol/mg of protein) and measuring the basal rates of efflux at the same external free $[\text{Ca}^{2+}]$ and internal Ca^{2+} contents used to calculate the rates of Ca^{2+} uptake (Fig. 1). The rates of Ca^{2+} uptake corrected for the basal rates of Ca^{2+} efflux are referred to as uniporter activities.

The inner-membrane potentials ($\Delta\psi$) were determined in parallel with the Ca^{2+} -influx measurements from the accumulation of TPP^{+} (Fig. 1). The uptake of TPP^{+} was followed with an electrode (Kamo *et al.*, 1979) as described previously (Kessar & Crompton, 1981). The electrode response was calibrated by adding standard amounts of TPP^{+} to the complete reaction medium containing mitochondria, but without succinate and ATP. This procedure allowed for any TPP^{+} binding to the mitochondria in the de-energized state. In order to check that the mitochondria were de-energized during calibration, 0.5 μM -CCCP was added; this caused no detectable release of TPP^{+} . The volumes of matrix water were determined in separate incubations as described previously (Crompton & Heid, 1978). Samples (1 ml) of the incubations were withdrawn, pipetted immediately into Eppendorf centrifuge tubes containing 50 μl of 200 mM-[U- ^{14}C]sucrose (0.05 μCi) and $^3\text{H}_2\text{O}$ (0.5 μCi), mixed, and centrifuged (for 1 min) about 10 s before the 1.5 μM external- Ca^{2+} point was reached.

Results

Effects of P_i on Ca^{2+} transport

In order to resolve the action of α -adrenergic agonists on the uniporter reaction, it was necessary to distinguish between effects brought about by changes in the driving force for Ca^{2+} uptake ($\Delta\tilde{\mu}_{\text{Ca}^{2+}}$) and an effect on the uniporter itself. A primary action on the uniporter would be evident as a changed uniporter activity at constant $\Delta\tilde{\mu}_{\text{Ca}^{2+}}$. Of the components of $\Delta\tilde{\mu}_{\text{Ca}^{2+}}$, $\Delta\psi$ may be estimated, but $\Delta[\text{Ca}^{2+}]$ cannot, since internal free $[\text{Ca}^{2+}]$ is not directly measurable. The latter problem was approached by looking for experimental conditions under which the uniporter reaction was not affected by changes in internal Ca^{2+} . In practice, as described below, it was found that the use of sufficient P_i satisfied this requirement. Although P_i has no direct effect on the uniporter of cardiac mitochondria (Crompton *et al.*, 1978a), P_i promotes Ca^{2+} influx by influencing both components of $\Delta\tilde{\mu}_{\text{Ca}^{2+}}$. P_i facilitates maintenance of $\Delta\psi$ during Ca^{2+} uptake

by dissipation of the pH gradient (for review, see Nicholls & Akerman, 1982). At sufficiently high concentrations, P_i may also decrease matrix free Ca^{2+} (Zoccarato & Nicholls, 1982). On these grounds, it was also essential to establish criteria by which the comparison of uniporter activities would not be influenced by variations in $[P_i]$.

Fig. 2 shows that added P_i caused an increase in both $\Delta\psi$ and the rate of Ca^{2+} uptake, with no plateau in either up to $400\mu M$ - P_i , under otherwise standard assay conditions. The degree to which the increased rate of Ca^{2+} uptake with added P_i resulted from the increase in $\Delta\psi$ was investigated with mitochondria from control perfused hearts, as reported in Fig. 3(a). In this case, at each $[P_i]$, $\Delta\psi$ was manipulated over a range of values by partial uncoupling with various amounts of CCCP. The amounts of CCCP were chosen by trial and error, so that all of the plots overlapped over some range of $\Delta\psi$. This enabled the influence of P_i on the Ca^{2+} -uptake rates to be seen independently of its influence on $\Delta\psi$.

