Regulation of the mitochondrial matrix volume *in vivo* and *in vitro*

The role of calcium

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1. The ability of α -adrenergic agonists and vasopressin to increase the mitochondrial volume in hepatocytes is dependent on the presence of extracellular Ca^{2+} . 2. Addition of Ca^{2+} to hormone-treated cells incubated in the absence of Ca^{2+} initiates mitochondrial swelling. 3. In the presence of extracellular Ca^{2+} , A23187 ($7.5 \mu\text{M}$) induces mitochondrial swelling and stimulates gluconeogenesis from L-lactate. 4. Isolated liver mitochondria incubated in KCl medium in the presence of 2.5 mM-phosphate undergo energy-dependent swelling, which is associated with electrogenic K^+ uptake and reaches an equilibrium when the volume has increased to about $1.3\text{--}1.5 \mu\text{l}/\text{mg}$ of protein. This K^+ -dependent swelling is stimulated by the presence of $0.3\text{--}1.0 \mu\text{M}\text{-Ca}^{2+}$, leading to an increase in matrix volume at equilibrium that is dependent on $[\text{Ca}^{2+}]$. 5. Ca^{2+} -activated K^+ -dependent swelling requires phosphate and shows a strong preference for K^+ over Na^+ , Li^+ or choline. It is not associated with either uncoupling of mitochondria or any non-specific permeability changes and cannot be produced by Ba^{2+} , Mn^{2+} or Sr^{2+} . 6. Ca^{2+} -activated K^+ -dependent swelling is not prevented by any known inhibitors of plasma-membrane ion-transport systems, nor by inhibitors of mitochondrial phospholipase A_2 . Swelling is inhibited by 65% and 35% by 1 mM-ATP and 100 μM -quinine respectively. 7. The effect of Ca^{2+} is blocked by Ruthenium Red ($5 \mu\text{g}/\text{ml}$) at low $[\text{Ca}^{2+}]$. Spermine (0.25 mM) enhanced the swelling seen on addition of Ca^{2+} , correlating with its ability to increase Ca^{2+} uptake into the mitochondria as measured by using Arsenazo-III. 8. Mitochondria derived from rats treated with glucagon showed less swelling than did control mitochondria. In the presence of Ruthenium Red and higher $[\text{Ca}^{2+}]$, the mitochondria from hormone-treated animals showed greater swelling than did control mitochondria. 9. These data imply that an increase in intramitochondrial $[\text{Ca}^{2+}]$ can increase the electrogenic flux of K^+ into mitochondria by an unknown mechanism and thereby cause swelling. It is proposed that this is the mechanism by which α -agonists and vasopressin cause an increase in mitochondrial volume *in situ*.

INTRODUCTION

The matrix volume of mitochondria influences many aspects of their metabolism. Thus an increase in matrix volume causes a stimulation of the oxidation of all substrates entering the respiratory chain before ubiquinone, an increase in the rate of pyruvate metabolism, elevated ATP concentrations, stimulated uncoupler-dependent ATPase activity and an activation of glutaminase [see Armston *et al.* (1982) and references therein]. These parameters are affected in the same way by glucagon treatment of rats or hepatocytes (see Halestrap, 1981; Halestrap *et al.*, 1983). Furthermore, we have provided evidence that gluconeogenic hormones such as glucagon, α -adrenergic agonists and vasopressin increase the mitochondrial volume of the hepatocyte, with a time course and hormone-concentration dependency similar to their stimulation of gluconeogenesis (Quinlan *et al.*, 1983). Valinomycin (1 nM), which increases the matrix volume within the cell by acting as a K^+ ionophore in the mitochondrial inner membrane, also increases gluconeogenesis from L-lactate (Haynes *et al.*, 1974; Tolbert & Fain, 1974; Friedman & Dambach, 1980; Thomas & Halestrap, 1981; Quinlan *et al.*, 1983).

Data from the laboratories of Brierley and Garlid have identified a K^+/H^+ antiporter which pumps out K^+ from the mitochondria and an electrogenic uniporter which mediates K^+ entry into the mitochondria. It is thought

that the steady-state rate of these two transporters is responsible for the regulation of both the K^+ content and the matrix volume of the mitochondria (Brierley, 1976; Garlid, 1979, 1980; Dordick *et al.*, 1980; Brierley *et al.*, 1984). Garlid has proposed that the activity of the antiporter may be regulated by the intramitochondrial $[\text{Mg}^{2+}]$ and that this offers a mechanism of volume homeostasis for the mitochondria (Garlid, 1980; Dordick *et al.*, 1980; Nakashima *et al.*, 1982). In the present paper we investigate the mechanism by which hormones can perturb this homeostatic mechanism to cause an increase in the steady-state mitochondrial volume. Our data indicate that mitochondria respond to a rise in intramitochondrial $[\text{Ca}^{2+}]$ (such as may be induced by the action of vasopressin or α -adrenergic agonists) by an increase in electrogenic K^+ permeability. The consequent entry of K^+ and a compensating anion can account for the increase in matrix volume.

EXPERIMENTAL

Materials

Rats. For the preparation of liver mitochondria, fed female Wistar rats (approx. 250 g body wt., fed *ad libitum* on Breeding Diet from Oxoid, Basingstoke, Hants., U.K.) were used, whereas for the preparation of liver cells 24 h-starved male Wistar rats (approx. 350 g body wt.) were employed.

Chemicals. The sources of all chemicals, biochemicals and radiochemicals, except those listed below, were the same as those given by Quinlan *et al.* (1983). Arsenazo-III, furosemide, apamin, tetracaine, quinine, spermine and glucagon were obtained from Sigma Chemical Co., Poole, Dorset, U.K. Mepacrine hydrochloride was from ICN Pharmaceuticals, Plainview, NY, U.S.A., and trifluoperazine dihydrochloride was given by Smith, Kline and French Laboratories, Welwyn Garden City, Herts, U.K. Inositol 1,4,5-trisphosphate was generously given by Dr. R. F. Irvine, Department of Biochemistry, A.R.C. Institute of Animal Physiology, Babraham, Cambridge CB2 4AT, U.K.

