The influence of nupercaine on Ca^{2+} transport by rat liver and Ehrlich ascites cell mitochondria

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1. INTRODUCTION

The resistance of tumor mitochondria to deleterious effects of Ca^{2+} is firmly established [1,2] (reviewed in [31]) but remains largely unexplained. Local anesthetics such as nupercaine inhibit mitochondrial phospholipase A_2 [4] and stabilize liver and heart mitochondria so as to facilitate their accumulation and retention of Ca^{2+} [5–7]. These agents are also purported to inhibit a specific pathway of Ca^{2+} efflux in liver mitochondria [8,9]. This pathway could participate in Ca^{2+} cycling and its uncoupling action. Here, the effects of nupercaine on Ca^{2+} uptake and release by rat liver and Ehrlich ascites cell mitochondria have been compared. The objective was to probe for possible differences in phospholipase A_2 activity and Ca^{2+} efflux in tumor mitochondria to explain how they are able to escape damage by Ca^{2+} [10].

The maximum mitochondrial Ca^{2+} uptake, which is a convenient quantitative measure of their resistance to damage [10], is increased 2.5-fold by nupercaine in rat liver. The local anesthetic does not affect Ca^{2+} uptake by isolated tumor mitochondria or those within digitonin-treated Ehrlich ascites cells. Tumor mitochondrial phospholipase A_2 may therefore be absent or altered. Nupercaine-sensitive Ca^{2+} efflux from Ehrlich cell mitochondria can be demonstrated but only under selected experimental conditions. Attenuation of these nupercaine sensitive processes can provide an explanation for the resistance of tumor mitochondria to damage by Ca^{2+} due to decreased phospholipase activation and Ca^{2+} cycling.

2. MATERIALS AND METHODS

Rat liver mitochondria were isolated from male Wistar rats by a modification of the method in [11]. Ehrlich ascites cells were harvested and washed [12] and mitochondria isolated as in [10].

Calcium fluxes were measured spectrophotometrically with murexide [13] or arsenazo III [14] by means of an Aminco DW-2 dual wavelength spectrophotometer. Other details of the conditions are provided in the legends. Protein was assayed as in [15] with bovine serum albumin as standard.

All chemicals were obtained from Sigma Chemical Co. or Fisher Scientific (St Louis MO). Ruthenium red was recrystallized as in [16] and concentrations determined spectrophotometrically [16]. Nupercaine was kindly provided by Ciba Pharmaceutical Co. (Summit NJ) and FCCP was a gift of Dr P.G. Heytler, E.I. Dupont de Nemours and Co. (Wilmington DE).

3. RESULTS

Exogenous Mg^{2+} enhances maximum Ca^{2+} accumulation by liver mitochondria by ~2-fold (table 1). Similarly, nupercaine stimulates ~2.5-times. The stimulation by Mg^{2+} is largely supplanted by exposing liver mitochondria to nupercaine. These results are similar to those in [6] for Ca^{2+} retention by liver mitochondria. The salutory influence of adenine nucleotides however is not affected by nupercaine. ATP (or ADP; not shown) enhances Ca^{2+} uptake by 1.5-fold with or without the local anesthetic (table 1).

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Table 1

Maximum Ca^{2+} uptake by rat liver mitochondria

Addition(s)	Ca^{2+} uptake (μequiv./mg protein)	
	− Nupercaine	+ Nupercaine
None	0.68	1.69
Mg^{2+}	1.25	1.86
Mg^{2+}, ATP	1.67	2.42

The reaction medium consisted of 250 mM sucrose, 5 mM Tris–succinate, 2.5 mM Tris–P_i, 20 mM Tris–HCl (pH 7.2), BSA (1 mg/ml) plus 60 μM murexide. Mitochondria were pretreated with rotenone (0.4 μg/mg) and added at ~0.35 mg/ml and preincubated 1 min before Ca^{2+} additions (100 μM each). The reaction mixtures were aerated for 5 s every 2–3 cycles of Ca^{2+} uptake. When included, nupercaine (250 μM), $MgCl_2$ (2 mM), and ATP (1 mM) were added initially as indicated. The values are means derived from at least 4 separate experiments

Unlike liver mitochondria, nupercaine has little effect on the maximum Ca^{2+} uptake of isolated tumor cell mitochondria (table 2). Stimulation by Mg^{2+} and ATP is observed as in liver; however, both are the same in the presence or absence of

Table 2

Effect of nupercaine on the maximum Ca^{2+} uptake by isolated tumor mitochondria

Addition(s)	Ca^{2+} uptake (μequiv./mg protein)	
	− Nupercaine	+ Nupercaine
None	1.88	1.86
Mg^{2+}	2.86	2.87
Mg^{2+}, ATP	3.66	3.70

The reaction medium, conditions of Ca^{2+} loading and concentrations of added substances were identical to those in table 1. The final concentration of tumor mitochondria was ~0.3 mg/ml and oligomycin (1.2 μg/ml) was included. The values are means for three or more separate preparations

nupercaine. The insensitivity of Ca^{2+} accumulation to nupercaine is also demonstrable with tumor mitochondria in situ; i.e., within digitonin treated Ehrlich cells [10].