The rates of Ca^{2+} uptake at 141 mV are replotted in Fig. 3(b) (lower curve). At constant $\Delta\psi$, Ca^{2+} influx was promoted by up to $60\mu M$ - P_i , but higher amounts of added P_i caused no further stimulation. Fig. 3(b) also shows data obtained similarly for mitochondria from methoxamine-pretreated hearts (upper curve). Methoxamine-treated mitochondria showed a similar response to P_i at constant $\Delta\psi$, although the absolute rates of Ca^{2+} uptake were greater than with control mitochondria. P_i ($60\mu M$)

Fig. 2. Influence of added P_i on the rate of Ca^{2+} uptake and the inner-membrane potential

The rates of Ca^{2+} uptake and $\Delta\psi$ of mitochondria from control perfused hearts were measured at $1.5\mu M$ external free Ca^{2+} and 37 nmol of intra-mitochondrial Ca^{2+} /mg of protein as indicated in Fig. 1, except that $[P_i]$ was varied. Symbols: $\bullet$, Ca^{2+} uptake; $\circ$, $\Delta\psi$.

yielded a maximal stimulation at constant $\Delta\psi$ of about 1.5-fold in both cases. Experiments were performed to determine the additional contribution of endogenous P_i to the total P_i of the incubations. These gave values (means $\pm$ s.e.m.; four determinations) for endogenous P_i of $18 \pm 4\mu M$ for control mitochondria and $22 \pm 3\mu M$ for methoxamine-treated mitochondria with 0.7 mg of protein/ml (standard conditions).

The $\Delta\psi$ -independent stimulation below $60\mu M$ - P_i may reflect P_i -induced changes in $\Delta[Ca^{2+}]$ over this range as indicated as follows: Fig. 4(a) shows the

Fig. 3. Interrelations between added P_i , the inner-membrane potential and the rate of Ca^{2+} uptake

The measurements were made at $1.5\mu M$ external free Ca^{2+} and 38 nmol of internal Ca^{2+} /mg of protein as indicated in Fig. 1, except that $[P_i]$ was varied and CCCP (0 – 70 pmol) added in order to manipulate $\Delta\psi$ over the ranges shown. (a) Control mitochondria: $\bullet$, zero P_i ; Δ , $10\mu M$ - P_i ; $\square$, $25\mu M$ - P_i ; $\circ$, $60\mu M$ - P_i ; $\blacksquare$, $125\mu M$ - P_i ; $\blacktriangle$, $200\mu M$ - P_i ; $\blacktriangledown$, $400\mu M$ - P_i . (b): $\diamond$, The values of the rates of Ca^{2+} uptake at 141 mV from Fig. 3(a) (control mitochondria) replotted versus added $[P_i]$; $\blacklozenge$, the rates of Ca^{2+} uptake at 141 mV by mitochondria from methoxamine-treated hearts plotted against added $[P_i]$ (internal Ca^{2+} , $37\text{ nmol}/\text{mg}$).

relations between the rates of Ca^{2+} uptake and $\Delta\psi$ in the absence of added P_i with internal Ca^{2+} contents of 8 and 43 nmol/mg of protein. Ca^{2+} was accumulated faster at the lower Ca^{2+} content. The basal rates of Ca^{2+} efflux were greater with increased Ca^{2+} content, but not sufficiently so to account for the differences observed in Ca^{2+} uptake. In contrast, with $200\mu\text{M-P}_i$ (Fig. 4b) the rates of Ca^{2+} uptake were unaffected by changed Ca^{2+} content at any value of $\Delta\psi$. Inclusion of P_i therefore rendered the uniporter reaction insensitive to changes in matrix Ca^{2+} under the standard assay conditions, at least over the range 8–43 nmol of matrix Ca^{2+} /mg. Conversely, $200\mu\text{M-P}_i$ did not detectably increase the rate of Ca^{2+} uptake at constant $\Delta\psi$ with 8 nmol of Ca^{2+} /mg (comparison of broken and continuous lines in Fig. 4b), whereas with 43 nmol of Ca^{2+} /mg the stimulation by P_i was very marked (e.g. 54% at 135 mV and 40% at 145 mV).

P_i also caused a decrease in the basal rates of Ca^{2+} efflux to values (0.15–0.30 nmol of Ca^{2+} /min per mg) that were negligible in comparison with the rates of Ca^{2+} uptake. Previous work (Crompton *et al.*, 1976b) showed that the basal rate of Ca^{2+} efflux from heart mitochondria (in the absence of Na^+) is

increased upon inhibition of the respiratory chain with antimycin a. However, the moderate decreases in $\Delta\psi$ used in the present study did not affect the basal rates significantly.