Methods

Preparation of isolated liver cells and mitochondria. Liver mitochondria were prepared in buffer containing 300 mM-sucrose, 2 mM-EGTA and 10 mM-Tris/HCl, pH 7.4, as described previously (Halestrap, 1975, 1978). Liver cells were prepared and incubated as described by Quinlan *et al.* (1983).

Measurement of light-scattering. All light-scattering measurements were performed at 540 nm with a computerized home-built split-beam spectrophotometer as described previously (Quinlan *et al.* 1983). Hepatocytes were preincubated for 20 min at 6 mg of protein/ml in bicarbonate-buffered saline (Krebs & Henseleit, 1932) containing 10 mg of dialysed (but not defatted) bovine serum albumin/ml, 10 mM-L-lactate and 1 mM-pyruvate. Portions (3.5 ml) of cell suspension were added to both sample and reference cuvettes, which were maintained at 37 °C and constantly stirred in an atmosphere of O₂/CO₂ (19:1). CaCl₂ was omitted from the buffer in 'Ca²⁺-free' experiments and 0.2 mM-EGTA added to both cuvettes 2 min before addition of hormone to the sample cuvette. In mitochondrial experiments the incubation buffer contained 125 mM-KCl, 10 mM-Mops, 7 mM-Tris base, 0.5 mM-EGTA, 2.5 mM-potassium phosphate, 2.5 mM-MgCl₂, 2.5 mM-Tris succinate, 5 mM-sodium glutamate (K⁺ salt was used in Na⁺-free experiments) and 1 mM-potassium L-malate and was adjusted to pH 7.2 at 37 °C. In some experiments the KCl was replaced by other osmotic supports of equivalent osmolarity, as indicated in the appropriate Figures, and all other K⁺ was replaced by Na⁺. Mitochondria were added to a final concentration of about 3.5 mg of protein/ml and thoroughly mixed before 4 ml portions were transferred to the sample and reference cuvettes. The mitochondrial suspension was thoroughly stirred in an atmosphere of pure O₂ and maintained at 37 °C while the A₅₄₀ was continuously monitored. The atmosphere of pure O₂ was required to maintain adequate oxygenation at the prevailing rates of O₂ uptake and gas exchange across the surface of the cuvette, but produced no signs of O₂ poisoning. Further additions were made to sample and reference cuvettes as indicated.

Measurement of mitochondrial volumes. Incubation of mitochondria was performed in the spectrophotometer as outlined above, but the buffer contained in addition ³H₂O (1 µCi/ml) and [¹⁴C]sucrose (0.1 µCi/ml). After completion of the light-scattering determinations, four 0.9 ml samples were transferred from both the reference and sample cuvettes to 1.5 ml centrifuge tubes and centrifuged at 9000 g for 1 min to sediment the mitochondria. An

additional 0.9 ml sample from the original mitochondrial suspension was centrifuged in parallel for measurement of the protein content of the mitochondrial pellet. Assay of the ³H and ¹⁴C content of both the pellet and the supernatant was used to calculate the intramitochondrial volume (Armston *et al.*, 1982; Halestrap & Quinlan, 1983). In experiments where [¹⁴C]mannitol was used in place of the [¹⁴C]sucrose, 1 mM-mannitol was added to the medium and the [¹⁴C]mannitol was not added until the light-scattering observations were complete. This avoids the problems associated with slow leakage of mannitol into mitochondria, especially at higher temperatures (Halestrap & Quinlan, 1983; Whipps & Halestrap, 1984).

Measurement of extramitochondrial free Ca²⁺ concentrations. An ion-selective Ca²⁺ electrode was found to be unsatisfactory for measurements of [Ca²⁺] of less than about 0.5 µM in our experiments, since the presence of EGTA produced a substantial baseline drift in the signal. Thus Arsenazo-III (40–100 µM) was added to the buffer and the computerized spectrophotometer was converted into double-beam mode (670 nm–685 nm) for measuring the [Ca²⁺] from the change in absorbance (Scarpa, 1979). Calibration was performed by using buffer without added EGTA and titration with either standardized CaCl₂ or EGTA. A small (0.05 unit) pH fall occurred on addition of the CaCl₂ to buffer containing 0.5 mM-EGTA. Since the Arsenazo III signal is sensitive to pH (Scarpa, 1979), addition of excess EGTA at the end of the incubation (which produced a negligible pH change) was used to obtain a true zero [Ca²⁺] signal.

RESULTS AND DISCUSSION

Role of Ca²⁺ in the regulation of mitochondrial volume in hepatocytes

Previous work from this laboratory has demonstrated that the increase in mitochondrial volume induced by treatment of liver cells with vasopressin or phenylephrine is dependent on the presence of extracellular Ca²⁺ (Quinlan *et al.*, 1983). The effect of glucagon was much less sensitive to removal of extracellular Ca²⁺. In Fig. 1 we demonstrate that, if 2.5 mM-Ca²⁺ was added back to the incubation medium, those cells that had been exposed to either vasopressin (25 nM) or phenylephrine (20 µM) gave a substantial decrease in light-scattering consistent with an increase in mitochondrial volume. This effect was not seen after glucagon treatment. Comparison of traces (iii) and (v) shows that the swelling induced by adding back Ca²⁺ to cells treated with vasopressin was very similar to that produced by vasopressin treatment of cells in the presence of 2.5 mM-Ca²⁺. These data are consistent with a rise in cytoplasmic [Ca²⁺] being responsible for the increase in mitochondrial volume. Further evidence for this is provided by the swelling produced by the addition of 7.5 µM-A23187, a Ca²⁺ ionophore, as shown in trace (iv). At this concentration A23187 produced a 21.6 ± 2.9% increase in gluconeogenesis from L-lactate (10 mM). The control rate of gluconeogenesis (mean ± S.E.M. for six observations on different batches of cells) was 5.32 ± 0.40 nmol of glucose/min per mg of cell protein, rising to 6.44 ± 0.46 in the presence of A23187. In Ca²⁺-free medium A23187 caused a slight (7%) inhibition of gluconeogenesis and no light-scattering response.

