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Ca^{2+} RELEASE FROM ENERGETICALLY COUPLED TUMOR MITOCHONDRIA

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A Na^+/Ca^{2+} exchange activity for Ca^{2+} efflux has been identified in isolated Ehrlich ascites tumor mitochondria. Further, under conditions favoring cycling of Ca^{2+} across the mitochondrial inner membrane, extramitochondrial [Ca^{2+}] also was shown to be Na^+-dependent. The Na^+/Ca^{2+} exchange showed sigmoidal kinetics with a mean (±SD) [Na^+] required for half maximal stimulation of Ca^{2+} efflux of 8.4 ± 3.8 mM and a Hill coefficient of 1.6. Na^+/Ca^{2+} exchange was very sensitive to inhibition by the Ca^{2+} antagonist diltiazem (56% inhibition at 7.5 nmoles · mg protein^{-1}) whereas a number of other compounds, including verapamil, nupercaine, and trifluoperazine were less effective in inhibiting Ca^{2+} efflux. These data demonstrate for the first time the presence of a pathway in tumor mitochondria for unidirectional Ca^{2+} efflux induced by Na^+, and provide a mechanism for regulation of tumor intra- and extramitochondrial [Ca^{2+}]. Results of the present study support the need for further study of intracellular Na^+ and its role in regulation of Ca^{2+} homeostasis in tumor cells.

Ca^{2+} is recognized as playing a key role in the transduction of diverse metabolic and hormonal signals (1,2). Consequently, the mechanisms of Ca^{2+} transport across biomembranes and of regulation of intracellular Ca^{2+} become of great importance in understanding the messenger role of Ca^{2+}.

In this communication we report the release of Ca^{2+} from EAT mitochondria by physiological concentrations of Na^+. These findings have not been reported heretofore and have a direct bearing on the previously held concept of an impairment in Ca^{2+} release from tumor mitochondria (3) and subsequent altered ability to regulate intracellular [Ca^{2+}] (4,5). Results from the present study also suggest that Na^+-induced release of mitochondrial Ca^{2+}, either under steady-state conditions of Ca^{2+} cycling across the membrane, or during unidirectional efflux, is not restricted solely to mitochondria from "excitable" cells.

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Abbreviations: EAT, Ehrlich ascites tumor; CCCP, carbonyl cyanide m-chlorophenyl hydrazone; RR, ruthenium red; TFP, trifluoperazine; FCCP, carbonyl cyanide p-trifluoromethoxyphenylhydrazone.

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MATERIALS AND METHODS

Arsenazo III (sodium salt, grade 1), rotenone, Hepes, EGTA, CCCP, succinic acid, and RR were obtained from Sigma. RR was purified according to Luft (6). Nupercaine was obtained from K and K Laboratories, calmodulin and A23187 were obtained from Calbiochem-Behring Corp.; TFP was a gift from Smith Kline and French; verapamil was a gift from Knoll Pharmaceuticals; and diltiazem was a gift from Marion Laboratories.

EAT cells (hyperdiploid Ehrlich-Lettré ascites carcinoma, originally from Dr. Leonard A. Sauer) were maintained in male Swiss-Webster mice (Charles River Breeding Laboratories) by weekly intraperitoneal injection of 0.2 ml of undiluted ascites fluid. Cells from five to six mice were harvested approximately one week following injection and washed twice in 150 mM NaCl, 6 mM KCl, and 10 mM Hepes (pH 7.4). Mitochondria were isolated as described by Sauer and Dauchy (7) using a Dounce homogenizer, but omitting albumin and EDTA.

Ca^{2+} movement and extramitochondrial $[Ca^{2+}]$ were determined spectrophotometrically as described previously (8). A standard solution of $CaCl_2$ was used for calibration. The basic incubation medium contained: 1 mg mitochondrial protein, 250 mM sucrose, 3 µM rotenone, 15 mM succinate, 0.5 mM K phosphate, 58 µM arsenazo III, and 10 mM Hepes (pH 7.4) in a total volume of 1 ml. Additions to the incubation medium are noted in the figure legends; temperature was maintained at 30°C. Endogenous free Ca^{2+} was measured as the amount of Ca^{2+} released from an aliquot of mitochondria upon addition of CCCP (or the Ca^{2+} ionophore A23187) followed by chelation with EGTA (3.2 mM). Exogenous Ca^{2+} was added to a final Ca^{2+} load of approximately 50 nmol Ca^{2+} · mg protein^{-1}. The rate of Ca^{2+} efflux was determined following the addition of 1.6 µM RR. Mitochondrial swelling was monitored by following the decrease in absorbance at 520 nm as described previously (9). Protein was determined colorimetrically using the Folin-phenol reagent with BSA as standard (10).

