Stimulation of calcium-ion efflux from liver mitochondria by sodium ions and its response to ADP and energy state

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The Ruthenium Red-insensitive efflux of Ca^{2+} from previously loaded rat liver mitochondria was studied as a function of the added Na^+ concentration and ADP present. Stimulation of Ca^{2+} efflux is sigmoidally dependent on the Na^+ concentration; maximal stimulation of efflux was observed with 12–15 mM-NaCl. Na^+ -stimulated Ca^{2+} efflux from liver mitochondria is about one-tenth that from cardiac mitochondria. No synergistic effect of K^+ on the Na^+ -stimulated efflux was found. The alkali-metal cations other than Na^+ did not stimulate efflux and did not prevent stimulation by Na^+ . In the absence of Na^+ , Ca^{2+} efflux was diminished by added ADP, but the Na^+ -stimulated efflux was made correspondingly greater as ADP concentration was increased to 16 μM . The Na^+ -stimulated Ca^{2+} efflux was inhibited by 70% by oligomycin and was not observed in the presence of antimycin. It is suggested that failure to observe Na^+ -stimulation of Ca^{2+} efflux from liver mitochondria by some investigators is attributable to a high basal efflux existing before addition of the Na^+ salt.

It has been shown that an additional efflux of Ca^{2+} is obtainable from Ca^{2+} -loaded liver mitochondria on addition of NaCl in the presence of ATP and Mg^{2+} in a sucrose-based efflux medium (Haworth *et al.*, 1980). The results sharply contrast with the reported absence of effect of NaCl on Ca^{2+} efflux from liver mitochondria (Crompton *et al.*, 1977, 1978) in a KCl-based efflux medium. Haworth *et al.* (1980) suggested that the reason why Na^+ -stimulated Ca^{2+} efflux from liver mitochondria was not previously observed might be that there was a rapid rate of Ruthenium Red-insensitive Ca^{2+} efflux even in the absence of Na^+ . They reported that addition of ATP and MgCl_2 to the medium minimized this efflux in their system. Previously, ADP has been shown to be more effective than ATP as a means of decreasing the basal Ca^{2+} efflux from cardiac mitochondria (Harris, 1979). Therefore we considered that ADP would probably be a more effective agent for demonstrating Na^+ -stimulated efflux of Ca^{2+} from liver mitochondria. The possibility was also considered that the Na^+ -stimulated efflux of Ca^{2+} from liver mitochondria might be inhibited in KCl-based media used by Crompton *et al.* (1977, 1978) compared with the sucrose-based medium used by Haworth *et al.* (1980).

We have studied the effect of ADP on Ca^{2+} efflux from liver mitochondria and compared some properties of the Na^+ -stimulated Ca^{2+} efflux with those of cardiac mitochondria reported before (Harris, 1979; Harris *et al.*, 1979).

Methods

Rat liver mitochondria were prepared as described by Harris *et al.* (1971), with 0.1% bovine serum albumin in the isolation media. The mitochondria were suspended finally in 0.3 M-mannitol/10 mM-Tris/HCl buffer, pH 7.4, at a concentration of 50 mg/ml; 2 mg of defatted and deionized albumin was added per ml of suspension. The experiments were conducted by following changes in free Ca^{2+} in KCl- or mannitol-based media containing 0.4 mg of mitochondrial protein/ml and 100 μM -Arsenazo III (Vallieres *et al.*, 1975). The media contained either 120 mM-KCl or 300 mM-mannitol with 20 mM-Tris/Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid], pH 7.4, as buffer, 0.02% bovine serum albumin, 1.2 mM-pyruvate, 1.2 mM-malate and 0.6 mM- P_i as the respective Tris salts. The mitochondria were loaded with 20–50 nmol of Ca^{2+} /mg of protein, and the near-completion of the process was monitored spectroscopically at the wavelength pair 685 nm–665 nm with an Aminco DW-2 dual-wavelength spectrophotometer at 20°C. Some 95–100% of the Ca^{2+} load was accumulated within

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1 min, at which time 2 nmol of Ruthenium Red was added to inhibit the Ca^{2+} -uptake carrier. The rate of Ca^{2+} release was then recorded at the desired sensitivity and was modified by small additions of ADP. Next, one or more additions of NaCl were made and the effects were recorded. Calibrations of the sensitivity of the system to free Ca^{2+} were made frequently during each run. In some experiments LiCl, KCl, RbCl, CsCl and inhibitors such as oligomycin, rotenone and antimycin A were added as noted in the text. The experiments were made at 20°C.