In summary, although an exact interpretation of the effects of P_i would be premature, the essential point with regard to the aims of this study is that inclusion of $200\mu\text{M-P}_i$ (standard assay conditions) is advantageous from three aspects. Firstly, $200\mu\text{M-P}_i$ most probably precludes changes in uniporter activity owing to changes in internal free Ca^{2+} , which may, or may not, occur (Fig. 4b). Secondly, with $200\mu\text{M-P}_i$ the uniporter activities of both control and methoxamine-pretreated mitochondria would not be influenced by the small changes encountered in the availability of P_i between preparations provided that comparison is made either between rates at the same $\Delta\psi$ value (Fig. 3b) or between uniporter conductances. Simple rate measurements, on the other hand, would be subject to small variations in $[\text{P}_i]$ (Fig. 2) and thereby some irreproducibility. Thirdly, P_i depresses the basal rates of Ca^{2+} efflux (Fig. 4) so that the corrections applied in calculating uniporter activities are minimized.

Fig. 4. Influence of internal Ca^{2+} content on the relation between Ca^{2+} -uptake velocity and inner-membrane potential in the presence and absence of added P_i

The rates of Ca^{2+} influx (closed symbols) and basal Ca^{2+} efflux (in the presence of Ruthenium Red; open symbols) were measured as indicated in Fig. 1 at $2.4\mu\text{M}$ external free Ca^{2+} and either 8 or 43 nmol of intramitochondrial Ca^{2+} /mg of protein ($\text{Ca}_{\text{in}}^{2+}$). CCCP was used to vary $\Delta\psi$ over the ranges shown. (a) No added P_i : $\blacktriangle$, Ca^{2+} influx with 8 nmol of $\text{Ca}_{\text{in}}^{2+}$; $\blacktriangledown$, Ca^{2+} influx with 43 nmol of $\text{Ca}_{\text{in}}^{2+}$; $\triangle$, basal Ca^{2+} efflux with 8 nmol of $\text{Ca}_{\text{in}}^{2+}$; $\triangledown$, basal Ca^{2+} efflux with 43 nmol of $\text{Ca}_{\text{in}}^{2+}$. (b) $200\mu\text{M}$ added P_i : $\bullet$, Ca^{2+} influx with 8 nmol of $\text{Ca}_{\text{in}}^{2+}$; $\blacksquare$, basal efflux with 43 nmol of $\text{Ca}_{\text{in}}^{2+}$; $\circ$, basal Ca^{2+} efflux with 8 nmol of $\text{Ca}_{\text{in}}^{2+}$; $\square$, basal efflux with 43 nmol of $\text{Ca}_{\text{in}}^{2+}$. For comparison, the $\blacktriangle$ data from Fig. 4(a) are reproduced in Fig. 4(b) as a broken line.

Effects of methoxamine pretreatment on uniporter activity

Fig. 1 reports the effects of methoxamine administration on the uptake of Ca^{2+} and TPP^{+} in the presence of $200\mu\text{M-P}_i$. Methoxamine pretreatment caused an increase in the rate of Ca^{2+} uptake, but no detectable change in either the basal rates of Ca^{2+} efflux, or the rates and extent of TPP^{+} uptake. In six analogous experiments each with mitochondria from control and methoxamine-pretreated hearts, methoxamine caused an average increase in uniporter activity of about 38% with both 9 and 40 nmol of matrix Ca^{2+} /mg (Table 1). The uniporter activities were essentially the same at the two Ca^{2+} contents with both control and methoxamine-pretreated mitochondria. Methoxamine pretreatment induced no significant changes in either $\Delta\psi$ or the basal rates of Ca^{2+} efflux.

Representative relations between uniporter activity and $\Delta\psi$ are given in Fig. 5. Essentially the same relations were observed with internal Ca^{2+} contents of 9 nmol/mg (Fig. 5a) and 36 nmol/mg (Fig. 5b); for comparison the plots of Fig. 5(a) are reproduced in Fig. 5(b) (broken lines). The rate/ $\Delta\psi$ relations conformed closely to straight lines over the ranges investigated, and their slopes may be termed apparent uniporter conductances. The use of conductances is advantageous, since it allows the degree of $\Delta\psi$ -independent activation of the uniporter to be expressed unambiguously. The data reveal methoxamine-induced increases in conductance of about 42% with 9 nmol of matrix Ca^{2+} /mg (from 95 to 135 pmol of $\text{Ca}^{2+}\cdot\text{mg}^{-1}\cdot\text{min}^{-1}\cdot\text{mV}^{-1}$) and 31% with 36 nmol of Ca^{2+} /mg (from 99 to 130 pmol of $\text{Ca}^{2+}\cdot\text{mg}^{-1}\cdot\text{min}^{-1}\cdot\text{mV}^{-1}$) over the ranges studied. In six such experiments each with control and agonist-treated mitochondria, methoxamine caused an average increase of 38–40% in apparent uniporter conductance irrespective of Ca^{2+} content (Table 1).

