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The α-adrenergic-mediated activation of Ca^{2+} influx into cardiac mitochondria

A possible mechanism for the regulation of intramitochondrial free Ca^{2+}

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Mitochondria isolated from rat hearts perfused with adrenaline, and from hearts excised from adrenaline-treated rats, showed an enhanced rate of respiration-dependent Ca^{2+} uptake. Adrenaline pretreatment did not change the activity of the Na^+/Ca^{2+}-antiporter of isolated heart mitochondria. Simultaneous measurements of the membrane potential revealed that perfusion with adrenaline has no significant effect on this parameter during Ca^{2+} accumulation. The activation of Ca^{2+} uptake was induced also by the α-adrenergic agonist, methoxamine, but not by the β-adrenergic agonist, isoprenaline. Methoxamine pretreatment also increased the sensitivity of α-oxoglutarate dehydrogenase in intact mitochondria to 10 nM–300 nM extramitochondrial Ca^{2+} during steady-state Ca^{2+} recycling across the inner membrane. Possible implications of these data for the adrenergic regulation of oxidative metabolism are discussed.

Recent evidence indicates that intramitochondrial Ca^{2+} may contribute to the regulation of oxidative metabolism in heart by activating several intramitochondrial dehydrogenases (Denton & McCormack, 1980). A possible means of controlling intramitochondrial $[Ca^{2+}]$ in accordance with the requirements of these dehydrogenases is provided by the Na^+/Ca^{2+} cycle, which establishes the steady-state distribution of Ca^{2+} across the inner membrane (Crompton *et al.*, 1976*a*; Crompton & Heid, 1978; Nicholls & Crompton, 1980; Denton & McCormack, 1980).

Administration of adrenaline to hearts stimulates the conversion of glucose into CO_2 (Williamson, 1964, 1966), and the question arises whether this respiratory increment reflects an action of adrenaline in modulating intramitochondrial $[Ca^{2+}]$, and thereby tricarboxylate-cycle flux, by inducing changes in Ca^{2+} transport across the mitochondrial inner membrane. Hiraoka *et al.* (1980) and McCormack & Denton (1981) have reported evidence that β-adrenergic-induced elevation of sarcoplasmic $[Ca^{2+}]$ is relayed to the intramitochondrial compartment and causes activation of pyruvate dehydrogenase. However, the possibility of an additional, more direct, adrenergic effect on cardiac mitochondrial Ca^{2+} fluxes is suggested by the observed α-adrenergic activation of Ca^{2+} influx into liver mitochondria (Taylor *et al.*, 1980).

Abbreviation used: TPP^+, tetraphenylphosphonium.

The present paper examines the effects of treating hearts with adrenergic and cholinergic agents on the properties of the Ca^{2+}-uniporter and Na^+/Ca^{2+}-antiporter, the two Ca^{2+}-transport systems of the Na^+/Ca^{2+} cycle. The data reveal a selective adrenergic activation of the uniporter, which is expressed via an α-adrenergic mechanism; the consequent elevation of intramitochondrial free $[Ca^{2+}]$ during Ca^{2+} recycling across the inner membrane may contribute to the adrenergic activation of oxidative metabolism.

Methods

Heart perfusion

Hearts from albino Sprague–Dawley rats were removed and washed in ice-cold medium that was identical with the perfusion medium (see below), except that 8.4 mM-$NaHCO_3$ was used and the medium was not gassed. The aorta was cannulated and the heart was perfused under hydrostatic pressure (85 cm of water) by the Langendorff technique without recirculation. The perfusion medium contained 120 mM-NaCl, 4.8 mM-KCl, 1.2 mM-KH_2PO_4, 0.6 mM-$MgSO_4$, 1.3 mM-$CaCl_2$, 25.3 mM-$NaHCO_3$ and 10 mM-glucose, and was pregassed at 37°C (O_2/CO_2, 19:1). After 25–30 min perfusion, either 1 μM-adrenaline, 1 μM-isoprenaline, 10 μM-methoxamine or 100 nM-acetylcholine was introduced into the perfusion medium and, 1–2 min later,

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the heart was removed and homogenized immediately with a Polytron homogenizer as stated below (see under 'Mitochondrial preparation'). Every heart was perfused in parallel with a control heart, which was treated similarly, except that the agonist was omitted.

Animal injection

Rats were given a single intraperitoneal injection of adrenaline (2.5 mg/kg body wt.; dissolved in slightly acidified water); sham-injected rats (controls) received the same volume of acidified water. At the times indicated in Fig. 2, the animals were killed and the hearts were excised and homogenized as described under 'Mitochondrial preparation'.

Mitochondrial preparation

Rat heart mitochondria were prepared by using a Polytron homogenizer (Kinematica G.m.b.H., Luzern, Switzerland) as described previously (Crompton *et al.*, 1976*a*). The protein content was determined by a modified biuret method (Kröger & Klingenberg, 1966) with bovine plasma albumin (Sigma Chemical Co., St. Louis, MO, U.S.A.) as standard.

Measurement of Ca^{2+} fluxes

Changes in the extramitochondrial concentration of Ca^{2+} were measured with a Ca^{2+}-selective electrode developed by Simon and his co-workers (Ammann *et al.*, 1975), unless stated otherwise. The electrode potentials were amplified by a Philips pH meter (PN 9409), which drove a Perkin–Elmer recorder (model 56). The electrode responses were calibrated and plotted on a linear scale of Δ Ca^{2+} as stated previously by Crompton *et al.* (1976*b*). The concentrations of free Ca^{2+} were determined by calibrating the ΔmV versus Δ [Ca^{2+} (free)] with Ca^{2+}/*N*-(2-hydroxyethyl)ethylenediamine-*NN'N'*-triacetic acid buffers as described in detail previously (Crompton *et al.*, 1976*b*).