RESULTS

Characterization of Ca^{2+} Movement. It has been reported previously that isolated, energized EAT mitochondria readily take up Ca^{2+} from the medium and retain it (4, 11-16). Fig. 1 shows representative tracings of the time course of Ca^{2+} uptake by EAT

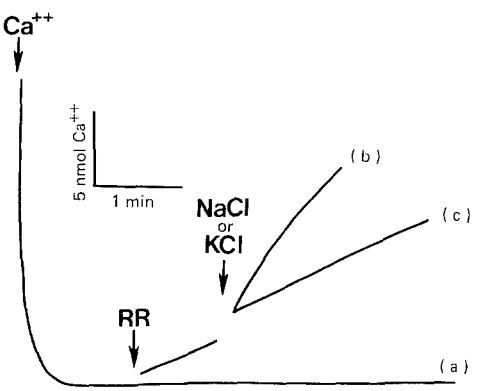

Fig. 1. **Na^+-induced release of mitochondrial Ca^{2+}.** Ca^{2+} (to give a final concentration of 50 nmol · mg protein^{-1}), RR (1.6 µM) and NaCl (or KCl) were added where indicated; all other conditions were as described under Materials and methods; (a) no further additions and in the absence of RR; (b) with 25 mM NaCl (3.4; 11.2); and (c) with 25 mM KCl (2.3; 2.8). The values in parentheses are initial Ca^{2+} efflux rates expressed as nmol · min^{-1} · mg^{-1}; the first value being the rate prior to addition of Na^+ (or K^+), and the second value, following the addition of Na^+ (or K^+). A downward deflection reflects Ca^{+2} uptake. The figure represents reproductions of typical recordings.

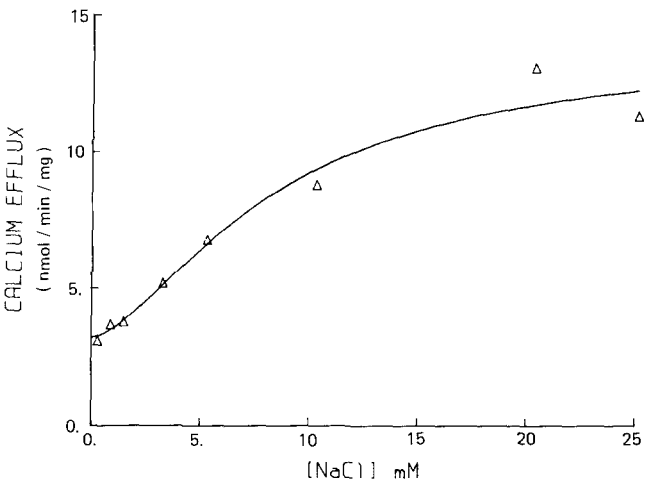

Fig. 2. **Dependence of Ca^{2+} efflux on $[Na^+]$.** Ca^{+2} efflux rates were determined as in Fig. 1. Data were obtained from eight separate experiments and plotted as means. The curve shown was obtained by fitting the data to the Hill equation using our modification (J.R.D. and J.W.L., unpublished) of a nonlinear least squares regression analysis (17) and inclusion of a y-intercept. The Na^+ concentrations include the Na^+ contributed by the arsenazo III (0.3 mM).

mitochondria and its subsequent release by the addition of Na^+ (Na^+-induced Ca^{2+} efflux). Uptake of Ca^{2+} was blocked with RR, indicating influx by the electrophoretic uniporter for Ca^{2+} (not shown). From a series of experiments, the Na^+-induced increase in the rate of efflux was approximately 4 times the rate observed in the absence of Na^+ (Na^+-independent Ca^{2+} efflux). As shown, K^+ could not substitute for Na^+. These data provide the first evidence for Ca^{2+} release from EAT mitochondria by Na^+.

The effect of Na^+ on Ca^{2+} efflux was concentration dependent and saturable, and displayed a sigmoidal relationship (Fig. 2). The mean (± S.D.) concentration of Na^+ required for half maximal stimulation of Ca^{2+} efflux was 8.4 ± 3.8 mM. Fitting of the data to the Hill equation yielded a Hill coefficient of 1.6. The data also show that in the absence of Na^+ (Fig. 2, y intercept) a slow rate of Ca^{2+} efflux was obtained (3.2 nmoles · min^{-1} · mg^{-1}). This is similar to the rate shown in Fig. 1 b (initial rate) which was not corrected for the small amount of Na^+ associated with arsenazo III. Thus, Ca^{2+} release from EAT mitochondria occurs as: (1) a slow basal Na^+-independent Ca^{2+} efflux; and (2) a significantly faster Na^+-induced Ca^{2+} efflux, designated as a Na^+/Ca^{2+} exchange.