Effects of adrenergic agonists on mitochondrial Ca^{2+} content

If the methoxamine-induced increase in uniporter conductance apparent *in vitro* reflects a similar activation *in vivo*, methoxamine would be predicted to cause a measurable increase *in vivo* of intramitochondrial Ca^{2+} . This prediction was tested, as reported in Fig. 6.

Mitochondria were isolated from perfused hearts essentially by the standard procedure, except that they were sedimented at intervals from the first supernatant of the homogenate and were not washed. EDTA (0.5 mM) was included in the homogenization medium to prevent uptake of Ca^{2+} . Curves were constructed of the relation between endogenous Ca^{2+} content and the time period that

elapsed between homogenization and sedimentation of the mitochondria. The time-courses enable estimates to be made of the losses of endogenous Ca^{2+} after homogenization.

The Ca^{2+} contents of mitochondria isolated from control perfused hearts were remarkably stable over the period 10–32 min after homogenization (average contents 1.84–1.94 nmol of Ca^{2+} /mg). Similarly, there were no detectable changes in the Ca^{2+} contents of mitochondria from methoxamine-pretreated hearts over the same time period (average contents 2.61–2.75 nmol of Ca^{2+} /mg). Methoxamine caused an increase in endogenous Ca^{2+} of 42–44%, which is similar in magnitude to the increase in uniporter activity *in vitro* (38–40%; Table 1).

As outlined in the introduction, hypotheses have been put forward that increases in heart mitochondrial Ca^{2+} may occur in response to both α -adrenergic and β -adrenergic stimuli, the latter via increased time-averaged cytoplasmic free Ca^{2+} . Accordingly, the endogenous Ca^{2+} contents were also measured after administration of the β -adrenergic agonists isoprenaline and adrenaline. Every perfusion with agonist and subsequent extraction and assay of Ca^{2+} was performed together with a control. The control values appertaining to each agonist did not differ significantly, e.g. the control values (nmol of Ca^{2+} /mg of protein; means $\pm$ S.E.M.) at 10 min were as follows: 1.81 ± 0.15 in 12 determinations (methoxamine), 1.91 ± 0.14 in four determinations (isoprenaline) and 1.96 ± 0.06 in four determinations (adrenaline). The data with different agonists may therefore be compared (Fig. 6).

Isoprenaline and adrenaline both caused significant increases in intramitochondrial Ca^{2+} , but in both cases slow losses of endogenous Ca^{2+} occurred with time. However the rates of Ca^{2+} loss are not sufficiently great to preclude extrapolation to zero time. The extrapolated plots yield the following values for endogenous Ca^{2+} (nmol/mg of protein) at the time of homogenization: adrenaline-treated, 4.2; isoprenaline-treated, 3.5; methoxamine-treated, 2.6; control, 1.75.

In summary, the largest increase in matrix Ca^{2+} was caused by adrenaline (140% from the extrapolated plots). The isoprenaline-induced increase (100%) was about twice that caused by methoxamine (48%). The sums of the separate isoprenaline-induced and methoxamine-induced increases were approximately equal to that obtained with adrenaline alone. The data therefore provide direct confirmation that the transport cycle is influenced by both β -adrenergic (Hiraoka *et al.*, 1980; McCormack & Denton, 1981) and α -adrenergic mechanisms (Kessar & Crompton, 1981), and that these may operate in concert to yield increased matrix Ca^{2+} (Hayat & Crompton, 1982).

Table 1. Ca^{2+} -transport properties of mitochondria isolated from hearts perfused in the presence and absence of methoxamine

The measurements were made as described in the Methods section with 200 μM - P_i and 1.5 μM external free Ca^{2+} . The uniporter activities (corrected for basal Ca^{2+} effluxes), the basal effluxes in the presence of Ruthenium Red, and the values of $\Delta\psi$ are those obtained in the absence of CCCP. The uniporter conductances were determined from the slopes of the dependencies of uniporter activity (corrected for basal Ca^{2+} effluxes) on $\Delta\psi$. These relations were obtained from measurements in the presence and absence of 50 pmol of CCCP (sufficient to change $\Delta\psi$ by 29–34 mV). All data are given as means $\pm$ S.E.M. for six separate experiments. The data were analysed by Student's unpaired *t*-test: **P* < 0.01; ***P* < 0.002; N. S., not significant.