Results

The stimulation of release of Ca^{2+} from liver mitochondria in response to additions of NaCl is illustrated in Fig. 1 for both mannitol- and KCl-based media. To lessen the Ca^{2+} efflux before the addition of NaCl, ADP (to 32 μM) was added. The relative stimulation was slightly more in mannitol medium than in 120 mM-KCl medium. With eight different preparations the ratio between efflux after addition of NaCl (to 10 mM) to that before was always greater than 2:1 and by adding ADP could be raised to about 3:1. Other experiments made with varied additions of KCl (to 2–30 mM) to the

mannitol medium failed to reveal any stimulatory effect. From a series of experiments the relation between Ca^{2+} efflux and added NaCl concentration was obtained (Fig. 2). The stimulation is sigmoidal with respect to concentration and maximal stimulation of Ca^{2+} efflux occurred at 12–15 mM-NaCl with different mitochondrial preparations. Significant stimulation could be seen with as little as 2 mM-NaCl. Similar results were obtained with Ca^{2+} loads of 20 and 50 nmol/mg of protein.

With increasing prior loading with Ca^{2+} , more ADP was required to lessen the basal efflux of Ca^{2+} to a given value (Fig. 3). In the condition of low efflux, the additional efflux obtained with NaCl was not proportionately increased with the Ca^{2+} load (Fig. 3). With our mitochondrial preparations 16 μM -ADP was the optimal concentration for demonstration of Na^{+} -stimulated Ca^{2+} efflux from liver mitochondria. The lessening of the basal efflux by ADP could be evaluated by making serial additions and was consistently observed, though the sensitivity varied.

Additions of the salts LiCl, NaCl, KCl, RbCl and CsCl at concentrations of 10 mM were made to test their respective effects on the Ruthenium Red-insensitive Ca^{2+} efflux. Of the salts tested, only NaCl significantly stimulated Ca^{2+} efflux from liver mito-

Fig. 1. Stimulation of Ca^{2+} efflux by Na^{+}

To the mitochondrial suspension having 1 mg of protein/2.5 ml of medium with 100 μM -Arsenazo III, a portion of 10 mM- CaCl_2 (to give 16 μM) was added; the changes of absorbance $\Delta(A_{685} - A_{665})$ in response to 40 nmol of Ca^{2+} and its subsequent removal from the medium by the mitochondria are shown at the left. When the absorbance had returned to near the pre- Ca^{2+} value, additions of Ruthenium Red (RR) to 0.8 μM and ADP to 32 μM were made. Inhibition of the Ca^{2+} influx by the Ruthenium Red leaves the efflux operative, and its rate is noted on the trace. Sufficient NaCl to provide 10 mM concentration was then added, and the new rate was measured. In the experiments calibrations of the sensitivity were interposed at intervals but have been omitted for clarity. Records of two experiments are shown: curve (a) was obtained in mannitol medium and curve (b) in the KCl medium. Compositions of the media are given in the Methods section. The numbers on the curves are efflux in nmol/min per mg of protein.

Fig. 2. Dependence on $[\text{NaCl}]$ of the Ca^{2+} efflux from mitochondria previously loaded with 40 nmol of Ca^{2+} /mg of protein measured after addition of Ruthenium Red ($0.8 \mu\text{M}$) and ADP ($16 \mu\text{M}$)

The rates of Ca^{2+} release were recorded as in Fig. 1, with due attention to the changes of sensitivity of the measuring system as the free $[\text{Ca}^{2+}]$ altered. A correction was applied to allow for depletion of the internal Ca^{2+} . The efflux was measured in the mannitol medium. The endogenous Ca^{2+} content was 9 nmol/mg of protein.

chondria. NaCl was also found to be effective if it followed a prior 10 mM addition of one of the other salts.

The question whether and to what extent the stimulation of Ca^{2+} efflux by NaCl is energy-dependent was found to be difficult to answer unequivocally. This is because endogenous sources of energy suffice to leave the process operative; for example, in the presence of rotenone liver mitochondria still take up as much as 100 nmol of Ca^{2+} /mg of protein, albeit at a diminished rate, and the efflux measured in the presence of Ruthenium Red is still stimulated by NaCl . To remove effectively the response to NaCl by liver mitochondria, it was necessary to use antimycin, perhaps because of endogenous flavin-linked substrates (Fig. 4). The Na^+ -stimulated Ca^{2+} efflux was inhibited by 70% without altering the basal efflux in the presence of $5 \mu\text{g}$ of oligomycin/mg of protein.