The standard reaction medium (volume 4 ml) contained 120 mM-KCl, 5 mM-4-(2-hydroxyethyl)-1-piperazine-ethanesulphonate [tris(hydroxyethyl)-aminomethane salt] (pH 7.0), 2 μg of rotenone, 0.25 mM-KH_2PO_4 (pH 7.0), 1.5 mg of mitochondrial protein and $CaCl_2$ as indicated in the legends.

The suspension was pre-incubated for 7–10 min at 25°C, after which the efflux of endogenous Ca^{2+} was essentially complete. Ca^{2+} uptake was started by addition of 2 mM-succinate (K^+ salt) as respiratory substrate in the presence or absence of 100 μM-ATP (K^+ salt) as indicated in the legends.

The Na^+-induced efflux of Ca^{2+} was measured by allowing the mitochondria to accumulate Ca^{2+}, as described above, after which further uptake was blocked by addition of 1 nmol of Ruthenium Red/mg of protein (Moore, 1971). About 1 min later, net efflux of Ca^{2+} was started by addition of 8 mM-NaCl.

In the experiment of Fig. 3, Ca^{2+} influx was measured by using the metallochromic Ca^{2+} indicater arsenazo III (Scarpa *et al.*, 1978). Arsenazo III (100 μM) was added to the standard reaction medium (volume 3 ml) and the $\Delta A_{675-685}$ nm was monitored with a Perkin–Elmer model 356 dual-wavelength spectrophotometer.

Measurement of the mitochondrial membrane potential

The inner-membrane potential was calculated from the accumulation of TPP^+, which was measured by using an electrode constructed as described by Kamo *et al.* (1979). The 90% response time of the electrode to changes in [TPP^+] was less than 1 s. TPP^+ (40 μM) was added to the experimental medium (see above) and the membrane potentials were calculated from the Nernst relation, taking into account the small degree of membrane binding of TPP^+, as described by Kamo *et al.* (1979). The volume of the matrix water [0.85 ± 0.02 μl/mg of protein (mean $\pm$ S.E.M. for 15 determinations)] was determined with ^3H_2O and [^{14}C]sucrose as described by Crompton & Heid (1978).

Measurement of mitochondrial respiration

Mitochondrial respiration was monitored with an oxygen electrode. The reaction medium (2 ml, pH 7.0) contained 120 mM-KCl, 10 mM-4-(2-hydroxyethyl)-1-piperazine-ethane sulphonate [tris-(hydroxyethyl)aminomethane salt], 2 mM-KH_2PO_4, 1 mg of mitochondrial protein and 5 mM-EGTA plus $CaCl_2$ to give the concentrations of free Ca^{2+} stated in Figs. 7 and 8. NaCl (10 mM) was included as indicated in the Figure legends. The suspension was pre-incubated for 20 min at 25°C, after which 0.5 mM-ADP was added and, 2 min later, 50 μM-α-oxoglutarate. The endogenous respiratory substrates were almost depleted during the pre-incubation period as judged by the fact that the rate of O_2 consumption in the presence of ADP was never greater than 6% of the rate seen in the presence of ADP and oxoglutarate.

Results

The effects of adrenaline on Ca^{2+} uptake and release by heart mitochondria

Fig. 1 compares the Ca^{2+}-transport activities of mitochondria prepared from rat hearts perfused in the presence and absence of 1 μM-adrenaline. The mitochondria were pre-incubated in the presence of rotenone, which inhibits the oxidation of endogenous substrates and allows the release of endogenous Ca^{2+} (about 18 nmol of Ca^{2+}/mg of protein).

In Fig. 1(*a*), the re-uptake of Ca^{2+} was started

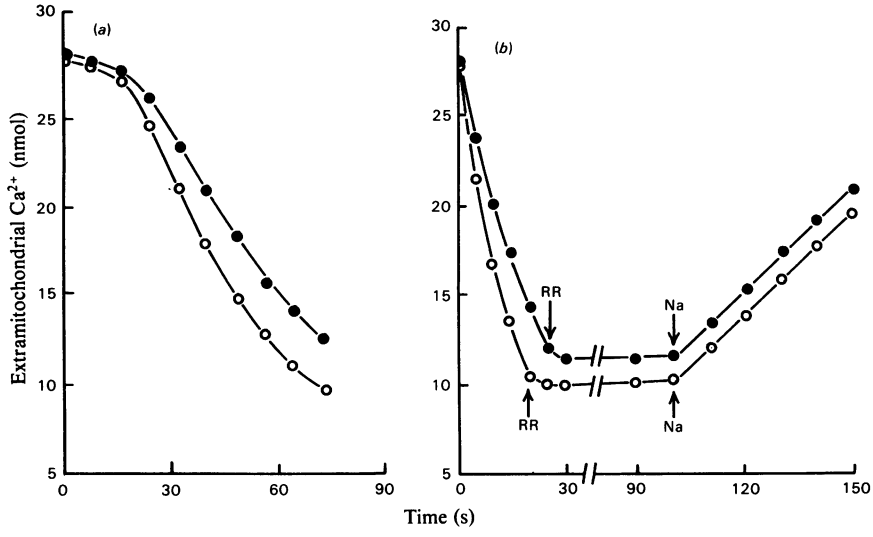

Fig. 1. *The uptake and Na^+-induced release of Ca^{2+} from mitochondria isolated from hearts perfused with and without adrenaline*

Mitochondria from adrenaline-treated and control hearts were prepared and assayed as described in the Methods section. After a pre-incubation period of 10 min, Ca^{2+} uptake was started at zero time by the addition of either 2 mM-succinate (*a*) or 2 mM-succinate plus 100 μM-ATP (*b*). At the times indicated, further uptake was stopped by the addition of 1 nmol of Ruthenium Red/mg of protein (RR). Ca^{2+} efflux was induced by the addition of 8 mM-NaCl (Na). Symbols: ●, control; ○, adrenaline-treated (1 μM, 1 min).

at zero time by the addition of succinate as respiratory substrate. The uptake of Ca^{2+} by both adrenaline-treated and control mitochondria exhibits an initial lag phase of about 20 s duration. A similar, pronounced lag phase in Ca^{2+} accumulation by mitochondria from non-perfused hearts oxidizing succinate has been noted previously (Crompton *et al.*, 1976*b*).

