Sub-micromolar concentrations of extramitochondrial Ca^{2+} stimulate the rate of citrulline synthesis by rat liver mitochondria

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1. In the presence of physiological concentrations of Na^+ and Mg^{2+} , the rate of citrulline synthesis by isolated rat liver mitochondria respiring on a range of substrates was stimulated by up to 60% when the extramitochondrial Ca^{2+} concentration was raised from 130 pM to 770 nM. 2. Our findings suggest that hormonal stimulation of the urea cycle may be mediated by Ca^{2+} .

INTRODUCTION

The liver is the main organ in which urea formation occurs (Meijer & Hensgens, 1982). The urea cycle is shared between the cytosol and mitochondria (Cohen & Hayano, 1948). Charles *et al.* (1967) demonstrated that isolated liver mitochondria can synthesize citrulline, an intermediate of the urea cycle, when supplied with ornithine, HCO_3^- , NH_4^+ and an oxidizable substrate. Carbamoyl phosphate is formed within the mitochondrial matrix from NH_4^+ , HCO_3^- and 2ATP by carbamoyl-phosphate synthase (ammonia). Ornithine enters mitochondria via electroneutral exchange with citrulline plus H^+ (McGivan *et al.*, 1977). Ornithine transcarbamoylase catalyses formation of citrulline from ornithine and carbamoyl phosphate. Citrulline exits from the mitochondria in order to continue the urea cycle.

The action of glucagon, catecholamines, vasopressin and angiotensin II on perfused liver (Miller, 1960; Mallette *et al.*, 1969; Williamson *et al.*, 1969) and on isolated liver cells (Hensgens *et al.*, 1980; Titheradge & Haynes, 1980; Corvera & Garcia-Sainz, 1982; Drew *et al.*, 1985) results in increased rates of citrulline and urea synthesis. In response to these hormones, the phosphorylation potential of the mitochondrial matrix may increase (Siess *et al.*, 1977; Bryla *et al.*, 1977). Mitochondria isolated from liver cells exposed to glucagon, α -adrenergic agonists, angiotensin II or vasopressin display an increase in citrulline production relative to controls (Yamazaki & Graetz, 1977; Bryla *et al.*, 1977; Hensgens *et al.*, 1980; Titheradge & Haynes, 1980; Rabier *et al.*, 1982; Corvera & Garcia-Sainz, 1982), leading Bryla *et al.* (1977) and Wanders *et al.* (1981) to suggest that the rate of citrulline synthesis by isolated mitochondria is correlated with intramitochondrial ATP or ATP/ADP ratio (but see Rajjman & Bartulis, 1979).

These hormones also give rise to an increase in cytoplasmic Ca^{2+} concentration from a basal value of 100 nM up to 600 nM (Murphy *et al.*, 1980; Charest *et al.*, 1983; Berthon *et al.*, 1984). Denton & McCormack (1980, 1985) and Hansford (1985) have proposed that the raised cytoplasmic Ca^{2+} concentration in response to hormonal stimuli leads to a raised Ca^{2+} concentration in the mitochondrial matrix, and that this leads to an activation of Ca^{2+} -sensitive dehydrogenases found within the mitochondrial matrix, thereby stimulating

mitochondrial metabolism independently of the matrix phosphorylation potential. Mitochondria isolated from hormonally treated animals contain more total Ca^{2+} than do controls (Assimakopoulos-Jeannet *et al.*, 1986), yet the rate of citrulline production by mitochondria isolated from untreated animals has been reported to decrease when these are exposed to Ca^{2+} , and to increase in the presence of EGTA (Meijer *et al.*, 1981; Lof *et al.*, 1983). In addition, carbamoyl-phosphate synthase (ammonia) is known to be inhibited by Ca^{2+} (Meijer *et al.*, 1981; Lof *et al.*, 1983; Cerdan *et al.*, 1984).

Meijer *et al.* (1981) employed mitochondria with > 7 nmol of Ca^{2+} /mg of protein. Somlyo *et al.* (1985) have reported that mitochondria *in vivo* may contain only about 1 nmol of Ca^{2+} /mg of protein. Using a technique which we have previously reported to produce a mitochondrial preparation with a low Ca^{2+} content (Johnston & Brand, 1986, 1987), we re-address the question: what effect does an increase in $[\text{Ca}^{2+}]_o$ within the physiological range have on the production rate of citrulline by isolated liver mitochondria?

EXPERIMENTAL

Mitochondria were prepared as described by Johnston & Brand (1986, 1987), except that mannitol was used as osmotic support in place of sucrose.