Mitochondrial Ca^{2+} uptake by digitonized cells (table 3) is stimulated only 12% at the optimum concentration of nupercaine (250 μM). Exogenous Mg^{2+} does not stimulate which is attributed to retention of Mg^{2+} by digitonized cells [10]. These

Table 3

Influence of nupercaine on the maximum Ca^{2+} uptake by mitochondria of digitonin-treated Ehrlich ascites cells

Addition(s)	Ca^{2+} uptake (μequiv./10^8 cells)	Mitochondrial Ca^{2+} uptake (μequiv./mg protein) calc.[a]
None	5.94	2.70
Nupercaine		
125 μM	5.20	2.36
250 μM	6.68	3.04
500 μM	6.38	2.90
Mg^{2+}, 2 mM	5.97	2.71
Nupercaine, 250 μM + Mg^{2+}, 2 mM	5.89	2.68

[a] Calculated for a mitrochondrial content of 2.2 mg protein/10^8 cells [10]

The reaction conditions were the same as in tables 1 and 2 except Ehrlich cells (at final conc. ~5.5×10^6/ml) were treated with digitonin (350 μg/ml) and oligomycin (3.0 μg/ml) in addition to rotenone (present at 1.6 μg/ml). The values are means from results with three different cell preparations

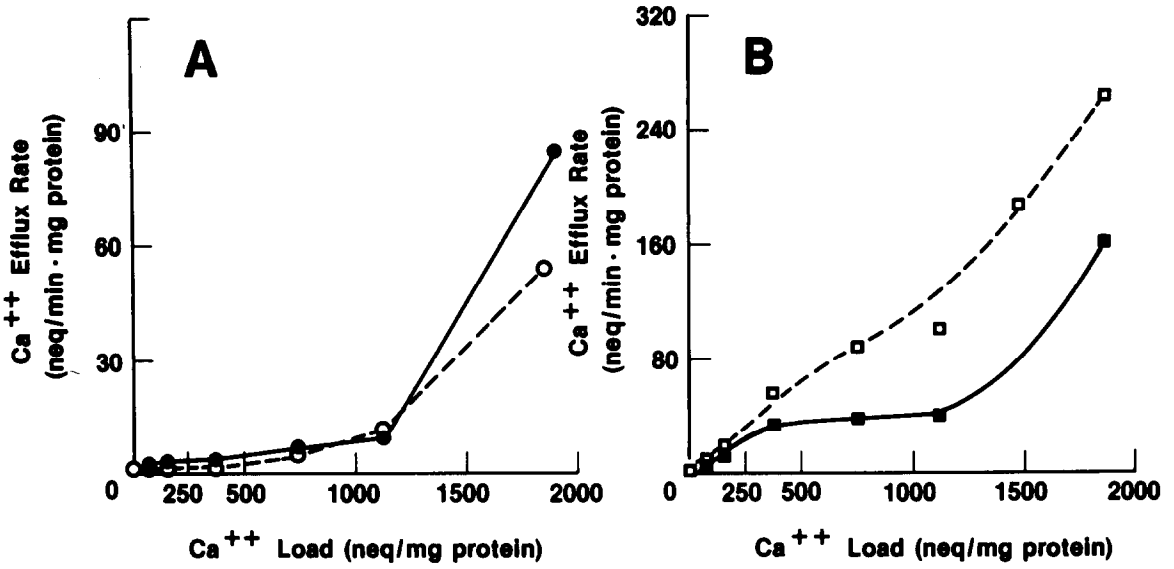

Fig.1(A,B). Nupercaine sensitivity of Ca^{2+} release from tumor mitochondria. The reaction medium was as in table 1 except arsenazo III (10 μM) was substituted for murexide. Mitochondria (pre-treated with rotenone and oligomycin, 0.4 μg/mg and 2.0 μg/mg, respectively) were at ~0.3 mg/ml and preloaded with the indicated quantities of Ca^{2+} as in the previous experiments. After achieving a steady state, Ca^{2+} efflux was initiated with ruthenium red (0.8 nmol/mg) in the presence and absence of nupercaine (250 μM) added 10 s before ruthenium red (A). When included, FCCP (2 μM) was added immediately after ruthenium red (B). The results are representative of 3 separate experiments (solid lines and symbols, with nupercaine).

observations indicate Ehrlich cell mitochondria are not altered during isolation to explain their insensitivity to nupercaine. Nupercaine was tested next for effects on Ca^{2+} efflux.