Studies on the regulation of cytoplasmic [Ca²⁺] by

Fig. 1. Effect of Ca²⁺ on light-scattering by hepatocytes

Rat liver cells were incubated in both sample and reference cuvettes of a split-beam spectrophotometer as described in the Experimental section. In traces (i)–(iii) the incubation medium contained no added Ca²⁺, and 0.2 mM-EGTA was added at the start of the recording. The 2.5 mM-CaCl₂ was added to both cuvettes as indicated; addition artifacts caused a break in the trace, which has been filled with the broken line. Where indicated, 0.1 μ M-glucagon (Gluc), 20 μ M-phenylephrine (Phen), 25 nM-vasopressin (Vaso) or 7.5 μ M-A23187 was added to the sample cuvette. The traces shown are typical of at least three similar experiments.

vasopressin and phenylephrine using perfused livers and isolated hepatocytes have indicated that the hormones initially release Ca²⁺ from the endoplasmic reticulum in a manner that is independent of extracellular [Ca²⁺] and dependent on the production of inositol 1,4,5-trisphosphate (Blackmore *et al.*, 1982, 1983; Charest *et al.*, 1983; Thomas *et al.*, 1983, 1984; Berthon *et al.*, 1984; Burgess *et al.*, 1984; Joseph *et al.*, 1984; Kleineke & Soling, 1985). However, this rise in cytoplasmic [Ca²⁺] is only transient unless extracellular Ca²⁺ is present, since Ca²⁺ is rapidly lost from the cell (Blackmore *et al.*, 1982, 1984; Berthon *et al.*, 1984; Reinhart *et al.*, 1982, 1984b,c,d). Addition of Ca²⁺ back to vasopressin- or phenylephrine-treated hepatocytes in Ca²⁺-free medium causes a rapid rise in cytoplasmic [Ca²⁺], phosphorylase activation, O₂ consumption and ketone-body output, which is not seen in control cells but which can be induced by low concentrations of A23187 (DeWitt & Putney, 1983; Reinhart *et al.*, 1982, 1983, 1984b; Williamson *et al.*, 1985). It is believed that vasopressin and phenylephrine, in addition to causing release of Ca²⁺ from the endoplasmic reticulum, exert an effect on the plasma-membrane Ca²⁺-transporting mechanisms (Blackmore

et al., 1984; Mauger *et al.*, 1984; Prpic *et al.*, 1984; Reinhart *et al.*, 1984b). The light-scattering responses shown in Fig. 1 show a pattern entirely consistent with these known effects of hormones on calcium homeostasis and suggest that, for significant swelling of the mitochondria to occur, the rise in cytoplasmic [Ca²⁺] must be maintained by the presence of extracellular Ca²⁺. In its absence only a very small and transient light-scattering response is seen (Fig. 1). Furthermore the synergistic effects of glucagon and phenylephrine on the increase in mitochondrial volume seen in hepatocytes (Quinlan *et al.*, 1983) are associated with synergistic effects on Ca²⁺ fluxes (Morgan *et al.*, 1983, 1984; McCormack, 1985b).

Regulation of mitochondrial volume by [Ca²⁺] in isolated liver mitochondria

We have confirmed (results not shown) that, when mitochondria are injected into KCl medium supplemented with respiratory substrates, they swell substantially in an

Fig. 2. Effects of various additions on the Ca²⁺- and valinomycin-dependent swelling of energized liver mitochondria in KCl medium

Mitochondria were incubated in KCl medium in both cuvettes of a split-beam spectrophotometer. The medium composition was as given in the Experimental section, except that for trace (v), where phosphate was omitted. Antimycin (1 μ g/ml) was present throughout the experiment recorded in trace (iv). Where indicated 0.43 mM-Ca²⁺, 1 nM-valinomycin (VAL) or 100 μ M-quinine was added to the sample cuvette only. For traces (i) and (ii) mitochondrial volumes were measured for the mitochondria from both cuvettes as detailed in the Experimental section. The ³H₂O-[¹⁴C]sucrose space (expressed as μ l/mg of protein; mean $\pm$ S.E.M. for four replicates) for the reference cuvettes was 1.347 ± 0.004 and 1.375 ± 0.012 for traces (i) and (ii) respectively and the corresponding experimental cuvettes gave values of 1.466 ± 0.008 and 1.522 ± 0.008 respectively. Parallel determinations of the matrix volume with [¹⁴C]mannitol gave values of 0.726 ± 0.017 and 0.668 ± 0.016 for the reference cuvettes and 0.876 ± 0.030 and 0.762 ± 0.017 for the sample cuvettes. Mean values from several experiments are given in the text.

energy-dependent manner as a result of the electrogenic uptake of K^+ (see Webster & Bronk, 1978). This swelling reaches completion in 6–8 min at 37 °C when the matrix volume (3H_2O – $[^{14}C]$ sucrose) is 1.3–1.5 μ l/mg (see below). However, further swelling of 10–20% can be induced by the addition of low concentrations of either valinomycin or Ca^{2+} , as shown in Fig. 2. Here the changes in A_{540} associated with the addition of either 1 nM-valinomycin or submicromolar Ca^{2+} to the sample cuvette of a split-beam spectrophotometer are shown in parallel with measurement of the matrix volume with 3H_2O and either $[^{14}C]$ sucrose or $[^{14}C]$ mannitol (Halestrap & Quinlan, 1983). The presence of the reference cuvette corrects for any light-scattering changes that are independent of the effector added. The concentrations of mitochondria, valinomycin and Ca^{2+} used were designed to mimic as closely as possible the effects of hormones and valinomycin on the mitochondrial light-scattering seen in intact hepatocytes (Quinlan *et al.*, 1983). The Ca^{2+} was added as small volumes of 250 mM- $CaCl_2$ to the incubation medium (which contains 0.5 mM-EGTA) to produce the required $[Ca^{2+}]$. This was measured in parallel experiments using Arsenazo-III, as considered below (Fig. 4). The rationale behind the approach was to mimic the hormonally induced release of Ca^{2+} from internal stores into a buffered cytoplasm as closely as possible. The concentrations of Ca^{2+} used were normally such that the final $[Ca^{2+}]$ was below that at which extramitochondrial Ca^{2+} is buffered (Nicholls, 1978; Fig. 4) and insufficient to cause uncoupling of the mitochondria or a decrease in the NAD(P)H fluorescence (Quinlan & Halestrap, 1986). The mean increase ($\pm$ S.E.M. for the number of mitochondrial preparations shown) in mitochondrial matrix volume (3H_2O – $[^{14}C]$ sucrose space) was $12.7 \pm 2.3\%$ ($n = 4$) with 1 nM-valinomycin and $10.4 \pm 2.1\%$ ($n = 6$) with Ca^{2+} (0.43 mM total concn.) from a control value of $1.262 \pm 0.050 \mu$ l/mg of protein. Both these changes and the magnitude of the light-scattering changes are similar to those seen after valinomycin, glucagon or phenylephrine treatment of hepatocytes (Quinlan *et al.*, 1983). The Ca^{2+} -induced swelling was energy-dependent, being abolished by the presence of antimycin (Fig. 2, trace iv) and, unlike that caused by valinomycin, was dependent on the presence of phosphate (Fig. 2, trace v). We have been unable to elicit swelling with addition of Sr^{2+} , Ba^{2+} or Mn^{2+} at concentrations well in excess of the $[Ca^{2+}]$ required to exert its effect (results not shown).