	Control	Methoxamine	Change caused by methoxamine (%)
(a) With 9 nmol of intramitochondrial Ca^{2+} /mg of protein			
Uniporter activity (nmol of Ca^{2+} /min per mg)	7.9 $\pm$ 0.4	11.0 $\pm$ 0.4	+38**
Basal Ca^{2+} efflux (nmol of Ca^{2+} /min per mg)	0.29 $\pm$ 0.09	0.44 $\pm$ 0.06	N.S.
$\Delta\psi$ (mV)	166 $\pm$ 3	163 $\pm$ 3	N.S.
Apparent uniporter conductance (pmol of Ca^{2+} · mg ⁻¹ · min ⁻¹ · mV ⁻¹)	102 $\pm$ 6	143 $\pm$ 7	+40**
(b) With 40 nmol of intramitochondrial Ca^{2+} /mg of protein			
Uniporter activity (nmol of Ca^{2+} /min per mg)	7.8 $\pm$ 0.4	10.8 $\pm$ 0.6	+38*
Basal Ca^{2+} efflux (nmol of Ca^{2+} /min per mg)	0.33 $\pm$ 0.10	0.47 $\pm$ 0.05	N.S.
$\Delta\psi$ (mV)	163 $\pm$ 4	161 $\pm$ 4	N.S.
Apparent uniporter conductance (pmol of Ca^{2+} · mg ⁻¹ · min ⁻¹ · mV ⁻¹)	101 $\pm$ 4	139 $\pm$ 5	+38**

Fig. 5. Relations between uniporter activity and inner-membrane potential in mitochondria from control and methoxamine-treated hearts

The measurements were made as indicated in Fig. 1 with 200 μM - P_i and 1.5 μM external free Ca^{2+} and either (a) 9 or (b) 36 nmol of intramitochondrial Ca^{2+} /mg (Ca_i^{2+}). The rates of Ca^{2+} influx are corrected for the basal rates of Ca^{2+} efflux in the presence of Ruthenium Red; these did not differ significantly with control and methoxamine-treated mitochondria (i.e. 0.2–0.3 nmol of Ca^{2+} /min per mg at 9 nmol of Ca_i^{2+} and 0.3–0.4 nmol/min per mg at 36 nmol of Ca_i^{2+} with both preparations of mitochondria). (a) 9 nmol of Ca_i^{2+} : ●, control; ○, methoxamine-treated. (b) 36 nmol of Ca_i^{2+} : ■, control; □, methoxamine-treated. For comparison, the curves of Fig. 5(a) are reproduced in Fig. 5(b) as broken lines.

Fig. 6. Endogenous Ca^{2+} contents of mitochondria from control and agonist-treated hearts

The determinations were made on mitochondria from perfused hearts as described in the Methods section. The abscissa values refer to the time periods between homogenization and sedimentation of mitochondria. Symbols: ●, control; △, methoxamine ($10\mu\text{M}$, 2 min); ○, isoprenaline ($1\mu\text{M}$, 2 min); □, adrenaline ($1\mu\text{M}$, 2 min). Bars indicate $\pm$ S.E.M.; the numbers of separate experiments are given in parentheses. The data were analysed by Student's unpaired *t*-test. With respect to control values, $P < 0.001$ for all points, except those indicated (*), where $P < 0.01$.

The relation of these changes to the sensitivities of matrix dehydrogenases to Ca^{2+} is considered in the Discussion section.

Discussion

Previous studies revealed that administration *in vivo* of the α -adrenergic agonist methoxamine to rat hearts induced a stable activation of mitochondrial Ca^{2+} uptake that was not readily accounted for as a secondary response to stimulation of the respiratory chain (oxidizing succinate), since this was not detectably affected (Kessar & Crompton, 1981). On the other hand, it was observed that the α -adrenergic agonist phenylephrine stimulated mitochondrial respiration in liver (Taylor *et al.*, 1980). Subsequent work in our laboratory (T. P. Goldstone & M.