Steady state Ca^{2+} release caused by inhibiting uptake with ruthenium red has been ascribed to a specific efflux pathway inhibited by local anesthetics [8,9]. Nupercaine did not inhibit ruthenium red-dependent Ca^{2+} efflux from tumor mitochondria (fig.1A). Nupercaine stimulated slightly presumably due to a slight uncoupling effect. In contrast, ruthenium red-dependent Ca^{2+} release elicited with uncoupling agent [17] is inhibited by nupercaine (fig.1B). The extent of inhibition ranges from 40–60% depending upon the Ca^{2+} load. This differential sensitivity to nupercaine implies the existence of 2 efflux pathways. Results obtained with rat liver mitochondria under comparable conditions are dealt with below.

4. DISCUSSION

The maximum Ca^{2+} uptake of tumor mitochondria is 2–3-times that of liver mitochondria depending upon whether they are supplemented with exogenous Mg^{2+} and Mg^{2+} plus ATP. Liver mitochondria treated with nupercaine take up the same amount of Ca^{2+} as tumor mitochondria (1.69 and 1.86 μequiv. Ca^{2+}/mg protein, respectively) or ~70% in the presence of Mg^{2+} with and without ATP. The close agreement between values (especially without Mg^{2+} and adenine nucleotide) could be fortuitous or correspond to the Ca^{2+} uptake capacity of mitochondria when phospholipase A_2 activity is minimal; i.e., inhibited by local anesthetic in rat liver [5–7] or 'deficient' in tumor mitochondria. Direct measurements of phospholipase activities during Ca^{2+} transport are necessary to substantiate this interpretation which is technically feasible with the recent development of sensitive and specific assay methods [18]. These studies suggest Mg^{2+} exerts different influences on Ca^{2+} transport, e.g., in liver, Mg^{2+} may prevent Ca^{2+} activation of mitochondrial phospholipase A_2 or permeability effects of lysophosphatides [7,18] since nupercaine largely eliminates Mg^{2+} stimulation.

The Mg^{2+} (and perhaps ATP) stimulation of tumor mitochondrial Ca^{2+} uptake (table 2) presumably occurs by a different or additional mechanism, e.g., non-specific membrane stabilization at extremely high intramitochondrial Ca^{2+} levels (>2 μequiv. Ca^{2+}/mg protein).

The mechanism of ruthenium red-insensitive Ca^{2+} efflux from mitochondria is not known nor is that by which local anesthetics inhibit this Ca^{2+} release [9]. Nupercaine inhibited Ca^{2+} release from tumor mitochondria elicited with uncoupling agent (fig.1B) but not the steady state release under metabolizing conditions (fig.1A). These may, therefore, represent different pathways of Ca^{2+} efflux. Coupled respiration may 'protect' against changes in membrane structure (transitions) necessary for Ca^{2+} efflux [20] that are facilitated by uncoupling agents [21]. Calcium release by this pathway may not occur in the aerobic steady state. In experiments with rat liver mitochondria not presented, nupercaine inhibited Ca^{2+} egress by both pathways, i.e., on addition of ruthenium red during the aerobic steady state (cf. [9]) and Ca^{2+} release with ruthenium red plus respiratory inhibitor (e.g., with antimycin, nupercaine inhibits by 70–20% as the Ca^{2+} load is increased from 200–800 nequiv. Ca^{2+}/mg protein, contrary to results cited in [8] although for very different conditions). The sluggishness and nupercaine insensitivity of aerobic Ca^{2+} efflux from tumor mitochondria at large Ca^{2+} loads may be quite germane to their peculiar ability to take up and retain Ca^{2+} (e.g., release by ruthenium red occurred at 1 nequiv./min.mg for 400 nequiv. Ca^{2+}/mg (fig.1A) compared to 10/min for rat liver; not shown). Steady state Ca^{2+} cycling via this pathway would be slow and therefore could cause minimal loss of internal Mg^{2+} and/or adenine nucleotides which is evidently crucial for maintaining the transmembrane electrical potential [19], hence the driving force sustaining Ca^{2+} uptake. An interesting possibility is that phospholipase A_2 activity is necessary for Ca^{2+} release via the nupercaine-sensitive efflux pathway operable during coupled electron flow. Therefore, this is not demonstrable in Ehrlich cell mitochondria as they are deficient in the enzyme.

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