Fig. 3 shows the effects of adding increasing concentrations of Ca^{2+} to energized mitochondria in media containing different osmotic supports. Each was present at 250 mosM, and all other additions were the same as for the KCl medium. In parallel oxygen-electrode studies it was shown that in all the media tested mitochondria remained well coupled for at least 10 min; no swelling occurs in sucrose or mannitol media at any of the $[Ca^{2+}]$ used, and very little response is observed in choline chloride medium. A limited response is seen in both the NaCl and LiCl media at the higher concentrations of Ca^{2+} , whereas RbCl medium gives a similar response to KCl medium. The simplest interpretation of the data of Fig. 3(a) is that Ca^{2+} , like valinomycin (Pressman, 1976), induces an electrogenic entry of K^+ or Rb^+ in preference to Na^+ and Li^+ . The data of Fig. 3(b) support this conclusion. Addition of 20 mM- K^+ to mitochondria incubated in sucrose media produced swelling as

Fig. 3. Effects of Ca^{2+} on the swelling of energized mitochondria incubated in media of different ionic composition

The experimental technique was essentially the same as that described for Fig. 2. In (a) the osmotic support of the medium was varied by replacing the 125 mM-KCl with 125 mM of the alternative ionic osmotic support or 250 mM-sucrose or -mannitol as indicated. All media contained 0.5 mM-EGTA. Ca^{2+} was sequentially added to the sample cuvette, to the final total concentration shown. In (b) sucrose medium was used and 20 mM-KCl added to the sample cuvette as indicated; for trace (i) 0.43 mM- Ca^{2+} was added before the KCl.

expected, and this was considerably enhanced by prior treatment with Ca^{2+} at a concentration that was without effect on swelling in the sucrose medium without added K^+ . Parallel experiments using a K^+ electrode showed a small loss of K^+ from the medium under these conditions, consistent with a stimulation of K^+ entry into the mitochondria (results not shown). However, our data do not preclude a significant effect of Ca^{2+} on the entry of Na^+ and Li^+ which is counterbalanced by a very active efflux mechanism for these cations. At equilibrium in an ionic medium, the matrix volume reached reflects the net uptake of the cation as determined by the activities of its electrogenic influx and protogenic efflux (Brierley, 1976; Garlid, 1980). It is known that there are both influx and efflux pathways for K^+ , Na^+ , Li^+ and choline (Mitchell & Moyle, 1969; Douglas & Cockerell, 1974; Rosen & Futai, 1980). Indeed, the Na^+/H^+ antiport is extremely active (Mitchell & Moyle, 1969; Douglas & Cockerell, 1974), and prevents energy-dependent swelling in NaCl media. Thus the smaller magnitude of Ca^{2+} -induced swelling in Na^+ medium could be a result of rapid removal from the mitochondria of the Na^+ entering as a result of Ca^{2+} treatment.

Influence of $[Ca^{2+}]$ and effectors of mitochondrial Ca^{2+} transport on Ca^{2+} -induced swelling

The data in Figs. 4 and 5 show the concentration-dependence on Ca^{2+} of mitochondrial swelling, and

Fig. 4. Effects of Ruthenium Red on the Ca²⁺-sensitive K⁺-dependent swelling and extramitochondrial [Ca²⁺] of energized liver mitochondria

In (a) mitochondrial swelling was monitored as described in Fig. 2 in the presence (trace i) or absence (trace ii) of Ruthenium Red (RR; 5 $\mu g/ml$). Sequential additions of Ca²⁺ were made to the sample cuvette at the concentrations (mM) indicated, followed by 1.0 mM-EGTA and 20 mM-NaCl. Note that the media all contained 0.5 mM-EGTA and 5 mM-sodium glutamate. In (b) the same mitochondria were incubated under conditions identical with those in (a), but the buffer was supplemented with 40 μM -Arsenazo-III. Measurements of [Ca²⁺] were made with a double-beam spectrophotometer recording absorbance changes at 670–685 nm. Calibration of the response with known [Ca²⁺] was performed in the absence of mitochondria and EGTA as described in the Experimental section. Note that addition of 0.25 mM-spermine produced an addition artefact and not a rise in [Ca²⁺].