Crompton, unpublished work) has confirmed the agonist-induced stimulation of the respiratory chain in liver (phenylephrine), but not in heart (methoxamine). Whether these contrasting responses reflect tissue diversity or the agonist used is not yet known. It seems, however, that the action of methoxamine on heart may offer a relatively unambiguous means to answer the question of whether the uniporter itself is susceptible to α -adrenergic modification. A critical examination of this question needs to take into consideration possible methoxamine-induced changes in (a) the driving force for Ca^{2+} influx, (b) co-anion availability and (c) nonspecific Ca^{2+} fluxes.

Data from a wide range of experimental approaches with intact mitochondria indicate that Ca^{2+} influx is driven solely by $\Delta\bar{\mu}_{\text{Ca}^{2+}}$ (reviewed by Nicholls & Akerman, 1982). Ca^{2+} entry in response to $\Delta\bar{\mu}_{\text{Ca}^{2+}}$ is also indicated by studies of purified glycoprotein and peptide components of heart mitochondria reconstituted into black-lipid membranes; these exhibited selective Ca^{2+} -permeability properties, which were inhibited by Ruthenium Red, and developed near-Nernstian potentials with applied Ca^{2+} gradients (Miranova *et al.*, 1982). On this basis, it appears that comparison of either uniporter conductances or rates of Ca^{2+} uptake at equipotential points, both at constant external Ca^{2+} , would provide a reliable estimation of the degree of uniporter activation, subject to the provision that the measurements were not affected by changes in internal free Ca^{2+} . The present study has not ruled out the possibility that hormone-induced changes in internal free Ca^{2+} occurred. However, if such changes did occur, they would not be predicted to influence the measurements, since the rates of Ca^{2+} influx were independent of Ca^{2+} content over the range used in the standard assay conditions (Figs. 4b and 5; Table 1).

Regarding co-anion availability, it was observed that the action of $>60\mu\text{M-P}_i$ on both control and treated mitochondria was entirely attributable to increase in $\Delta\psi$ (Figs. 2 and 3), so that with sufficient P_i comparison of conductances (or rates at equipotential points) discriminates against this factor (Fig. 5, Table 1). Alternatively, the data may be compared at relatively low internal $[\text{Ca}^{2+}]$ (e.g. $9\text{ nmol of Ca}^{2+}/\text{mg}$; Fig. 5a, Table 1a), since under these conditions the rates at constant $\Delta\psi$ were not influenced by addition of co-anion ($200\mu\text{M-P}_i$; Fig. 4b).

Finally, Ruthenium Red, a selective inhibitor of the uniporter (reviewed by Nicholls & Akerman, 1982), was used to estimate Ca^{2+} fluxes via a non-uniport route(s), so that true uniporter activities could be obtained by correction. The basal rates of Ca^{2+} efflux in the presence of Ruthenium Red (Na^+ -independent efflux; Crompton *et al.*, 1978b) were negligible under standard conditions, and were

not significantly altered by methoxamine pretreatment (Fig. 1, Table 1). In summary, therefore, taking account of these three factors, the data are consistent with a methoxamine-induced modification of the uniporter.

The two Ca^{2+} carriers of the transport cycle are considered to catalyse unidirectional Ca^{2+} fluxes (Nicholls & Crompton, 1980). Unidirectional influx via the uniporter will be imposed by the magnitude *in vivo* of $\Delta\psi$ (Puskin *et al.*, 1976; Crompton *et al.*, 1976b; Nicholls, 1978; Crompton & Heid, 1978). The energy-dependence of Ca^{2+} efflux via the $\text{Na}^+-\text{Ca}^{2+}$ carrier may ensure that this also operates unidirectionally (Crompton *et al.*, 1976b, 1977). It follows that the steady-state distribution of Ca^{2+} will be determined by the relative kinetic properties of the two opposing transport processes and, in the present context, that uniporter activation would be predicted to displace the Ca^{2+} distribution to a new steady state with increased matrix Ca^{2+} . This prediction was confirmed by direct measurement of endogenous Ca^{2+} . In fact, the increase in matrix Ca^{2+} (about 48% from the extrapolated plots) matched reasonably well with the degree of uniporter activation by methoxamine (38–40%). A linear relation between these two parameters, as observed, would only follow if changes in matrix Ca^{2+} elicit proportional changes in $\text{Na}^+-\text{Ca}^{2+}$ carrier activity, as discussed previously (Nicholls & Crompton, 1980). This condition would appertain when total matrix Ca^{2+} provides a free matrix $[\text{Ca}^{2+}]$ that is less than the K_m value of the $\text{Na}^+-\text{Ca}^{2+}$ carrier. Available data indicate that the K_m value would not be exceeded at the endogenous Ca^{2+} contents revealed in the present study. Hansford & Castro (1982) observed a hyperbolic dependence of $\text{Na}^+-\text{Ca}^{2+}$ carrier activity on matrix $[\text{Ca}^{2+}]$, with half-maximal activity at about 6 nmol of Ca^{2+} /mg in the presence of 5 mM- P_i . A comparable value has been obtained in our laboratory under identical conditions, i.e. 8.2 ± 0.24 nmol of Ca^{2+} /mg (mean $\pm$ S.E.M.; four determinations. P. Kessar & M. Crompton, unpublished work). A similar value (about 8 nmol of Ca^{2+} /mg) may be calculated from the data of Coll *et al.* (1982).