Fig. 1(*a*) shows that adrenaline-treated mitochondria accumulate Ca^{2+} more rapidly than control mitochondria, e.g. the adrenaline-treated mitochondria decreased the extramitochondrial Ca^{2+} from 25 nmol to 13 nmol in 31 s, whereas control mitochondria accomplished the same accumulation in 44 s. However, a quantitative interpretation of the effects of adrenaline is made difficult by the lag phase and, therefore, conditions were sought that eliminate it.

Fig. 1(*b*) reports that there is no detectable lag phase when uptake is started with succinate plus 100 μM-ATP. In addition, the rates of Ca^{2+} accumulation by both adrenaline-treated and control mitochondria are higher than those observed in the absence of ATP, but the activation of Ca^{2+} uptake by adrenaline-pretreatment is still observed.

The reason for the stimulation of Ca^{2+} uptake by ATP is not known. There is evidence that adenine nucleotides may 'protect' the mitochondria against Ca^{2+}-induced damage (for review, see Nicholls & Crompton, 1980). However, this phenomenon cannot apply to the observed elimination of the lag phase by ATP, since, at this stage, minimal Ca^{2+} accumulation has occurred.

Control experiments were carried out to ensure that there was no contamination of the mitochondrial preparations by sarcoplasmic reticulum that would contribute to the observed uptake of Ca^{2+}. In these experiments (results not shown), the accumulation of Ca^{2+} was measured on addition of 100 μM-ATP plus 50 μM-atractylate to inhibit the mitochondrial adenine nucleotide exchange (Pfaff *et al.*, 1965) and, also, on addition of 100 μM-ATP plus 3 μg of oligomycin/mg of protein. In neither experimental condition was there any detectable uptake of Ca^{2+} (i.e. <1% of the uptake reported in Fig. 1*b*).

Fig. 1(*b*) also shows the inhibition of Ca^{2+} uptake by Ruthenium Red, an inhibitor of the Ca^{2+}-uniporter (Moore, 1971), and the net efflux of Ca^{2+} on the addition of Na^+, catalysed by an Na^+/Ca^{2+}-antiporter (Crompton *et al.*, 1976*a*; Crompton *et al.*, 1977; Nicholls & Crompton, 1980). Adrenaline pretreatment does not cause any detectable difference in the rate of Na^+-induced efflux of Ca^{2+} [from a number (*n*) of separate experiments, the rates of Ca^{2+} efflux induced by 10 mM-Na^+ (nmol

of Ca^{2+}/mg of protein per min) were: control, $11.2 + 0.6$ ($n = 5$); adrenaline pretreated, $10.5 + 0.8$, $n = 5$ (means ± S.E.M.)].

Fig. 2 shows that the rate of Ca^{2+} uptake by isolated heart mitochondria is increased also when adrenaline is administered to rats before killing. The data reveal that maximal activation occurred within 2 min after injection of adrenaline. It is concluded from the data of Figs. 1 and 2 that, whereas adrenaline administration brings about an increase in the rate of Ca^{2+} uptake via the Ca^{2+}-uniporter, there is no change in the Na^+/Ca^{2+}-antiporter that survives the mitochondrial isolation procedure.

The effects of adrenaline on the driving force for Ca^{2+} uptake and the properties of the Ca^{2+}-uniporter

An increase in the rate of Ca^{2+} accumulation may be brought about either by a change in the properties of the uniporter or by an increase in the driving force for Ca^{2+} influx, and experiments were performed to discriminate between these possibilities.

Regarding the driving force for Ca^{2+} influx, there is considerable evidence that Ca^{2+} enters passively down its gradient of electrochemical potential (Selwyn *et al.*, 1970; Scarpa & Azzone, 1970; Rottenberg & Scarpa, 1974; Nicholls, 1978; Crompton & Heid, 1978; Saris & Åkerman, 1980). If influx rates are compared at the same concentrations of extramitochondrial Ca^{2+}, it seems reasonable to assume that any variation in the driving force would be represented as a variation of the membrane potential. The inner-membrane potentials, as judged by the distribution of TPP^+ across the inner membrane, are shown in Fig. 3. In the presence of EGTA, which prevents Ca^{2+} uptake, the membrane potentials of mitochondria isolated from both adrenaline-treated and control hearts attained a maximum of 169–170 mV after 20 s respiration. In the absence of EGTA, when Ca^{2+} uptake occurs, at least 60 s elapsed before the maximal membrane potentials were reached. Some depression of the membrane potential during Ca^{2+} uptake would be predicted; indeed, this has been noted in liver mitochondria (Åkerman, 1978). Therefore, a meaningful correlation between Ca^{2+} influx and membrane potential is achieved only when the two

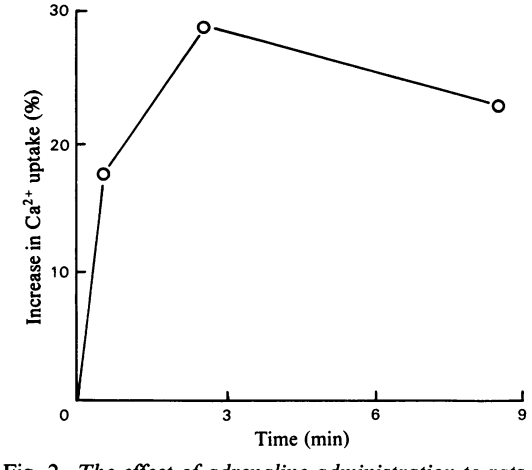

Fig. 2. *The effect of adrenaline administration to rats in vivo on the rate of Ca^{2+} uptake by heart mitochondria*
Adrenaline was administered to rats and hearts mitochondria were isolated as described in the Methods section. The rates of re-uptake of endogenous Ca^{2+} on addition of 2 mM-succinate plus 100 μM-ATP were measured as stated for Fig. 1(*b*). The graph shows the percentage increase in the initial rates of Ca^{2+} uptake of mitochondria isolated from adrenaline-treated rats with respect to control mitochondria. The values on the abscissa are the time period that elapsed between injection of adrenaline and killing of each rat.