whether the Ca²⁺ acts from the outside of the mitochondrial inner membrane or from within the matrix. In Fig. 4(a) light-scattering responses at various concentrations of added Ca²⁺ were studied in the presence and absence of 5 μg of Ruthenium Red/ml to inhibit Ca²⁺ entry into the mitochondria via the electrogenic uniporter. Arsenazo-III was used in parallel experiments to determine the extramitochondrial [Ca²⁺] under identical conditions (Fig. 4b). The addition of 0.36 mM- and 0.43 mM-Ca²⁺ produced a [Ca²⁺] of about 0.3 and 0.8 μM respectively and gave significant light-scattering changes. The submicromolar concentrations of Ca²⁺ are difficult to measure with accuracy, because the small pH changes (< 0.1) occurring on binding of Ca²⁺ by EGTA cause perturbation of the Arsenazo-III signal (Scarpa, 1979). Nevertheless it is clear that significant swelling occurs at a [Ca²⁺] below that at which the mitochondria buffer the external Ca²⁺ (see Nicholls, 1978). These conditions were reached on addition of 0.5 mM-Ca²⁺ in the experiments shown in Fig. 4(a). If Ruthenium Red (5 $\mu g/ml$) was added to inhibit Ca²⁺ entry, a very much higher [Ca²⁺] was required to produce swelling (trace i). These data imply that Ca²⁺ must enter the mitochondria for swelling to occur. This conclusion is further supported

by the effects of adding 0.25 mM-spermine to increase the rate of Ca²⁺ entry into the mitochondria (Nicchitta & Williamson, 1984) and 20 mM-Na⁺ to increase the efflux of Ca²⁺ from the mitochondria (Nicholls & Akerman, 1982; Nedergaard, 1984; McCormack, 1985b). Some additional uptake of Ca²⁺ from the medium is seen after spermine addition, as indicated in Fig. 4(b). Under these conditions additional mitochondrial swelling was observed, as demonstrated in Fig. 5. In experiments on three different batches of mitochondria, 0.25 mM-spermine decreased the mean ($\pm$ S.E.M.) mitochondrial 'set point' for Ca²⁺ from 0.87 ± 0.04 to 0.46 ± 0.08 μM while the magnitude of the Ca²⁺-induced light-scattering change increased by $117 \pm 17\%$. Spermine had no effect on the light-scattering in the absence of Ca²⁺. An effect of adding 20 mM-NaCl to activate the efflux pathway was sometimes but not always apparent on the swelling response, as shown in Fig. 5. The Na⁺/Ca²⁺ antiporter of liver mitochondria is hormonally regulated (Goldstone *et al.*, 1983) and thus the variability in the effect of Na⁺ may be a result of the hormonal status of the rats used. However, the partial reversal of the light-scattering change that occurred on removal of Ca²⁺ with excess EGTA was enhanced by the presence of Na⁺. Under

Fig. 5. Effects of spermine and Na^+ on mitochondrial swelling and Ca^{2+} fluxes

Light-scattering by energized mitochondria incubated in KCl medium in the presence of 1 mM-MgATP was measured as described in the legend to Fig. 2. Sodium glutamate was replaced by potassium glutamate in all incubations, and the medium was supplemented with 20 mM-NaCl in (ii) and (iii) and with 0.25 mM-spermine in (iii). Further additions of 0.25 mM-spermine, 20 mM-NaCl and 1 mM-EGTA were made to the sample cuvette where indicated.

these conditions Ca^{2+} efflux from the mitochondria was largely Na^+ -dependent (results not shown), in agreement with others (Nedergaard, 1984; McCormack, 1985b).

Effects of various agents on Ca^{2+} -dependent swelling

Various potential effectors of the Ca^{2+} -induced K^+ swelling were investigated, and the following were found to be without appreciable effect; 250 μM -mepacrine, 500 μM -tetracaine and 60 μM -trifluoperazine, all of which have been shown to inhibit the mitochondrial phospholipase A_2 responsible for the massive swelling and damage seen with large uptake of Ca^{2+} (Halestrap, 1981; Broekemeier *et al.*, 1985), and 0.1 μM -apamin, 200 μM -furosemide and 5 mM-tetraethylammonium chloride, which inhibit a variety of plasma-membrane K^+ -transport mechanisms (Palfrey & Greengard, 1981; Schwarz & Passow, 1983; Seager *et al.*, 1984). Neomycin (200 μM), a polyaminoglycoside which inhibits phospholipase C (Downes & Michell, 1981), was without appreciable effect except under conditions of high extramitochondrial $[\text{Ca}^{2+}]$ in the presence of Ruthenium Red, as shown in Fig. 6. On seven different preparations of mitochondria, the mean ($\pm$ S.E.M.) inhibition of the initial rate of swelling was $64.8 \pm 6.6\%$. Addition of 1 mM-MgATP (or 1 mM-MgADP; results not shown) inhibited the Ca^{2+} -induced swelling seen in the presence or absence of Ruthenium Red, as shown in Fig. 6. In the absence of Ruthenium Red

Fig. 6. Effects of MgATP and neomycin on the Ca^{2+} -activated mitochondrial swelling in the presence and the absence of Ruthenium Red

Mitochondrial light-scattering was measured as described in Fig. 2 in the presence (continuous lines) or absence (broken lines) of Ruthenium Red (RR; 5 $\mu\text{g}/\text{ml}$); 1 mM-MgATP was present (in both reference and sample cuvettes) only in the experiments recorded in traces (i) and (iv). Neomycin (100 μM) was added to both cuvettes in the experiment recorded in trace (ii).

the extent of inhibition was $67.4 \pm 6.8\%$ ($n = 3$) and in its presence $64.5 \pm 7.1\%$ ($n = 9$). It should be noted that the normal experimental technique included the presence of MgATP in order to protect the mitochondria from massive Ca^{2+} -induced swelling and damage. Addition of 100 μM -quinine produced some swelling in its own right (Fig. 2, trace iii), as would be expected from its ability to inhibit the K^+/H^+ antiporter (Nakashima & Garlid, 1982; Jung *et al.*, 1984), but it also inhibited the ability of Ca^{2+} to cause swelling. In experiments on four different preparations of mitochondria, a $33.6 \pm 7.0\%$ inhibition of the initial rate of swelling was observed. No such effect of quinine on the swelling induced by higher $[\text{Ca}^{2+}]$ in the presence of Ruthenium Red was observed. There are reports that quinine may have additional inhibitory effects on the electrogenic K^+ -uniporter activity (Nakashima & Garlid, 1982; Jung & Brierley, 1984), and this may indicate that the Ca^{2+} -activated K^+ influx occurs by the same channel. This concentration of quinine would be expected to inhibit almost totally the Ca^{2+} -activated K^+ pump of the red blood cell (Reichstein & Rothstein, 1981). In some experiments (results not shown), the effect of removing most of the plasma-membrane and microsomal contamination from the mitochondria by Percoll-density-gradient centrifugation (Armstrong & Halestrap, 1984) was studied. No differences were observed between these mitochondria and those usually used, demonstrating that products liberated from

contaminating membranes by Ca²⁺ were not responsible for the swelling. Addition of 10 μ M-inositol 1,4,5-trisphosphate, which releases Ca²⁺ from the endoplasmic reticulum (Joseph *et al.*, 1984), produced no mitochondrial swelling.

