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A STUDY OF CALCIUM PUMP ACTIVITY OF LYSOSOMES FROM RAT RENAL CORTEX

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SUMMARY

Fractions rich in either primary or secondary lysosomes were prepared from rat renal cortex by differential centrifugation and evaluated for their capacity for net calcium uptake. No uptake was observed in the absence of ATP. A vigorous uptake did take place in the presence of ATP but it was largely prevented by azide and other inhibitors of mitochondrial calcium uptake, suggesting that it was attributable to contamination by mitochondria. Evidence was obtained for an inhibitory influence of the secondary lysosomal fraction on mitochondrial calcium uptake.

INTRODUCTION

ATP-dependent calcium uptake has been described in mitochondria [1], plasma membrane [2], and microsomes [2] obtained from rat kidney tissue by differential centrifugation. All of these fractions are likely to be contaminated to some degree by either primary or secondary lysosomes. Lysosomes contain an excess of anionic groups and should possess a considerable capacity for binding of divalent cations which might reach the vesicle interior. Studies on lysosomal calcium uptake from kidney tissue have not previously been reported.

In the present study, we have measured net calcium uptake by primary and secondary lysosomal fractions from rat renal cortex. Net calcium uptake was observed but it was substantially prevented by agents or conditions which also prevented calcium uptake by mitochondria. The presence of secondary lysosomes in the mitochondrial fraction was found to have an inhibitory effect on calcium uptake by mitochondria.

MATERIALS AND METHODS

Male Sprague-Dawley rats weighing 90–130 g were the source of the kidney tissue. The rats were killed by decapitation. After removal of the capsule and medul-

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lary region, the sliced cortical tissue was placed in an ice-cold 0.45 M sucrose, 0.68 mM EDTA medium and homogenized with five up and down strokes each of the loose- and tight-fitting Dounce homogenizer pestles. The resulting homogenate was separated by differential centrifugation according to either the method of Shibko and Tappel [3] or of Maunsbach [4], so as to yield mitochondrial and secondary lysosomal fractions. The relative purity of each fraction was determined by assay of the marker enzymes cytochrome oxidase [5] and acid phosphatase [5], expressed as specific activity following protein analysis [6]. A fraction rich in primary lysosomes was obtained by differential centrifugation using the procedure of Wattiaux-de Coninck et al. [5] as modified slightly by us [7].

Calcium uptake was measured at 37 °C in the following medium: 56 mM Tris · HCl (pH 7.2), 40 mM KCl, 5 mM $MgCl_2$, 5 mM Na_2ATP, 5 mM KH_2PO_4, and 100 μM $CaCl_2$. Calcium uptake was determined both by a radioactive Ca^{2+} procedure and by atomic absorption analysis. In the former, 0.04–0.06 μCi of ^{45}Ca was included in the incubation mixture. 2.5 ml of the medium were preincubated at 37 °C and the assay was initiated by addition of 0.5 ml of a subcellular fraction supplying up to 0.2 mg protein per ml of incubation medium. Uptake was terminated by rapid filtration of a 1-ml aliquot under vacuum through a 0.45 μm Millipore filter. The filters were pre-soaked in 0.25 M KCl and washed with 10 ml deionized water just before use. Samples were filtered and washed with 1 ml of 0.25 M sucrose. The filter was dried and placed in 10 ml of scintillation fluid (6 g PPO in 1 l toluene) and the filtrate was diluted with 10 ml of Aquasol (New England Nuclear). The samples were counted in a Beckman liquid scintillation counter, Model 133. When Ca^{2+} was to be determined by direct analysis, the assay was begun by addition of a 2-ml aliquot of the fraction to 7 ml of medium, and terminated by immersion of the reaction tube in an ice bath. The contents of the tube were then centrifuged at 170 000 $\times g$ at 4 °C for 20 min. The pellet was wet-ashed with a mixture of HNO_3 and $HClO_4$ and both the pellet and the supernatant were analyzed for their calcium content using a Perkin-Elmer Model 303 atomic absorption spectrophotometer. In either procedure, uptake data were calculated based upon the analysis of the retentate or the pellet and verified by analysis of the filtrate or supernatant.

RESULTS

Fraction purity

The relative distribution of the marker enzymes is shown in Table I. The classical light mitochondrial fraction from kidney is composed of primary lysosomes and small mitochondria. Acid phosphatase activity was enriched 5-fold as compared to the corresponding mitochondrial fraction but cytochrome oxidase activity was only slightly reduced.