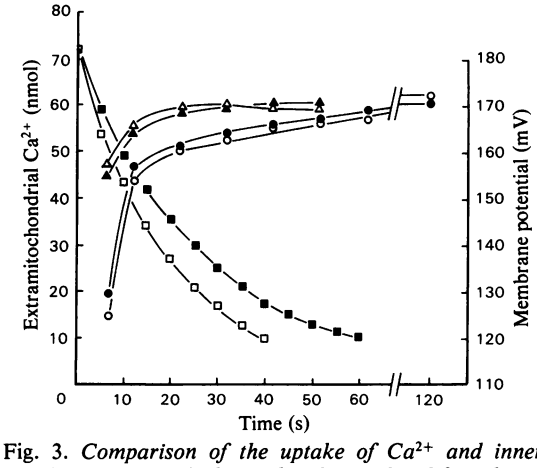

Fig. 3. *Comparison of the uptake of Ca^{2+} and inner-membrane potential of mitochondria isolated from hearts perfused with and without adrenaline*
The time courses of Ca^{2+} uptake and inner-membrane potential after the addition of 2 mM-succinate plus 100 μM-ATP to control and adrenaline-treated (1 μM, 1 min) mitochondria were measured as described in the Methods section. $CaCl_2$ (45 nmol) was added to the incubation medium at the beginning of the pre-incubation period. Ca^{2+} uptake by adrenaline-treated (□) and control (■) mitochondria was measured. The inner-membrane potentials of adrenaline-treated (○) and control (●) mitochondria during Ca^{2+} uptake and the inner-membrane potentials of adrenaline-treated (△) and control (▲) mitochondria in the presence of 1 mM-EGTA were also measured.

parameters are determined over the same time period.

The rates of Ca^{2+} influx reported in Fig. 3 decreased with time; presumably, this is due mainly to the decrease in extramitochondrial [Ca^{2+}] relative to the K_m value of the uniporter for Ca^{2+} (8–15 μM; Crompton *et al.*, 1976*b*; Fig. 5). Comparison of the rates of Ca^{2+} influx was made by calculating the rates from the time taken to decrease the extramitochondrial Ca^{2+} from 25 nmol to 15 nmol (6.2 to 3.8 μM), an arbitrary span when the membrane potentials changed slowly and at a near constant rate with time. The overall rates of Ca^{2+} influx calculated in this way were related to the average value of the membrane potential during this period. The values of the rates of Ca^{2+} influx and the membrane potentials calculated from the data of Fig. 3 are as follows: control mitochondria, 25 nmol of Ca^{2+}/mg per min, 165 mV; adrenaline-treated mitochondria, 36 nmol of Ca^{2+}/mg per min, 161 mV.

The rates of Ca^{2+} influx and the membrane potentials from several preparations of adrenaline-treated and control mitochondria are compared in Fig. 4.

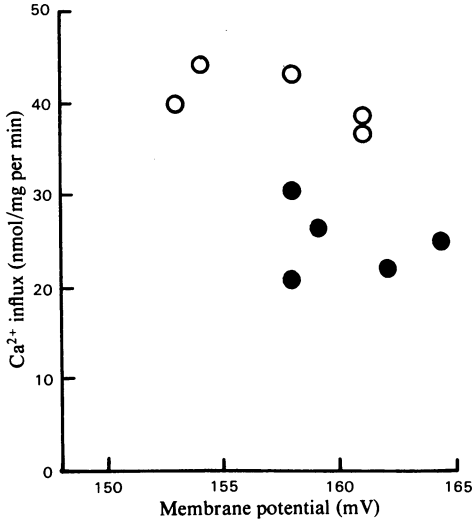

Fig. 4. *The relation between the rates of Ca^{2+} uptake and the inner-membrane potential in mitochondria from adrenaline-treated and control hearts*

The rates of Ca^{2+} uptake and the inner-membrane potential were derived from curves of the type shown in Fig. 3. The rates of Ca^{2+} uptake were calculated from the time taken for the mitochondria to decrease the extramitochondrial Ca^{2+} from 25 nmol to 15 nmol, and, in each case, the membrane potential was the average value over the same time interval. Symbols: O, adrenaline-treated (1 μM, 1 min) mitochondria; ●, control mitochondria.

The rates of Ca^{2+} influx into adrenaline-treated mitochondria clustered within the range 36–44 nmol of Ca^{2+}/mg per min, whereas the rates observed with control mitochondria lay within the range 21–31 nmol of Ca^{2+}/mg per min. The membrane potentials of the two types of mitochondria were not significantly different [adrenaline-treated mitochondria, 157 ± 3.8 mV (means ± S.D.); control mitochondria, 160 ± 2.7 mV (mean ± S.D.)].

It may be concluded that the increased rate of Ca^{2+} influx brought about by adrenaline pretreatment reflects an activation of the uniporter rather than an increased driving force for Ca^{2+} accumulation. This is supported by the data of Fig. 5, which report the degree of uniporter activation at different concentrations of extramitochondrial free Ca^{2+}. The effect of adrenaline pretreatment is greater at lower concentrations of Ca^{2+}, e.g., the degrees of activation were about 15% at 20 μM-Ca^{2+} and about 60% at 3 μM-Ca^{2+}, which suggests that the affinity of the uniporter for Ca^{2+} is increased by adrenaline.