Mitochondrial protein was determined by a modified biuret method (Gornall *et al.*, 1949) with bovine serum albumin as standard.

Mitochondrial total Ca^{2+} content was measured with a Varian SPECTRAA-40 atomic absorption spectrophotometer (with kind permission of Dr. P. Fletcher and Dr. M. Keall, Schlumberger Research, Cambridge) after mitochondrial Ca^{2+} had been extracted with 5% (w/v) trichloroacetic acid/1.5% (w/v) LaCl_3 (final concns.) and the denatured protein removed by centrifugation. Mitochondrial preparations contained 0.84 ± 0.07 nmol of Ca^{2+} /mg of protein (mean $\pm$ S.E.M. for 12 preparations).

The basic incubation medium for mitochondrial citrulline production contained 50 mM-KCl, 25 mM-mannitol, 20 mM-Hepes, 10 mM-NTA, 20 mM- KHCO_3 , 10 mM- NH_4Cl , 2 mM- KH_2PO_4 , 4 mM- MgCl_2 (free $[\text{Mg}^{2+}] = 0.34$ mM), 10 mM-NaCl and 10 mM-ornithine monohydro-

chloride, at 37 °C, adjusted to pH 7.25 with KOH. This basic medium contained 1.2 μM total endogenous Ca^{2+} , determined by atomic absorption spectrophotometry; 60 mM-KCl was used in media without 10 mM-NaCl. Media not containing MgCl_2 had a total endogenous Ca^{2+} content of 0.5 μM . CaCl_2 was added from 10 mM stock solution to give the free $[\text{Ca}^{2+}]$ required. The medium was placed in an open-top reaction chamber kept at constant temperature by a water jacket connected to a thermostatically controlled water bath. The medium was gassed with O_2/CO_2 (19:1) for 5 min before mitochondria were added. Mitochondria were incubated at 0.5 mg of mitochondrial protein/ml in a final volume of 5 ml. Throughout the procedure the medium was stirred with a magnetic flea, O_2/CO_2 (19:1) was blown over the surface and pH was monitored. Endogenous rates of citrulline production were about 8 nmol/min per mg of protein; this varied from incubation to incubation, and was not noticeably affected by Ca^{2+} . Substrate was added after 5 min, and 1 ml samples were removed at 0, 1, 2 and 3 min after addition of substrate. These were added to 0.3 ml of 10% trichloroacetic acid and the denatured protein was removed by bench centrifugation. The citrulline content of the supernatant was measured by the method of Archibald (1944).

Apparent stability constants at pH 7.25 for Ca-NTA ($9.52 \times 10^3 \text{ M}^{-1}$), Ca-EGTA ($9.56 \times 10^6 \text{ M}^{-1}$), Mg-NTA ($1.69 \times 10^3 \text{ M}^{-1}$) and Mg-EGTA (82 M^{-1}) were calculated from the absolute stability constants (Sillen & Martell, 1964). Free Ca^{2+} and free Mg^{2+} concentrations were calculated by using programs 1 and 2 of Fabiato & Fabiato (1979), which were adapted to be run under Pascal/VS, an IBM-derived compiler and run-time system available under the Phoenix/MVS mainframe operating system at Cambridge University. Endogenous Ca^{2+} was measured and taken into account when calculating free Ca^{2+} concentrations.

Mannitol, Hepes, NTA, standard CaCl_2 , MgCl_2 , KH_2PO_4 , KHCO_3 , NH_4Cl , L-citrulline, L-ornithine monohydrochloride, diacetylmonoxime, glutamic acid, H_3PO_4 , H_2SO_4 , trichloroacetic acid and LaCl_3 were obtained from BDH, Dagenham, Essex, U.K. EGTA, rotenone, Ruthenium Red and sodium arsenite were obtained from Sigma Chemical Co., Poole, Dorset, U.K. KOH, KCl, NaCl and succinic acid were obtained from Fisons, Loughborough, Leics., U.K. L-Malic acid was obtained from Koch-Light, Colnbrook, Bucks., U.K. O_2/CO_2 (19:1) was from British Oxygen Company, Guildford, Surrey, U.K.

Student's *t* test for matched pairs was used to assess the statistical significance of an increase in citrulline synthesis with respect to citrulline synthesis at 130 pM- Ca^{2+} . For clarity, significance is shown only for experiments performed with both Na^+ and Mg^{2+} present.

