PHOSPHATE-DEPENDENT, TRIFLUOPERAZINE-SENSITIVE Ca^{2+} EFFLUX FROM RAT LIVER MITOCHONDRIA

Modulation by a cytosol factor

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1. Introduction

Mitochondria possess separate pathways for the uptake and release of Ca^{2+}: an electrophoretic uniporter for Ca^{2+} influx, and an independent electroneutral efflux mechanism operational when a high membrane potential is retained (reviews [1,2]). Ca^{2+} efflux from heart and brain mitochondria appears to be directly coupled to Na^+ entry [3,4]; in liver mitochondria, however, the mechanism(s) which regulates the release (or retention) of Ca^{2+} is unclear. The role of P_i in this process is uncertain as, under diverse experimental conditions, P_i has been reported to stimulate [5], inhibit [6] or efflux in parallel with Ca^{2+} efflux [7].

These apparently conflicting reports regarding P_i prompted us to re-evaluate its effect on Ca^{2+} efflux in liver mitochondria. In our hands, P_i stimulated mitochondrial Ca^{2+} efflux. We have discovered a heat-stable factor in liver cytosol which significantly enhanced P_i-dependent Ca^{2+} efflux. This enhanced Ca^{2+} efflux was effectively blocked by the phenothiazine antipsychotic drug, TFP. These results suggest involvement of a cytosol factor in the modulation of Ca^{2+} release from rat liver mitochondria.

2. Materials and methods

Arsenazo III (sodium salt grade 1), rotenone, Hepes and ruthenium red were obtained from Sigma. Ruthenium red was further purified according to [8]. Calmodulin was obtained from Calbiochem-Behring Corp., and trifluoperazine was a gift from Smith Kline and French (Philadelphia PA).

Sprague-Dawley rats (175–250 g) were used without fasting. Unless noted otherwise, all procedures were carried out at 4°C. Livers were homogenized as in [9] in 70 mM sucrose, 220 mM mannitol containing 10 mM Hepes (pH 7.4) (SMH). The mitochondrial fraction was isolated by differential centrifugation [9] and washed twice by resuspension in SMH and centrifugation at 95 000 × *g*. The cytosol was obtained by centrifugation of the post-mitochondrial supernate at 100 000 × *g* for 1 h.

Ca^{2+} movement was determined spectrophotometrically at 675–685 nm [10] with an Aminco DW2a using arsenazo III. The basic incubation medium contained: 250 mM sucrose; 7.3 mM mannitol; 10 mM Hepes (pH 7.4); 3 μM rotenone; 16.7 mM succinate; and 58 μM arsenazo III. Additions to the basic incubation medium are noted in section 3; the final volume was 3.0 ml. Ca^{2+} efflux was determined in the presence of 1.6 μM ruthenium red. Here, Ca^{2+} efflux refers to the ruthenium red-insensitive Ca^{2+} efflux. Membrane potential was determined using the fluorescent voltage-dependent dye, diS-C_3-(5), as described for liver mitochondria [11]. Swelling was monitored by following the change in absorbance at 540 nm. Protein was determined by a modification of the Folin phenol method [12]. The data were fitted to the Hill equation using a modification (J. W. L., unpublished) of the method in [13].

Abbreviations: P_i, inorganic phosphate; TFP, trifluoperazine; diS-C_3-(5); 3,3'-dipropylthiodicarbocyanine iodide; CDR, calcium-dependent regulatory protein

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3. Results

3.1. *Characterization of mitochondrial integrity and Ca^{2+} movement*

Ca^{2+} was taken up rapidly and retained (net flux equal to zero) by respiring mitochondria for ⩾5 min, the time frame over which all other measurements were made (fig.1A). Addition of ruthenium red to block uptake after Ca^{2+} had been accumulated resulted in a steady efflux of Ca^{2+} (fig.1B). This was presumed to represent the electroneutral Ca^{2+} efflux pathway which is operative at high membrane potential and has been described in [1,2]. The mitochondria were capable of generating a large membrane potential when energized with succinate. This hyperpolarized state was maintained for ⩾5 min at ~−160 mV as determined by the null point method in [14] and is similar to values reported for liver mitochondria using this [11,15] and other [15] methods. Addition of Ca^{2+} (54 nmol/mg mitochondrial protein) resulted in a partial depolarization followed by rapid repolarization within 20 s (not shown) consistent with observations using the safranine method [16]. Furthermore, no swelling of mitochondria was observed under conditions identical to those employed in the experiments with arsenazo III (not shown).