The effects of adrenergic and cholinergic agents on the activity of the Ca^{2+}-uniporter

Fig. 6 compares the effects on uniporter activity of pretreatment of hearts with 1 μM-adrenaline for 1 min and for 2 min. Administration of adrenaline for the two time periods yielded the same activation of the uniporter (34%). It appears, therefore, that activation of the uniporter is complete within 1 min, which is in agreement with the data of Fig. 2.

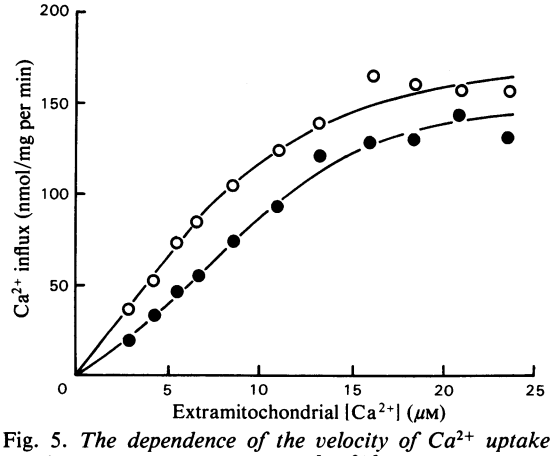

Fig. 5. *The dependence of the velocity of Ca^{2+} uptake on the extramitochondrial free [Ca^{2+}] by mitochondria from adrenaline-treated and control hearts*

The rates of Ca^{2+} uptake and the extramitochondrial free [Ca^{2+}] were determined as described in the Methods section. Ca^{2+} uptake was started by the addition of 2 mM-succinate plus 100 μM-ATP. Symbols: ●, control mitochondria; O, adrenaline-treated (1 μM, 1 min) mitochondria.

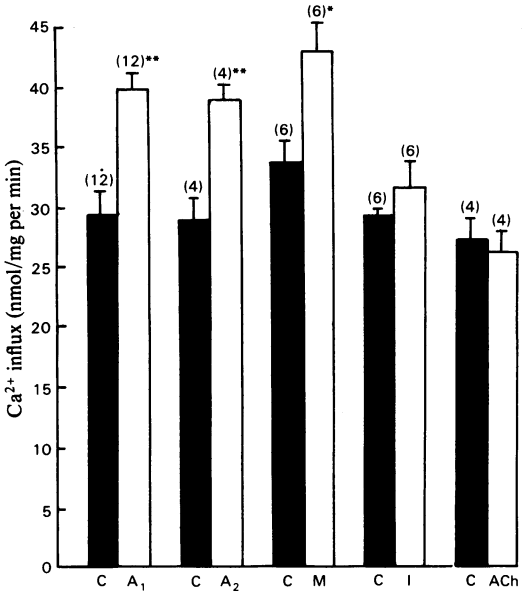

Fig. 6. *The effects of adrenergic and cholinergic agents on the rate of Ca^{2+} uptake by cardiac mitochondria*
The rates of Ca^{2+} uptake by mitochondria isolated from perfused hearts were calculated over the range 15–25 nmol of extramitochondrial Ca^{2+} as in Fig. 4. Each heart perfusion with agonist was carried out in parallel with a control perfusion without agonist, and data from agonist-treated and control hearts are reported in adjacent columns. Abbreviations used: C, control; A_1, 1 μM-adrenaline for 1 min; A_2, 1 μM-adrenaline for 2 min; M, 10 μM-methoxamine for 2 min; I, 1 μM-isoprenaline for 1 min; ACh, 100 nM-acetylcholine for 2 min. The number of mitochondrial preparations tested in each case is given in parentheses. Bars indicate +S.E.M. The data were analysed by Student's unpaired t test: *, $P<0.02$; **, $P<0.01$.

Heart contains both α- and β-adrenergic receptors (Benfey & Varma, 1967; Govier, 1968; Schümann *et al.*, 1975), and the ability of α- and β-adrenergic agonists to activate the uniporter were compared with the activation by adrenaline. The α-agonist used was methoxamine, which causes no detectable change in the cyclic AMP content of hearts in the short term (5–10 min perfusion) and a slight decrease thereafter (Schümann *et al.*, 1975). Fig. 6 shows that perfusion for 2 min with 10 μM-methoxamine gave a mean uniporter activity of 43 nmol of Ca^{2+}/mg per min, when assayed under the prescribed conditions, which may be compared with the activity of 33.5 nmol of Ca^{2+}/mg per min in control mitochondria prepared and assayed in parallel. The degree of activation by the α-agonist (28%) approaches the activation caused by adrenaline (34%).

In contrast, perfusion with isoprenaline, a β-agonist (Mayer, 1974; Dobson *et al.*, 1976) did not change uniporter activity significantly. In addition, administration of acetylcholine, which induces a large increase in the cyclic GMP content of heart, and a small decrease in cyclic AMP (LaRaia & Sonnenblick, 1971; George *et al.*, 1972; Kuo *et al.*, 1972) caused no significant change in uniporter activity. On this basis, it seems that changes in the myocardial content of cyclic nucleotides do not bring about any change in uniporter activity that can survive the mitochondrial isolation procedure. The lasting activation induced by methoxamine, however, suggests that the action of adrenaline on the uniporter may be mediated via an α-adrenergic mechanism. Other experiments (results not shown) revealed no significant effect of either methoxamine, isoprenaline or acetylcholine on the activity of the Na^+/Ca^{2+} antiporter assayed as described in Fig. 1.