A Na^{2+}-independent Ca^{2+} efflux from EAT mitochondria previously has been described (18,19); however, release was elicited with uncoupler (FCCP) making it difficult

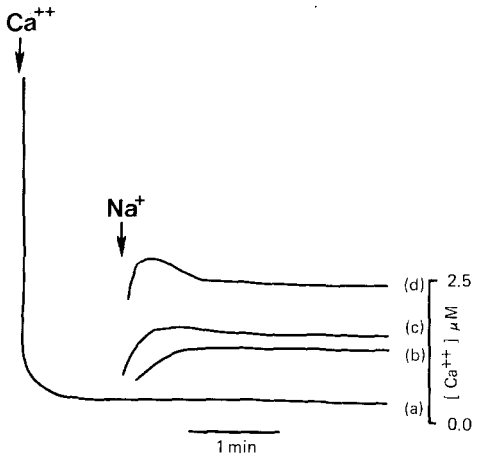

Fig. 3. **Na^+-induced increase in the extramitochondrial [Ca^{2+}].** Ca^{2+} (to give a final concentration of 50 nmol · mg^{-1}) and Na^+ were added where indicated; all other conditions were as described under Materials and methods; (a) no further additions and in the absence of added NaCl; (b) with 10 mM NaCl; (c) with 15 mM NaCl; and (d) with 20 mM NaCl. EGTA (3.2 mM) was added to achieve zero free [Ca^{2+}]. The figure represents reproductions of typical recordings.

to interpret whether efflux occured by reversal of the uniporter, or by a separate pathway (also see from (20)).

Na^+-Induced Increase in Extramitochondrial Ca^{2+} Concentration. In the absence of RR, the Na^+/Ca^{2+} exchange activity of energized EAT mitochondria resulted in an increase in extramitochondrial Ca^{2+} (Fig. 3) which could be maintained constant for the time period studied (usually 5 min.). The addition of 20 mM Na^+ increased the extramitochondrial Ca^{2+} from a mean (± S.D.) of 0.50 ± 0.06 μM to 1.96 ± 0.64 μM. Ca^{2+} cycling across the mitochondrial inner membrane occurred in the absence of RR resulting in a Na^+-dependent change in the steady state concentration of extramitochondrial Ca^{2+} (Fig. 3; compare curves b-d). Little or no mitochondrial swelling was observed under the conditions of Ca^{2+} cycling (not shown). Taken together, these observations support the conclusion that the Na^+/Ca^{2+} exchange activity of EAT mitochondria is physiologically relevant.

Effect of Various Agents on Release of Mitochondrial Ca^{2+}. Since both a Na^+-independent efflux, as well as a Na^+/Ca^{2+} exchange was exhibited by EAT mitochondria, the question arose as to whether these were two distinct pathways. Thus, to further characterize the release of Ca^{2+} from EAT mitochondria, a variety of agents known to affect Ca^{2+} efflux in other systems was studied. The presence of both Na^+-independent

TABLE 1 THE EFFECT OF VARIOUS AGENTS ON RELEASE OF MITOCHONDRIAL Ca^{2+}

Addition	Ca^{2+} Efflux ($nmol \cdot min^{-1} \cdot mg^{-1}$)		Inhibition (%)	
	$-Na^+$	$+Na^+$	$-Na^+$	$+Na^+$
none[a]	2.7 ± 0.5	13.1 ± 0.9	—	—
Mg^{2+} (0.5 mM)	1.8 ± 0.2	9.6 ± 0.6	26.9 ± 4.2	42.2 ± 4.2
Mg^{2+} (1.0 mM)	1.6 ± 0.2	6.2 ± 0.3	38.9 ± 2.5	62.9 ± 2.0
nupercaine (200 μM)	1.7 ± 0.2	10.3 ± 0.7	33.2 ± 1.3	38.0 ± 4.9
verapamil (200 μM)	1.6 ± 0.1	10.3 ± 1.5	36.6 ± 4.8	38.6 ± 9.6
TFP (60 μM)	2.3 ± 0.7	8.4 ± 0.8	36.9 ± 6.5	32.8 ± 3.6
diltiazem (7.5 μM)	3.2 ± 0.6	6.2 ± 1.2	0	56.1 ± 4.2

Ca^{2+} efflux rates were determined as described in Fig. 1. The values for percent inhibition were based on paired observations. When included, Na^+ was 20 mM. Values given represent the mean ± SEM of two or more separate experiments.