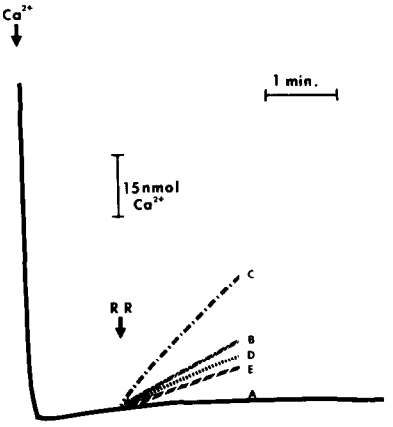

Fig.1. Effect of cytosol and TFP on mitochondrial Ca^{2+} efflux. Mitochondria (1.0 mg/ml) and 1.5 mM P_i were added to the basic incubation medium. Ca^{2+} (54 nmol/mg) and ruthenium red (RR) (1.6 μM) were added where indicated; (A) no further additions, and in the absence of RR; (B) no further additions (3.6); (C) 3.2 mg cytosol (7.7); (D) 3.2 mg cytosol and 20 μM TFP (2.0); (E) 20 μM TFP (1.7). The values in parentheses are initial Ca^{2+} efflux rates expressed as nmol . min^{-1} . mg^{-1}. RR was added in (B–E). A downward deflection reflects Ca^{2+} uptake.

3.2. *Effect of cytosol and TFP on Ca^{2+} movement*

The addition of cytosol did not affect the rate of Ca^{2+} influx, or the ability of mitochondria to retain Ca^{2+} (tracing similar to fig.1A), but in the presence of ruthenium red, stimulated Ca^{2+} efflux (fig.1C). This did not appear to be a damage-induced leak via reversal of the Ca^{2+} uniporter since, under these conditions, the cytosol had no effect on swelling or membrane potential of energized mitochondria. The cytosol-stimulated Ca^{2+} efflux was inhibited by TFP (fig.1D), a drug known to interfere with the interaction between the CDR, calmodulin, and its target systems [17]. TFP also had a small inhibitory effect on Ca^{2+} efflux in the absence of cytosol (fig.1E), probably due to association of residual cytosol factor with the mitochondria. This small inhibition was insufficient to explain the large inhibitory effect observed in the presence of cytosol. The effect of cytosol on Ca^{2+} efflux was concentration-dependent and appeared to be saturable (fig.2).

The inhibitory effect of TFP on the cytosol-stimulated mitochondrial Ca^{2+} efflux was also concentration dependent (fig.3). The concentration of TFP required for half-maximal inhibition was 8.6 ± 3.1 μM, similar to values reported for TFP inhibition of calmodulin [17–19] and other CDR-mediated processes [20,21]. In addition, a small rate of Ca^{2+} efflux (1–2 nmol . min^{-1} . mg^{-1}) insensitive to TFP (50 μM) always was observed irrespective of the addition of cytosol or P_i.

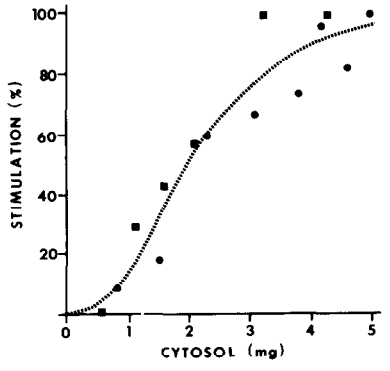

Fig.2. The effect of cytosol on mitochondrial Ca^{2+} efflux. Ca^{2+} efflux rates were determines as in fig.1: (●,■) data were obtained from 2 separate mitochondrial and cytosol preparations; (– – –) line of best fit obtained from computer analysis of the data. Results are expressed as % maximum stimulation of Ca^{2+} efflux.

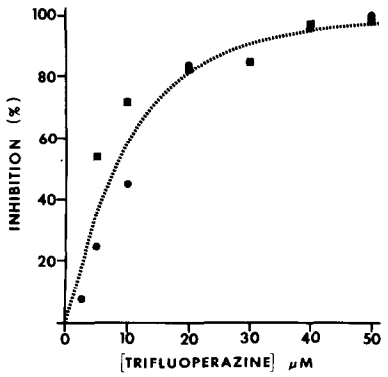

Fig.3. The inhibitory effect of TFP on cytosol-stimulated mitochondrial Ca^{2+} efflux. Ca^{2+} efflux rates were determined as in fig.1 with cytosol (3.0 and 3.8 mg) and TFP: (•,▪) data obtained from 2 separate mitochondrial and cytosol preparations; (– – –) line of best fit obtained from computer analysis of the data. Results are expressed as % maximum inhibition of Ca^{2+} efflux.