Effects of hormone treatment of animals in the Ca²⁺-induced K⁺-dependent swelling of liver mitochondria

The data of Table 1 show the effects of hormone treatment of rats on the rate of Ca²⁺-induced swelling of subsequently isolated liver mitochondria. Under conditions where Ca²⁺ entry into the mitochondria is permitted, the mitochondria from glucagon-treated animals show a consistent decrease in both the extent and initial rate of Ca²⁺-induced swelling, of $54 \pm 5.5\%$ and $59 \pm 4.7\%$ respectively. However in the presence of Ruthenium Red and higher [Ca²⁺], the glucagon-treated mitochondria swell more rapidly ($80.1 \pm 10.9\%$ faster) and to a greater extent ($58.9 \pm 10.4\%$) than the control mitochondria. Mitochondria from adrenaline- and phenylephrine-treated animals show a similar effect. The difference between the hormone effect seen in the presence or absence of Ruthenium Red may be related to the different effects of neomycin under both conditions. Two explanations for these contrasting effects may be offered: either the effects of Ca²⁺ in the presence of Ruthenium Red are exerted at an extramitochondrial locus distinct from the intramitochondrial locus for Ca²⁺ action seen in the absence of Ruthenium Red, or in the presence of Ruthenium Red Ca²⁺ enters the mitochondria by a pathway usually used for efflux. This reversal of the efflux pathway would have to be activated by hormone treatment and inhibited by neomycin. We have no evidence to support either of these possibilities, although activation of the Na⁺-dependent Ca²⁺-efflux mechanism by β -agonists, but not α -agonists, has been reported (Goldstone *et al.*, 1983).

Table 1. Effects of hormone treatment of rats on the Ca²⁺-activated K⁺-dependent swelling of isolated liver mitochondria

Swelling was measured in the presence or absence of Ruthenium Red (5 μ g/ml) as described in the legend to Fig. 6. The swelling was initiated by addition of 0.43 mM- or 0.57 mM-CaCl₂ in the presence or absence of 1 mM-ATPMg or Ruthenium Red respectively. The initial extramitochondrial [Ca²⁺] were 0.8 μ M and 7.5 μ M respectively. Hormone treatment of animals was carried out as described in the Experimental section. Values are given as means $\pm$ S.E.M. for the numbers of different mitochondrial preparations shown in parentheses. All values for mitochondria from hormonally treated animals are significantly different from control ($P < 0.01$ by paired Student's *t* test): n.d., not determined.

Hormone treatment	Initial rates of swelling (ΔA_{540} /min per g of protein)	
	Ruthenium Red + 0.5 mM-CaCl ₂	1 mM-ATPMg + 0.43 mM-CaCl ₂
Control	4.48 ± 0.39 (18)	16.02 ± 3.02 (10)
Glucagon	7.59 ± 0.60 (18)	7.35 ± 1.93 (10)
Adrenaline	7.80 ± 1.40 (10)	n.d.
Phenylephrine	7.72 ± 1.50 (10)	n.d.

Conclusions

The data of Fig. 1 clearly indicate that vasopressin and α -agonists exert their effects on the mitochondrial volume through a Ca²⁺-dependent mechanism. Furthermore, for the effect to be significant and sustained, Ca²⁺ must be present in the extracellular medium. As discussed above, this is also necessary to maintain the hormonal elevation of cytoplasmic [Ca²⁺]. The effect of glucagon on the mitochondrial volume is much less dependent on the presence of extracellular Ca²⁺ and is considerably slower in onset (Quinlan *et al.*, 1983). Measurements of the mitochondrial Ca²⁺ content after treatment of isolated hepatocytes or perfused livers with α -agonists or vasopressin have yielded conflicting results (see Reinhart *et al.*, 1984d). However, the most recent measurements, where care has been taken to avoid contamination of the mitochondrial fraction with either extraneous Ca²⁺ or non-mitochondrial membrane vesicles, show a rise in mitochondrial Ca²⁺ content after hormone treatment (Reinhart *et al.*, 1984a; Shears & Kirk, 1984; Kleineke & Soling, 1985; McCormack, 1985b). The increase in mitochondrial Ca²⁺ content is particularly pronounced when glucagon and an α -agonist are added together (Morgan *et al.*, 1983, 1984; McCormack, 1985b); it is under these conditions that maximal swelling of mitochondria is seen in the hepatocytes (Quinlan *et al.*, 1983). Hormonally induced increases in matrix [Ca²⁺] are also supported by less direct evidence, involving the activity of intramitochondrial Ca²⁺-sensitive enzymes (Oviasu & Whitton, 1984; McCormack, 1985a,b; Staddon & McGivan, 1985).

Isolated mitochondria can be made to swell in KCl medium when the extramitochondrial [Ca²⁺] is elevated. Swelling of mitochondria of the same order as is detected in hepatocytes treated with hormones (Quinlan *et al.*, 1983) can be seen at 0.2–1.0 μ M-Ca²⁺, which is similar to the measured rise in cytoplasmic [Ca²⁺] after hormone treatment (Blackmore *et al.*, 1982; Charest *et al.*, 1983; Thomas *et al.*, 1984). Significant swelling is detected below the concentration of Ca²⁺ at which the efflux pathway for Ca²⁺ is saturated and the mitochondria buffer the extramitochondrial [Ca²⁺]. The effect of Ca²⁺ is intramitochondrial, dependent on the mitochondria being energized, and involves stimulation of the electrogenic entry of K⁺ into the mitochondria, followed by entry of a compensating anion. Phosphate is essential for the swelling to occur at physiological [Ca²⁺], and appears to have a function other than as a compensating anion, since valinomycin-induced swelling is independent of phosphate under these conditions. Mitochondria remain totally coupled during the swelling, and the effect is quite distinct from the massive swelling that occurs on overloading mitochondria with Ca²⁺ in the presence of phosphate, when a general increase in permeability is seen (see Beatrice *et al.*, 1980; Armston *et al.*, 1982). However, it is of interest that the presence of adenine nucleotides and pretreatment with glucagon inhibits both types of swelling, which suggests some feature(s) common to both phenomena.