The effect of methoxamine on the intramitochondrial [Ca^{2+}] during steady-state Ca^{2+} recycling

There is considerable evidence that the physiological role of the Ca^{2+}-uniporter in heart is the catalysis of unidirectional Ca^{2+} influx as part of the Na^+/Ca^{2+} cycle, which establishes the distribution of Ca^{2+} across the inner membrane (Crompton *et al.*, 1976*a*; Crompton & Heid, 1978; Nicholls & Crompton, 1980). It would be predicted, therefore, that activation of the Ca^{2+}-uniporter by α-adrenergic agonists would lead to an elevated intramitochondrial free [Ca^{2+}] during steady-state Ca^{2+} recycling across the inner membrane.

This prediction was tested at physiological concentrations of extramitochondrial Ca^{2+} and Na^+ by using the activity of α-oxoglutarate dehydrogenase as an index of the intramitochondrial free [Ca^{2+}]. This technique was introduced by Denton and his co-workers after their discovery that α-oxoglutarate dehydrogenase and some other intramitochondrial enzymes are activated by free Ca^{2+} over the range 0.1–20 μM-Ca^{2+} (Denton & McCormack, 1980). For α-oxoglutarate dehydrogenase the activation by Ca^{2+} is due to a large decrease in the K_m for oxoglutarate (McCormack & Denton, 1979). Measurement of α-oxoglutarate oxidation in intact mitochondria gave apparent K_m values with respect to extramitochondrial α-oxoglutarate of about 30 μM in the presence of about 20 nmol of endogenous Ca^{2+}/mg of protein and about 1 mM in the presence of 5 mM-EGTA to prevent Ca^{2+} uptake. In the experiments reported below, 50 μM-oxoglutarate was used to permit a sensitive response to changes in [Ca^{2+}].

The utility of this system to assess the performance of the Na^+/Ca^{2+} cycle was investigated as

report in Fig. 7. In the absence of Na^+, the rate of oxoglutarate oxidation is increased markedly when the extramitochondrial free $[Ca^{2+}]$ is increased from 10nM to 100nM. In the presence of Na^+, the activation occurs over the range 10nM–1μM-Ca^{2+}. These results are similar to those reported by Denton *et al.*, (1980). The displacement of the activation curve by Na^+ may be interpreted to reflect a large increase in the rate of efflux due to operation of the Na^+/Ca^{2+}-antiporter, so that restoration of the intramitochondrial free $[Ca^{2+}]$ in the presence of Na^+ requires a higher activity of the uniporter, i.e. a higher extramitochondrial free $[Ca^{2+}]$. At very high concentrations of extramitochondrial Ca^{2+}, the rate of oxoglutarate oxidation is depressed severely. It has been suggested that the use of Ca^{2+} buffers that yield high concentrations of free Ca^{2+} may cause mitochondrial damage (Denton *et al.*, 1980), due to excessive Ca^{2+} accumulation, and this possibility is considered further in the Discussion section. The essential point, however, is that oxoglutarate oxidation does appear to respond to changes in the activities of the Ca^{2+}-uniporter and Na^+/Ca^{2+}-antiporter over the approximate range 10–500nM extramitochondrial Ca^{2+}.

Two further aspects of the system need to be considered. First, the maximal rate of O_2 consumption, 44ng-atoms of O/mg per min, reflects an oxidation rate of 22nmol of oxoglutarate/mg per min at 25°C, if it is assumed that malate is the end product. Comparison of this value with the rate of oxoglutarate transport by rat heart mitochondria (60nmol/mg per min with 50μM-oxoglutarate at 2°C; Sluse *et al.*, 1975) indicates that oxoglutarate transport was not rate limiting. Secondly, the maximal respiratory rates with optimal concentrations of extramitochondrial Ca^{2+} and 50μM-oxoglutarate were never greater than 25% of the rates observed with saturating [oxoglutarate] (5mM). One may assume, therefore, that the respiratory chain did not exert any limitation on the rates of oxidation with 50μM-oxoglutarate.

The experimental system was used to investigate the effects of methoxamine perfusion. Fig. 8 shows

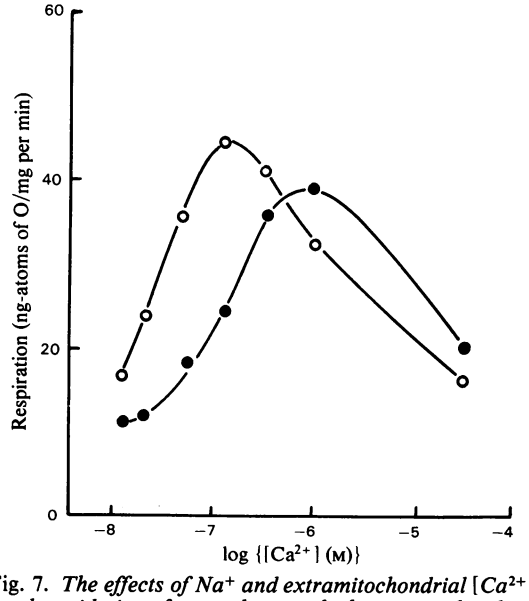

Fig. 7. *The effects of Na^+ and extramitochondrial $[Ca^{2+}]$ on the oxidation of α-oxoglutarate by heart mitochondria*
The rates of oxidation of 50μM-α-oxoglutarate by mitochondria isolated from non-perfused hearts were measured as described in the Methods section in the presence of Ca^{2+}-buffers, which gave the concentrations of free Ca^{2+} stated. Symbols: ●, with 10mM-Na^+ present; ○, without Na^+.