3.3. *P_i dependence of the effect of cytosol and TFP on Ca^{2+} efflux*

P_i was essential for the effects of both TFP and cytosol on Ca^{2+} efflux. Exogenous P_i, in the absence of cytosol, stimulated Ca^{2+} efflux to a small but significant extent (table 1). TFP inhibited Ca^{2+} efflux to a small but significant extent in the presence of P_i whereas, in its absence, TFP showed minimal or no effect. The cytosol-stimulated Ca^{2+} efflux was observed in the presence of P_i, with little or no stimulation in its absence. It was therefore concluded that the component of Ca^{2+} efflux from mitochondria, which is stimulated by cytosol and inhibited by TFP, is dependent on the presence of P_i.

Table 1
P_i dependence of the effect of cytosol and TFP on Ca^{2+} efflux

P_i (mM)	nmol . min^{-1} . mg^{-1}		
	No additions	TFP (20 μM)	Cytosol[a]
0	2.3 ± 0.21 (9)	2.0 ± 0.16 (9)	2.2 ± 0.32 (6)
1.5	3.3 ± 0.25 (9)	1.5 ± 0.08 (8)	5.4 ± 0.63 (7)

[a] Protein was 3.0 ± 0.51 mg/ml

Values given represent the mean ± SEM. The number of experiments is given in parenthesis. Ca^{2+} efflux rates were determined as in fig.1

3.4. *Preliminary characterization of the cytosol factor*

Attempts were made to ascertain certain physicochemical characteristics of the putative cytosol factor responsible for stimulating mitochondrial Ca^{2+} efflux. Subjection of cytosol to boiling for 5 min had no effect on its stimulatory capacity. Extraction with ether resulted in no change in its stimulatory effect on Ca^{2+} efflux suggesting that the active component was not lipid. The supernate obtained by centrifugation (100 000 × *g* for 60 min) of the boiled cytosol was passed through an ultrafilter (Amicon YM10) which retains species > 10 000 M_r. The entire Ca^{2+} efflux stimulatory activity could be recovered in the retentate. The factor present in the cytosol therefore, was: (1) heat stable; (2) not lipid; and (3) had $M_r > 10\,000$. Although these properties are similar to those of calmodulin, the addition of purified calmodulin to the incubation medium (0.34–34 μg/ml) had no effect on Ca^{2+} efflux in the presence or absence of P_i. Thus the cytosol factor which modulates Ca^{2+} efflux does not appear to be calmodulin. In [22,23] calmodulin also had no effect on mitochondrial Ca^{2+} movement.

4. Discussion

TFP binds to Ca^{2+}-dependent proteins other than calmodulin [17,20,21]. TFP also has been shown to disrupt mitochondrial energy production [24], increase membrane stabilization [25], and antagonize the binding of α adrenergic receptor agonists in hepatocytes [26].

Here, the observed effect of cytosol on Ca^{2+} efflux was prevented by TFP. The concentration range of TFP (2–20 μM) which was most effective was similar to that observed for calmodulin-dependent systems [17–19], and CDR-independent systems [17,21]. Although the inhibitory action of TFP on mitochondrial Ca^{2+} efflux may be due to its membrane stabilizing effect [25]; this does not appear to be the case since Ca^{2+} efflux is dependent on the presence of both the cytosol factor and P_i. The possibility that the cytosol factor is a Ca^{2+}-binding protein similar to calmodulin remains to be established but this is supported by the physicochemical findings and the sensitivity to TFP.

Our observations suggest that the cytosol factor identified in this study may participate in a messenger system capable of modulating intracellular free Ca^{2+} concentration at the level of the mitochondrion, a phe-

nomenon which, heretofore, has not been described. A number of unrelated hormones have been reported to mediate their effects via an increase in intracellular free Ca^{2+} (reviews [18,27]). These include insulin [28] and oxytocin [29] which appear to increase intracellular free Ca^{2+} via an effect at the level of the plasma membrane. Alternatively α-adrenergic agonists such as vasopressin, angiotensin II and catecholamines appear to activate hepatic cells through increased intracellular free Ca^{2+} by promotion of Ca^{2+} release from mitochondria and/or other intracellular stores [27,30]. The nature of the signal generated, however, is unknown [27]. The cytosol factor described here could constitute a possible mechanism whereby an extracellular hormone–receptor interaction (α-adrenergic) could be coupled to mitochondrial Ca^{2+} release.

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