Mitochondria are known to possess an electrogenic K⁺-influx pathway and a separate protogenic efflux mechanism (see the Introduction section). The former has a K_m of 6–12 mM and is activated by thiol reagents, phosphate and high pH (Jung *et al.*, 1977; Chang & Diwan, 1982). In addition, it shows activation by

atractylate and adenine-nucleotide depletion, and inhibition by ADP, suggesting some association with the ATP/ADP translocate (Jung *et al.*, 1977; Panov *et al.*, 1980; Jung & Brierley, 1981, 1984). This is of particular interest in view of the effects of ATP and ADP to inhibit the Ca^{2+} -induced swelling reported here and the well-documented effect of hormone treatment to increase the adenine nucleotide content of mitochondria (Bryla *et al.*, 1977; Siess *et al.*, 1977; Titheradge & Haynes, 1980; Aprille *et al.*, 1982). Thus our data are consistent with vasopressin and α -agonists stimulating the electrogenic influx of K^+ into mitochondria via a Ca^{2+} -dependent stimulation (either direct or indirect) of the K^+ uniporter. If this is in some way associated with the adenine nucleotide transporter, then glucagon could exert its Ca^{2+} -independent effect by increasing cytoplasmic fatty acyl-CoA (Christiansen, 1977), which is known to bind to the adenine nucleotide transporter at the same site as atractylate (Vignais, 1976) and to induce mitochondrial swelling at concentrations below those exerting detergent-like properties (Harris *et al.*, 1979; Beatrice *et al.*, 1980).