The estimates of mitochondrial Ca^{2+} *in vivo* bear directly on the critical question of whether the concentration of matrix Ca^{2+} in heart is appropriate for regulation of oxidative metabolism. The values obtained (1.75–4.20 nmol of Ca^{2+} /mg, depending on hormonal state) favour a regulatory function when considered with other published data on the Ca^{2+} -sensitivity of α -oxoglutarate dehydrogenase in intact heart mitochondria. Both Coll *et al.* (1982) and Hansford & Castro (1982) reported Ca^{2+} -sensitivity of this enzyme over a range of matrix Ca^{2+} contents with an approximate upper limit at 2–3 nmol of Ca^{2+} /mg. Those investigators also obtained esti-

mates of the percentage of total matrix Ca^{2+} that is free by use of a null-point titration technique (Williamson & Murphy, 1980). Application of these estimates to the present data yields intramitochondrial free $[\text{Ca}^{2+}]$ of 1.1–1.4 μM in control perfused mitochondria (1.75 nmol of total Ca^{2+} /mg) and more in agonist-treated mitochondria according to the proportions indicated in Fig. 6. These extrapolations, although tenuous, concur nevertheless with the concept that matrix free Ca^{2+} *in vivo* may be hormonally varied within the range (0.1–10 μM) required for regulation of pyruvate dehydrogenase phosphate phosphatase (Denton *et al.*, 1972; Pettit *et al.*, 1972; Severson *et al.*, 1974; Hansford, 1981), α -oxoglutarate dehydrogenase (McCormack & Denton, 1979) and NAD-linked isocitrate dehydrogenase (Denton *et al.*, 1978, 1980; Hansford & Castro, 1981).

The relative capacities of isoprenaline and methoxamine to increase matrix Ca^{2+} revealed that the β -agonist was more potent. The β -adrenergic influence may be reasonably interpreted to reflect increased cytoplasmic Ca^{2+} (time averaged), since neither the uniporter nor the $\text{Na}^+-\text{Ca}^{2+}$ carrier of isolated heart mitochondria was affected by tissue pretreatment with β -adrenergic agonists (Kessar & Crompton, 1981). An increase in peak cytosolic free Ca^{2+} after β -adrenergic stimulation has been shown directly (Allen & Kurihara, 1980). An increase in time-averaged free Ca^{2+} will result also from the positive chronotropic effect of β -adrenergic stimulation. It seems likely that the relay behaviour in response to increased cytoplasmic Ca^{2+} may involve a proportionally larger increase in matrix Ca^{2+} in the new steady state. Such amplification would follow from the signoidal kinetic properties of the uniporter (Scarpa & Graziotti, 1973; Crompton *et al.*, 1976a) and inhibition of the $\text{Na}^+-\text{Ca}^{2+}$ carrier by external Ca^{2+} (Hayat & Crompton, 1982). The additional amplification in the relay affected by the α -adrenergic activation of the uniporter may perhaps be related to other α -adrenergic responses in heart. Although the mechanical response of heart is under β -adrenergic control (reviewed by Tsien, 1977), there is increasing evidence for the involvement of α -adrenergic receptors in certain biochemical events. These include the stimulation of glucose uptake (Keely *et al.*, 1977), the activation of phosphofructokinase (Patten *et al.*, 1983, and references therein) and the inactivation of glycogen synthase (Ramachandran *et al.*, 1983). α -Adrenergic mechanisms therefore emerge as a possible means of co-ordinating changes in both glycolytic production of pyruvate and its oxidation, the latter via modification of the mitochondrial uniporter.

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