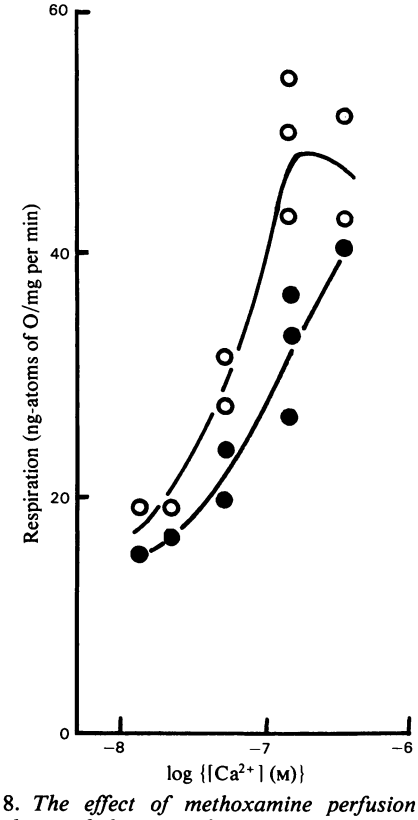

Fig. 8. *The effect of methoxamine perfusion on the dependence of the rate of α-oxoglutarate oxidation by cardiac mitochondria on the extramitochondrial $[Ca^{2+}]$*
The oxidation of 50μM-α-oxoglutarate by cardiac mitochondria was measured in the presence of 10mM-Na^+ and Ca^{2+}-buffers as described in the Methods section. The data were obtained from three mitochondrial preparations from methoxamine-treated (10μM for 2min) hearts and three control preparations. Symbols: ●, control mitochondria; ○, methoxamine-treated mitochondria.

that methoxamine pretreatment leads to a displacement of the activation curve towards lower extramitochondrial concentrations of Ca^{2+} during steady-state Ca^{2+} recycling. The shift in the Ca^{2+} activation curve for α-oxoglutarate oxidation is predicted from the selective activation of the uniporter by methoxamine, since this would permit activation of α-oxoglutarate dehydrogenase by intramitochondrial Ca^{2+} to be achieved at lower concentrations of extramitochondrial Ca^{2+}. The value of this experimental system in allowing methoxamine action to be evaluated at physiological concentrations of Ca^{2+} is considered in the Discussion section.

Other experiments have revealed no detectable effect of methoxamine pretreatment on the rate of succinate oxidation by heart mitochondria. In these experiments, succinate oxidation was measured under the same experimental conditions as described for the oxidation of oxoglutarate except that 5mM-succinate, 1mM-EGTA and 1μg of rotenone were substituted for oxoglutarate and the Ca^{2+}-EGTA buffers. The rates of succinate oxidation (ng-atoms of O/mg per min at 25°C) were as follows: control mitochondria (five preparations), 114 ± 8 (mean ± S.D.); methoxamine-treated mitochondria (three preparations), 114 ± 6 (mean ± S.D.). The same preparations of methoxamine-treated mitochondria oxidized 50μM-oxoglutarate (at 130nM-Ca^{2+}) at rates that were 60%, 62% and 47% higher than those of the control mitochondria, which were prepared and assayed in parallel. These data support the conclusion that the activation of α-oxoglutarate oxidation by methoxamine reflects an increased activation of oxoglutarate dehydrogenase by Ca^{2+} rather than an effect on the respiratory chain.

Discussion

Data in the present paper show that exposure of rat hearts to 1μM-adrenaline for 1 or 2min leads to a significant increase in the rate of Ca^{2+} uptake by isolated mitochondria. In considering possible explanations for this phenomenon, it is important to make the broad distinction between possible changes in the kinetic properties of the uniporter and possible changes in the driving force for Ca^{2+} accumulation. Regarding the latter possibility, parallel measurements revealed that adrenaline pretreatment did not affect significantly the inner-membrane potential attained during Ca^{2+} influx (Fig. 3); it is relevant to note that the net rate of Ca^{2+} uptake by liver and heart mitochondria is directly proportional to the magnitude of the inner-membrane potential (Åkerman, 1978; Crompton, 1980), so that, in the experiment of Fig. 3, for example, an increase in membrane potential of about 40% would be required to attribute the enhanced influx to an increased membrane potential. It is also relevant to consider the possibility, in principle, that adrenaline pretreatment may promote Ca^{2+} influx by causing enhanced binding of the accumulated Ca^{2+} to intramitochondrial constituents. This suggestion is probably not applicable since the adrenaline pretreatment increases the initial rates of Ca^{2+} influx. Moreover, the data of Fig. 8 indicate that methoxamine administration yields an increased intramitochondrial free [Ca^{2+}] during Ca^{2+} recycling. On the basis of these considerations it is suggested that the adrenergic stimulation of Ca^{2+} uptake reflects a change in the kinetic properties of the uniporter rather than an increase in conjugate driving force. The apparent increase in Ca^{2+} affinity of the uniporter (Fig. 5) agrees with this interpretation.

A change in the manifest properties of the uniporter may arise either by an alteration in the uniporter itself, or from a changed concentration of a regulator (of the uniporter), the concentration of which is determined by an adrenaline-sensitive process. Further work is needed to discriminate between these two possibilities. In any event, data (Fig. 6) indicate an α-adrenergic mechanism as the basis of this phenomenon.

Taylor *et al.* (1980) showed recently that Ca^{2+} uptake by isolated liver mitochondria is promoted when the liver is pretreated with α-adrenergic agonists. However, in contrast with cardiac mitochondria (the present paper), the α-agonist used, phenylephrine, also increased the membrane potential in liver mitochondria oxidizing succinate. The extent to which the observed stimulation of Ca^{2+} influx into liver mitochondria was a consequence of the increased membrane potential cannot be estimated, since simultaneous measurements of Ca^{2+} influx and membrane potential were not performed.