The effect of temperature on Ca^{2+} efflux (Heffron & Harris, 1981) may be summarized as follows: increasing the temperature from 20°C to 30°C or 37°C increases the basal efflux more than the Na^+ -stimulated efflux. Hence higher temperature simulates use of a higher Ca^{2+} load without additional ADP, and Na^+ -stimulation of efflux appears less obvious as the temperature is elevated. In agreement with other reports, the addition of

Fig. 3. Dependence on $[\text{ADP}]$ of the net Na^+ -stimulated and basal Ruthenium Red-insensitive Ca^{2+} efflux from mitochondria loaded with 20 nmol or with 50 nmol of Ca^{2+} /mg of protein

To stimulate the efflux, measured in the mannitol medium, NaCl (to 20 mM) was added. The other conditions were similar to those used in the mannitol experiment of Fig. 1. Curves: (a) 20 nmol of Ca^{2+} /mg of protein, basal efflux; (b) 50 nmol of Ca^{2+} /mg of protein, basal efflux; (c) 20 nmol of Ca^{2+} /mg of protein, Na^+ -stimulated efflux; (d) 50 nmol of Ca^{2+} /mg of protein, Na^+ -stimulated efflux. The total efflux values for each experiment are obtained respectively by summing values from (a) and (c) for the one and from (b) and (d) for the other.

uncoupling agent led to prompt release of the Ca^{2+} whether or not Ruthenium Red had been added.

Discussion

Our results show that Na^+ -stimulated Ca^{2+} efflux from isolated liver mitochondria can be readily demonstrated, particularly when low concentrations of ADP are added to minimize the basal Ruthenium Red-insensitive Ca^{2+} efflux. This latter efflux was similar in magnitude to the efflux from cardiac mitochondria under similar conditions; however, the Na^+ -stimulated Ca^{2+} efflux from liver mitochondria is only about 10% of the efflux from cardiac mitochondria. The efflux in response to 20 mM- NaCl observed here at 20°C is slightly greater than that

Fig. 4. Abolition of Na^+ -stimulation of Ca^{2+} efflux by antimycin

The mitochondria were loaded with 20 nmol of Ca^{2+} /mg of protein, and then Ruthenium Red (RR) ($0.8 \mu\text{M}$) and ADP ($8 \mu\text{M}$) were added in the mannitol medium. After observation of the basal efflux, either (a) antimycin (Am) at $2 \mu\text{g}/\text{mg}$ of protein was added and later followed by NaCl to 10 mM or (b) NaCl to 10 mM was added. The values of efflux in nmol/min per mg of protein are marked on the curves. The protein concentration was $0.6 \text{ mg}/\text{ml}$.

observed by Haworth *et al.* (1980) at 30°C by using the ^{45}Ca method for measuring Ca^{2+} efflux. These workers used ATP and MgCl_2 to inhibit maximally the basal Ruthenium Red-insensitive efflux, but did not specifically give release rates. In the present work, ADP was added to minimize the basal Ca^{2+} efflux, since previous studies had shown that it was more effective than ATP in lessening basal Ca^{2+} efflux from cardiac mitochondria (Harris, 1979). There does not appear to be any effect of ionic strength on Na^+ -stimulated Ca^{2+} efflux, as indicated by the similarity of response to Na^+ in the KCl- and mannitol-based media. Unlike the situation in cardiac- (Crompton *et al.*, 1980) and skeletal-muscle (J. J. A. Heffron & E. J. Harris, unpublished work) mitochondria, K^+ did not potentiate the Na^+ -stimulated Ca^{2+} efflux from liver mitochondria. The sensitivity of the Na^+ -stimulated Ca^{2+} -efflux system of liver mitochondria to the Na^+ concentration is similar to that reported for cardiac mitochondria (Harris, 1979). A significant increment of Ca^{2+} efflux was obtained with 1–2 mM- NaCl , indicating the probable physiological importance of the process

in liver-cell function, since cytoplasmic Na^+ concentration is 5–6 mM (Lee & Fozzard, 1975). Maximal Ca^{2+} efflux is obtained with about 15 mM- NaCl , in agreement with Haworth *et al.* (1980). The efflux system is responsive to Na^+ and not to any of its congeners.

The difficulty met in demonstrating the energy requirement for Na^+ stimulation of Ca^{2+} efflux from liver mitochondria is likely to stem from their high content of endogenous substrates, which appear to be fatty acids (Bryła *et al.*, 1967). As with cardiac mitochondria, the Na^+ -stimulated Ca^{2+} efflux from liver mitochondria requires energization.

It is not immediately clear to us why others have been unable to show Na^+ -stimulated Ca^{2+} efflux from liver mitochondria. The most likely possibility is that the basal Ca^{2+} efflux was very high in other studies, rendering it difficult to measure the rather small Na^+ -stimulated Ca^{2+} efflux (compared with heart). This suggestion is indeed made more likely by the report by Dawson & Fulton (1980), which showed that up to 50% of the accumulated Ca^{2+} is released from liver mitochondria within 3 min of

addition of Ruthenium Red. This basal Ca^{2+} efflux is very high when compared with our data in Figs. 1 and 3. Similar elevated Ca^{2+} efflux from liver mitochondria after Ruthenium Red addition was observed by Crompton *et al.* (1978). It is also likely that the method of monitoring the release of Ca^{2+} plays a role in detecting small fluxes, for instance, Ca^{2+} -specific electrodes are sluggish in response compared with direct spectroscopic monitoring of free Ca^{2+} with Arsenazo III.

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