RESULTS AND DISCUSSION

The aim of this work was to investigate the effect of an increase in $[\text{Ca}^{2+}]_o$ within the physiological range on the rate of citrulline synthesis by isolated mitochondria.

Figs. 1 and 2 demonstrate that the rate of citrulline synthesis by mitochondria is a function of $[\text{Ca}^{2+}]_o$ with 10 mM-succinate and 10 mM-glutamate/1 mM-malate as oxidizable substrates respectively. Citrulline synthesis was linear with time and protein concentration. In the presence of Na^+ and Mg^{2+} the rate of citrulline synthesis

Fig. 1. Effect of $[\text{Ca}^{2+}]_o$ on the rate of citrulline synthesis by isolated mitochondria using succinate as oxidizable substrate

Mitochondria were incubated as described in the Experimental section: 5 nmol of rotenone/mg of protein was added concomitantly with 10 mM-succinate. Citrulline was measured at 0, 1, 2 and 3 min after addition of substrate. Results are means $\pm$ S.E.M. for four different preparations: ●, 10 mM- Na^+ and 0.34 mM- Mg^{2+} free present; ■, no Na^+ or Mg^{2+} present; ▼, 10 mM- Na^+ present; ▲, 0.34 mM- Mg^{2+} free present; white symbols, as black symbols, but also 1 mM-EGTA present. Total $[\text{Ca}^{2+}]$ of media, with free $[\text{Ca}^{2+}]$ in parentheses, are: (a) Mg^{2+} -containing media: 1.2 μM plus 1 mM-EGTA (130 pM), 17 μM plus 1 mM-EGTA (1.75 nM), 47 μM plus 1 mM-EGTA (5.3 nM), 1.2 μM (20 nM), 17 μM (277 nM), 32 μM (525 nM), 47 μM (770 nM), 62 μM (1015 nM), 142 μM (2345 nM); (b) no Mg^{2+} in media: 0.5 μM plus 1 mM-EGTA (50 pM), 0.5 μM (5 nM), 25 μM (261 nM) and 72.5 μM (759 nM). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.005$.

increases as $[\text{Ca}^{2+}]_o$ increases from 1.75 nM- to 277 nM- Ca^{2+} for succinate and to 770 nM- Ca^{2+} for glutamate/malate. At higher $[\text{Ca}^{2+}]_o$ citrulline synthesis declines, which presumably reflects Ca^{2+} -induced derangement of mitochondrial function (Nicholls & Akerman, 1982).

When total $[\text{Ca}^{2+}]_o$ is increased in the presence of 1 mM-EGTA, which chelates Ca^{2+} and maintains a low free $[\text{Ca}^{2+}]_o$, an increase in citrulline synthesis was not observed, suggesting that free Ca^{2+} , not total Ca^{2+} , is the operative agent.

Movement of Ca^{2+} across the inner membrane of mitochondria maintaining a high transmembrane potential involves separate entry and exit pathways. Uptake is via an electrogenic uniporter and is inhibited by Mg^{2+} (Crompton, 1985). Efflux occurs on electroneutral $2\text{H}^+/\text{Ca}^{2+}$ and $2\text{Na}^+/\text{Ca}^{2+}$ antiporters (Goldstone & Crompton, 1982; Brand, 1985). Thus, at a given $[\text{Ca}^{2+}]_o$, Mg^{2+} lowers intramitochondrial free $[\text{Ca}^{2+}]$ by inhibiting Ca^{2+} uptake, and Na^+ lowers it by promoting Ca^{2+} efflux. In the absence of Na^+ and Mg^{2+} , stimulation and/or decline of citrulline synthesis is observed at $[\text{Ca}^{2+}]_o$ in the lower nanomolar range, whereas the presence of either Na^+ or Mg^{2+} results in stimulation and/or decline of citrulline

Fig. 2. Effect of $[\text{Ca}^{2+}]_0$ on the rate of citrulline synthesis by isolated mitochondria using glutamate/malate as oxidizable substrates

Mitochondria were incubated as described in the Experimental section. Citrulline was measured at 0, 1, 2 and 3 min after addition of 10 mM-glutamate plus 1 mM-malate. Results are means $\pm$ S.E.M. for four different preparations. Symbols are as in Fig. 1. Total $[\text{Ca}^{2+}]_0$ in media are as given for Fig. 1.

production at $[\text{Ca}^{2+}]_0$ intermediate to those observed in the presence of both Na^+ and Mg^{2+} .