Taylor *et al.* (1980) suggest that the adrenergic promotion of Ca^{2+} uptake by liver mitochondria may be responsible for the restoration of normal mitochondrial Ca^{2+} content after adrenergic stimulation. In the present study, the possibility has been explored that the α-adrenergic activation of the cardiac mitochondrial uniporter may contribute towards the stimulation by adrenaline of cardiac respiration, which accompanies the enhanced contractility (Williamson, 1964, 1966). Denton and his co-workers have published considerable evidence that the intramitochondrial enzymes α-oxoglutarate dehydrogenase, NAD^+-linked isocitrate dehydrogenase and pyruvate dehydrogenase phosphate phosphatase of rat heart are activated by free Ca^{2+} over the range 0.1–20μM (for review see Denton & McCormack, 1980). Yamada *et al.* (1980) have shown that about 1μM-Ca^{2+} greatly activates F_1-ATPase of skeletal muscle by promoting the

dissociation of the inhibitor protein, although the effect is reversed at higher $[Ca^{2+}]$. Intramitochondrial Ca^{2+}, therefore, may be a significant regulator of oxidative metabolism (Denton *et al.*, 1980). The control of intramitochondrial free Ca^{2+} in accordance with the regulatory requirements of intramitochondrial enzymes may be achieved by the Na^+/Ca^{2+} cycle of the inner membrane (Crompton, 1980; Denton *et al.*, 1980), and Denton *et al.* (1980) have proposed that the cycle may permit hormonal information to be relayed into the mitochondria. This concept is reinforced by the observation, reported here, that pretreatment with methoxamine displaces the Ca^{2+} activation curve of α-oxoglutarate dehydrogenase to lower extramitochondrial concentrations of Ca^{2+}, so that an activation by methoxamine occurs over the approximate range 10nM–500nM-Ca^{2+} (Fig. 8). This behaviour is predicted from the selective activation of the influx component of the Na^+/Ca^{2+} cycle by α-adrenergic agonists.

Figure 8 reveals that at about 100nM-Ca^{2+} the displacement of the activation curve is equivalent to a change in $[Ca^{2+}]$ of about 2.5-fold. If it is assumed that an approximately linear relationship exists between uniporter activity and $[Ca^{2+}]$ close to 100nM-Ca^{2+} (the K_m value of the uniporter for Ca^{2+} is 8–15μM; Crompton *et al.*, 1976*b*; Fig. 5), the curve-shift suggests the possibility that methoxamine causes an activation of about 2.5-fold in uniporter activity at 100nM-Ca^{2+}.

The use of α-oxoglutarate dehydrogenase as an index of the intramitochondrial free $[Ca^{2+}]$ revealed that relatively high extramitochondrial concentrations of buffered Ca^{2+} inhibit respiration severely (Fig. 7). Since relatively high concentrations of Ca^{2+} do not inhibit isolated α-oxoglutarate dehydrogenase (McCormack & Denton, 1979), it appears likely that Ca^{2+}-induced damage of the mitochondria may have occurred. In this connection, Nicholls & Crompton (1980) have discussed in detail the dependence of the mitochondrial capacity for net Ca^{2+} accumulation, i.e. the Ca^{2+}-buffering capacity, on the extramitochondrial $[Ca^{2+}]$. In essence (see Nicholls & Crompton, 1980; Nicholls & Scott, 1980) mitochondria behave as almost perfect buffers of extramitochondrial Ca^{2+} when this increases above a limiting concentration of about 1μM during steady-state Ca^{2+} recycling. However, the introduction of Ca^{2+} buffers may lead to excessive Ca^{2+} accumulation by the mitochondria, which attempt to restore the extramitochondrial free $[Ca^{2+}]$ to about 1μM. This interpretation is supported by other experiments (P. Kessar & M. Crompton, unpublished work), conducted with Ca^{2+}-EGTA buffers and 10mM-Na^+ under the same experimental conditions as in Fig. 7, which revealed considerable mitochondrial swelling with free $[Ca^{2+}]$ greater than 3μM and no detectable swelling with free $[Ca^{2+}]$ less than 1μM.

The suggested α-adrenergic activation of the uniporter provides a mechanism for the kinetic regulation of the intramitochondrial free Ca^{2+} via the Na^+/Ca^{2+} cycle. The possible value of kinetic control of the cycle in permitting Ca^{2+} distribution across the inner membrane to be changed without change in membrane potential has been discussed by Nicholls & Crompton (1980). Clearly kinetic control of the uniporter may also arise via changes in cytoplasmic free $[Ca^{2+}]$. In this case, the slow response of the cycle may permit the large-scale oscillations in sarcoplasmic $[Ca^{2+}]$ to be transmitted into a largely damped-out ripple of intramitochondrial $[Ca^{2+}]$, the mean value of which will be a function of the time-average sarcoplasmic $[Ca^{2+}]$ (Crompton, 1980; McCormack & Denton, 1981). In this connection, Hiraoka *et al.* (1980) and McCormack & Denton (1981) have shown that perfusion of rat hearts with β-adrenergic agonists increases the amount of active form of pyruvate dehydrogenase, and they provided evidence that this activation is mediated via an increased cytoplasmic $[Ca^{2+}]$; presumably, this is relayed into the mitochondria and activates pyruvate dehydrogenase phosphate phosphatase. The present studies lend some support to this interpretation to the extent that no permanent effects of β-agonists on the activities of the Ca^{2+}-uniporter and Na^+/Ca^{2+}-antiporter were detected.

In conclusion, the adrenaline-induced stimulation of cardiac respiration may be brought about, at least in part, by both α-adrenergic and β-adrenergic stimulation of Ca^{2+} influx into mitochondria and consequent increase in intramitochondrial free $[Ca^{2+}]$. The β-adrenergic component may lead to increased uniporter activity via activation of the slow inward Ca^{2+} current across the cardiac sarcolemma (Reuter & Scholz, 1977) and increased cytoplasmic Ca^{2+}. The α-adrenergic component may cause uniporter activation. Moreover, these two actions conceivably reinforce each other and, thereby, render the Na^+/Ca^{2+} cycle a sensitive locus for the control of oxidative metabolism.

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