[a]In the absence of phosphate, the Na^+-independent rate was 7.7 ± 1.1, and the Na^+/Ca^{2+} exchange activity was 14.5 ± 0.5 $nmol \cdot min^{-1} \cdot mg^{-1}$.

efflux and Na^+/Ca^{2+} exchange activity in EAT mitochondria is supported by the differential sensitivity of these rates to the various agents studied, as shown in Table 1. Mg^{2+} caused a greater decrease in the Na^+/Ca^{2+} exchange activity than in the Na^+-independent rate of Ca^{2+} efflux. However, nupercaine and verapamil inhibited both efflux rates to the same extent. Cockrell (19) failed to observe inhibition of a Na^+-independent Ca^{2+} efflux from EAT by mitochondria by nupercaine; however, the assay conditions (Ca^{2+} load, mitochondrial protein, oligomycin, and FCCP) were considerably different. At relatively high concentrations the antipsychotic drug, TFP, also inhibited both efflux rates to approximately the same extent. In agreement with the relatively non-specific effect of TFP on EAT mitochondria, calmodulin (1-5 μg · mg protein^{-1}) had no effect on Ca^{2+} efflux in either the absence or presence of Na^+ (not shown). At very low concentrations, only diltiazem, a Ca^{2+} antagonist, significantly and specifically inhibited the Na^+/Ca^{2+} exchange activity of EAT mitochondria (7.5 nmoles · mg mitochondrial protein^{-1} caused approximately 56% inhibition).

DISCUSSION

Two possible mechanisms for the unidirectional release of Ca^{2+} from mitochondria of a variety of tissues have been proposed, namely, a H^+/Ca^{2+} exchange (21), or a Na^+/Ca^{2+} exchange (22). These pathways, together with an electrophoretic uniporter for mitochondrial Ca^{2+} influx (23), have been implicated in maintenance of cellular Ca^{2+} homeostasis (24). While it is generally accepted that the mechanism of Ca^{2+} influx is similar for mitochondria from normal tissues and tumors (11,25), the abnormally high retention of Ca^{2+} by tumor mitochondria has been attributed, at least for EAT mitochondria, to the absence of a H^+/Ca^{2+} exchange mechanism for Ca^{2+} efflux (18). Na^+/Ca^{2+} exchange activity in tumor mitochondria has not been reported. Results from the present study show that Ca^{2+} is released from EAT mitochondria by Na^+, regulating extramitochondrial free $[Ca^{2+}]$. These results suggest that mitochondria may play a significant role in control of the free $[Ca^{2+}]$ in the EAT cell as suggested for other cells (24).

The Na^+/Ca^{2+} exchange mechanism for release of Ca^{2+} from EAT mitochondria appears to have properties in common with those reported for heart and other excitable tissues (22, 26-29), namely: (a) relatively rapid rate of Ca^{2+} release, (b) similar $[Na^+]$ required for half maximum velocity (8.4 mM vs 8-10 mM (22,26,28)), (c) regulation of extramitochondrial Ca^{2+} (22), and (d) similar sensitivity to the Ca^{2+} antagonist, diltiazem (30). In addition, both extramitochondrial Ca^{2+} and phosphate appear to regulate the exchange carrier for EAT mitochondria (see Fig 1, and Table 1) as well as for heart mitochondria (31,32). The expression of cooperative Na^+ sites in the Na^+/Ca^{2+} exchange of EAT mitochondria was slightly different from that reported for heart mitochondria (Hill coeffient of 1.6 vs 2-3.4 (22,32,33)), which may reflect differences between tissues, or anion requirements (32).

Our findings with EAT mitochondria suggest the need to reevaluate Ca^{2+} release mechanism(s) in other tumors. EAT mitochondria, as demonstrated in the present study, appear to have a putative role in the regulation of intramitochondrial Ca^{2+} and of intracellular free Ca^{2+}. Since intracellular free Ca^{2+} acts as a second messenger in a variety of cellular processes, it is essential to determine factors influencing this pool of Ca^{2+} in tumor cells and the role of mitochondria in its modulation. Finally, our

observation of Ca^{2+} release from EAT mitochondria by a Na^{+}/Ca^{2+} exchange mechanism suggests new importance for Na^{+} regulation in EAT cells

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