An increase in the rate of citrulline synthesis in response to an increase in $[\text{Ca}^{2+}]_0$ was also found with 10 mM-malate (23 nmol of citrulline/min per mg of protein at 130 pM- Ca^{2+}_0 , 36 nmol of citrulline/min per mg of protein at 770 nM- Ca^{2+}_0), 10 mM-2-oxoglutarate/1 mM-malate (40 nmol of citrulline/min per mg of protein at 130 pM- Ca^{2+}_0 , 52 nmol of citrulline/min per mg of protein at 770 nM- Ca^{2+}_0), and 10 mM-pyruvate/1 mM-malate (26 nmol of citrulline/min per mg of protein at 130 pM- Ca^{2+}_0 , 31 nmol of citrulline/min per mg of protein at 770 nM- Ca^{2+}_0). A similar pattern of response to omission of Na^+ and/or Mg^{2+} from the medium was also found with these substrates. Citrulline synthesis by coupled mitochondria was not supported by exogenous ATP alone (results not shown).

The pattern of response of citrulline production to Na^+ and Mg^{2+} , in conjunction with the prevention of the stimulation of citrulline synthesis by EGTA, is consistent with a rise in the rate of citrulline synthesis secondary to an increase in $[\text{Ca}^{2+}]$ of the mitochondrial matrix. Ruthenium Red, an inhibitor of mitochondrial Ca^{2+} uptake (Moore, 1971), interfered with the colour produced by the assay for citrulline and could not be used. Partial uncoupling of mitochondria results in a decline in citrulline synthesis (Williamson *et al.*, 1981), suggesting that stimulation of citrulline production in response to Ca^{2+} is not due to partial uncoupling. Meijer *et al.* (1981) observed an inhibition of citrulline synthesis by isolated mitochondria caused by Ca^{2+} (using succinate as oxidizable substrate), but they performed their experiments in the absence of Na^+ and Mg^{2+} . Under similar conditions to those of Meijer *et al.* (1981), we too observed an

inhibition of citrulline synthesis (Fig. 1). In the presence of 1 mM-arsenite, an inhibitor of 2-oxoglutarate dehydrogenase, citrulline synthesis driven by oxidation of 10 mM-glutamate/1 mM-malate was about 20 nmol/min per mg of protein, and sub-micromolar Ca^{2+}_0 did not stimulate citrulline production (results not shown).

The only intramitochondrial enzymes known to be stimulated by sub-micromolar Ca^{2+}_0 are pyruvate dehydrogenase via its phosphatase and kinase (Denton *et al.*, 1972; Cooper *et al.*, 1974; McCormack, 1985), 2-oxoglutarate dehydrogenase (Brenner-Holzach & Raaflaub, 1956; McCormack & Denton, 1979; McCormack, 1985), NAD-linked *threo*-(D₂)-isocitrate dehydrogenase (Denton *et al.*, 1978; McCormack, 1985) and branched-chain 2-oxoacid dehydrogenase in a manner analogous to that of pyruvate dehydrogenase (Patel & Olson, 1982; Paxton & Harris, 1982). The effects on citrulline synthesis reported here are unlikely to be secondary to stimulation of these enzymes by an increase in matrix $[\text{Ca}^{2+}]$ with concomitant increase in matrix [ATP], since stimulation of citrulline synthesis was observed with succinate as substrate in the presence of rotenone, under which circumstances there should not be any flux through the known Ca^{2+} -sensitive intramitochondrial dehydrogenases, and respiration is not stimulated (Johnston & Brand, 1987) (but see Moreno-Sanchez, 1985). In addition, we have used saturating concentrations of oxidizable substrates, whereas demonstration of the Ca^{2+} -sensitivity of the intramitochondrial dehydrogenases requires sub-saturating concentrations of substrate (Denton & McCormack, 1980, 1985; Hansford, 1985). The mechanism by which Ca^{2+} may stimulate citrulline synthesis has yet to be elucidated. One possible mechanism is that stimulation occurs via alterations in mitochondrial volume (Armstrong *et al.*, 1982).

We conclude that in the presence of physiological concentrations of Na^+ and Mg^{2+} the rate of citrulline synthesis by isolated liver mitochondria may be stimulated by $[\text{Ca}^{2+}]_0$ in the physiological range. This is consistent with the hormonal stimulation of citrulline and urea production by liver cells being associated with a rise in the cytoplasmic concentration of free calcium and a rise in the free calcium concentration of the mitochondrial matrix. The stimulation of citrulline synthesis by Ca^{2+} may be an important element in the hormonal stimulation of the urea cycle.

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