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REFERENCES

- Aprille, J. R., Nosek, M. T. & Brennan, W. A. (1982) *Biochem. Biophys. Res. Commun.* **108**, 834–839
- Armstrong, A. E. & Halestrap, A. P. (1984) *Biosci. Rep.* **4**, 903–908
- Armstrong, A. E., Halestrap, A. P. & Scott, R. D. (1982) *Biochim. Biophys. Acta* **681**, 429–439
- Beatrice, M. C., Palmer, J. W. & Pfeiffer, D. R. (1980) *J. Biol. Chem.* **255**, 8663–8671
- Berthon, B., Binat, A., Mauger, J.-P. & Claret, M. (1984) *FEBS Lett.* **167**, 19–24
- Blackmore, P. F., Hughes, B. P., Shuman, E. A. & Exton, J. H. (1982) *J. Biol. Chem.* **257**, 190–197
- Blackmore, P. F., Hughes, B. P., Charest, R., Shuman, E. A. & Exton, J. H. (1983) *J. Biol. Chem.* **258**, 10488–10494
- Blackmore, P. F., Waynick, L. E., Blackman, G. E., Graham, C. W. & Sherry, R. S. (1984) *J. Biol. Chem.* **259**, 12322–12325
- Brierley, G. P. (1976) *Mol. Cell. Biochem.* **10**, 41–62
- Brierley, G. P., Jurkowitz, M. S., Farooqui, T. & Jung, D. W. (1984) *J. Biol. Chem.* **259**, 14672–14678
- Broekemeier, K. M., Schmid, P. C., Schmid, H. H. O. & Pfeiffer, D. R. (1985) *J. Biol. Chem.* **260**, 105–113
- Bryla, J., Harris, E. J. & Plumb, J. A. (1977) *FEBS Lett.* **80**, 443–448
- Burgess, G. M., Irvine, R. F., Berridge, M. J., McKinney, J. S. & Putney, J. W. (1984) *Biochem. J.* **224**, 741–746
- Chang, H.-S. & Diwan, J. J. (1982) *Biochim. Biophys. Acta* **681**, 220–223
- Charest, R., Blackmore, P. F., Berthon, B. & Exton, J. H. (1983) *J. Biol. Chem.* **258**, 8769–8773
- Christiansen, R. Z. (1977) *Biochim. Biophys. Acta* **488**, 249–262
- DeWitt, L. M. & Putney, J. W. (1983) *FEBS Lett.* **160**, 259–263
- Dordick, R. S., Brierley, G. P. & Garlid, K. D. (1980) *J. Biol. Chem.* **255**, 10299–10303
- Douglas, M. G. & Cockerell, R. S. (1974) *J. Biol. Chem.* **249**, 5464–5471
- Downes, C. P. & Michell, R. H. (1981) *Biochem. J.* **198**, 133–140
- Friedman, N. & Dambach, G. (1980) *Biochim. Biophys. Acta* **596**, 180–185
- Garlid, K. D. (1979) *Biochem. Biophys. Res. Commun.* **87**, 842–849
- Garlid, K. D. (1980) *J. Biol. Chem.* **255**, 11273–11279
- Goldstone, T. P., Duddridge, R. J. & Crompton, M. (1983) *Biochem. J.* **210**, 463–472
- Halestrap, A. P. (1975) *Biochem. J.* **148**, 85–96
- Halestrap, A. P. (1978) *Biochem. J.* **172**, 389–398
- Halestrap, A. P. (1981) in *Short-Term Regulation of Liver Metabolism* (Hue, L. & Van de Werve, G., eds.), pp. 389–409, Elsevier/North-Holland, Amsterdam
- Halestrap, A. P. & Quinlan, P. T. (1983) *Biochem. J.* **214**, 387–393
- Halestrap, A. P., Armston, A. E., Quinlan, P. T. & Whipps, D. E. (1983) *Horm. Cell Regul.* **7**, 185–200
- Harris, E. J., Al-Shakhaly, M. & Baum, H. (1979) *Biochem. J.* **182**, 455–464
- Haynes, R. C., Garrison, J. & Yamazaki, R. K. (1974) *Mol. Pharmacol.* **10**, 381–388
- Joseph, S. K., Thomas, A. P., Williams, R. J., Irvine, R. F. & Williamson, J. R. (1984) *J. Biol. Chem.* **259**, 3077–3081
- Jung, D. W. & Brierley, G. P. (1981) *J. Biol. Chem.* **256**, 10490–10496
- Jung, D. W. & Brierley, G. P. (1984) *J. Biol. Chem.* **259**, 6904–6911
- Jung, D. W., Chavez, E. & Brierley, G. P. (1977) *Arch. Biochem. Biophys.* **183**, 452–459
- Jung, D. W., Farooqui, T., Utz, E. & Brierley, G. P. (1984) *J. Bioenerg. Biomembr.* **16**, 379–387
- Kleineke, J. & Soling, H. D. (1985) *J. Biol. Chem.* **260**, 1040–1045
- Krebs, H. A. & Henseleit, K. (1932) *Hoppe-Seyler's Z. Physiol. Chem.* **210**, 33–66
- Mauger, J.-P., Poggioli, J., Guesdon, F. & Claret, M. (1984) *Biochem. J.* **221**, 121–127
- McCormack, J. G. (1985a) *FEBS Lett.* **180**, 259–264
- McCormack, J. G. (1985b) *Biochem. J.* **231**, 597–608
- Mitchell, P. & Moyle, J. (1969) *Eur. J. Biochem.* **9**, 149–156
- Morgan, N. G., Blackmore, P. F. & Exton, J. H. (1983) *J. Biol. Chem.* **258**, 5110–5116
- Morgan, N. G., Charest, R., Blackmore, P. F. & Exton, J. H. (1984) *Proc. Natl. Acad. Sci. U.S.A.* **81**, 4208–4212
- Nakashima, R. A. & Garlid, K. D. (1982) *J. Biol. Chem.* **257**, 9252–9254
- Nakashima, R. A., Dordick, R. S. & Garlid, K. D. (1982) *J. Biol. Chem.* **257**, 12540–12545
- Nedergaard, J. (1984) *Eur. J. Biochem.* **144**, 159–168
- Nicchitta, C. V. & Williamson, J. R. (1984) *J. Biol. Chem.* **259**, 12978–12983
- Nicholls, D. G. (1978) *Biochem. J.* **176**, 463–474
- Nicholls, D. G. & Akerman, K. E. O. (1982) *Biochim. Biophys. Acta* **683**, 57–88
- Oviasu, O. A. & Whitton, P. D. (1984) *Biochem. J.* **224**, 181–186
- Palfrey, H. C. & Greengard, P. (1981) *Ann. N.Y. Acad. Sci.* **372**, 291–307
- Panov, A., Filippova, S. & Lyakhovich, V. (1980) *Arch. Biochem. Biophys.* **199**, 420–426
- Pressman, B. C. (1976) *Annu. Rev. Biochem.* **45**, 501–530
- Prpic, V., Green, K. C., Blackmore, P. F. & Exton, J. H. (1984) *J. Biol. Chem.* **259**, 1382–1385
- Quinlan, P. T. & Halestrap, A. P. (1986) *Biochem. J.* **236**, 789–800
- Quinlan, P. T., Thomas, A. P., Armstrong, A. E. & Halestrap, A. P. (1983) *Biochem. J.* **214**, 395–404
- Reichstein, E. & Rothstein, A. (1981) *J. Membr. Biol.* **59**, 57–63
- Reinhart, P. H., Taylor, W. M. & Bygrave, F. L. (1982) *Biochem. J.* **208**, 619–630
- Reinhart, P. H., Taylor, W. M. & Bygrave, F. L. (1983) *Biochem. J.* **214**, 405–412
- Reinhart, P. H., Van De Pol, E., Taylor, W. M. & Bygrave, F. L. (1984a) *Biochem. J.* **218**, 415–420
- Reinhart, P. H., Taylor, W. M. & Bygrave, F. L. (1984b) *Biochem. J.* **220**, 35–42
- Reinhart, P. H., Taylor, W. M. & Bygrave, F. L. (1984c) *Biochem. J.* **220**, 43–50

- Reinhart, P. H., Taylor, W. M. & Bygrave, F. L. (1984d) *Biochem. J.* **223**, 1–13
- Rosen, B. P. & Futai, M. (1980) *FEBS Lett.* **117**, 39–43
- Scarpa, A. (1979) *Methods Enzymol.* **56**, 301–352
- Schwarz, W. & Passow, H. (1983) *Annu. Rev. Physiol.* **45**, 359–374
- Seager, M. J., Granier, C. & Couraud, F. (1984) *J. Biol. Chem.* **259**, 1491–1495
- Shears, S. B. & Kirk, C. J. (1984) *Biochem. J.* **219**, 383–389
- Siess, E. A., Brocks, G., Lattke, H. K. & Wieland, O. H. (1977) *Biochem. J.* **166**, 225–235
- Staddon, J. M. & McGivan, J. D. (1985) *Biochem. J.* **225**, 327–333
- Thomas, A. P. & Halestrap, A. P. (1981) *Biochem. J.* **198**, 551–564
- Thomas, A. P., Marks, J. S., Coll, K. E. & Williamson, J. R. (1983) *J. Biol. Chem.* **258**, 5716–5725
- Thomas, A. P., Alexander, J. & Williamson, J. R. (1984) *J. Biol. Chem.* **259**, 5574–5584
- Titheradge, M. A. & Haynes, R. C. (1980) *J. Biol. Chem.* **255**, 1471–1477
- Tolbert, M. E. M. & Fain, J. N. (1974) *J. Biol. Chem.* **249**, 1162–1166
- Vignais, P. V. (1976) *Biochim. Biophys. Acta* **456**, 1–38
- Webster, K. A. & Bronk, J. R. (1978) *J. Bioenerg. Biomembr.* **10**, 23–44
- Whipps, D. E. & Halestrap, A. P. (1984) *Biochem. J.* **221**, 147–152
- Williamson, J. R., Cooper, R. H., Joseph, S. K. & Thomas, A. P. (1985) *Am. J. Physiol.* **248**, C203–C216

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