The procedures of Shibko and Tappel [3] and Maunsbach [4] for the isolation of kidney lysosomes yield fractions containing particles heavier than the average mitochondria. When examined with an electron microscope, dense homogeneous bodies corresponding to type II cytoplasmic bodies, also known as heterolysosomes or secondary lysosomes, were seen, but typical fields also contained about 10 large mitochondria per 25 secondary lysosomes. The lysosomal fraction from the Shibko and Tappel procedure contained an 8.5-fold enrichment in acid phosphatase and a

TABLE I

DISTRIBUTIONS OF MARKER ENZYMES

Fraction	Acid phosphatase[a]		Cytochrome oxidase[a] (units/mg protein)
	Total (units/mg protein)	Percentage free[b]	
Primary lysosomal fraction			
Wattiaux-de Coninck et al. [5] procedure	0.21±0.03		0.20±0.04
Secondary lysosomal fraction			
Shibko and Tappel [3] procedure	1.88±0.22	28±14	0.05±0.01
Maunsbach [4] procedure	1.50±0.80	82±15	0.07±0.11
Mitochondrial fraction			
Shibko and Tappel [3] procedure	0.22±0.01		0.25±0.03
Maunsbach [4] procedure	0.04±0.03		0.19±0.06
Wattiaux-de Coninck et al. [5] procedure	0.04±0.02		0.25±0.04

[a] Each value is mean ±S.D. from at least three determinations. One unit of acid phosphatase activity is defined as that amount of enzyme which released 1 μmol of inorganic phosphate from β-glycerophosphate per min per mg of protein [5]. One unit of cytochrome oxidase activity is defined as that amount of enzyme which catalyzes the oxidation of 90 % of the reduced cytochrome present in 1 min and in 100 ml of solution [5].

[b] Percentage of activity obtained in absence of Triton X-100.

5-fold dilution of cytochrome oxidase vis à vis the mitochondrial fraction. A low degree of latency in the lysosomes obtained with the Maunsbach procedure suggested extensive damage during isolation. Therefore, only the Shibko and Tappel procedure was used for the further studies on Ca^{2+} uptake.

Net calcium uptake

The secondary lysosomal fraction was found to have a vigorous capacity for net calcium uptake in the presence of ATP. Omission of either ATP or Mg^{2+} reduced the net uptake to zero. A major difficulty faced in these studies was the need to distinguish between uptake by the secondary lysosomes as opposed to contaminating mitochondria. One approach involved the testing of several known mitochondrial inhibitors (Table II). NaN_3, 2,4-dinitrophenol, and ruthenium red all essentially prevented net calcium uptake by either the lysosomal or the mitochondrial fractions. Oligomycin B and Triton X-100 were more effective in the lysosomal fraction than in the mitochondrial fraction. Since the concentration of total protein was held constant, the ratio of inhibitor to mitochondria was 4–5 times greater in the lysosomal fraction than in the mitochondrial fraction. The differential effect of these two compounds could thus be explained either as an indication of the existence of two different uptake

TABLE II

THE EFFECT OF INHIBITORS ON CALCIUM UPTAKE

Inhibitor	Concentration	Incubation time (min)	Inhibition % "L"[a]	Inhibition % "M"[a]
2,4-Dinitrophenol	0.25 mM	30	92	97
	0.50 mM	30	97	99
Sodium azide	2.5 mM	30	97	98
	5.0 mM	30	97	99
Ruthenium red	1 μM	30	72	60
	10 μM	30	99	99
Triton X-100	0.01 %	30	99	62
	0.1 %	30	99	88
Oligomycin B	10^{-3} mM	20	29	3
	10^{-3} mM	40	20	7

[a] Protein concentration was 0.05–0.20 for "M" fraction and 0.10–0.25 for "L" fraction in this series.

TABLE III

EFFECT OF NITROGEN VS. AIR ON NET CALCIUM UPTAKE

Fraction	Analytical technique	Total protein concentration (mg/ml)	Estimated mitochondrial protein[a] (mg/ml)	Net calcium uptake[b] (nmol/mg total protein) Aerobic	Net calcium uptake[b] (nmol/mg total protein) Anaerobic	Inhibition (%)
M	Atomic absorption	1.44		44	0	100
M	Atomic absorption	1.20		28	0	100
M	Atomic absorption	1.03		67	0	100
M	^{45}Ca	0.86		101	9	91
M	^{45}Ca	0.67		144	66	54
M	^{45}Ca	0.055		84	87	0
M	^{45}Ca	0.053		191	181	5
M	^{45}Ca	0.053		96	102	0
L	^{45}Ca	0.30	(0.075)	116	63	46
L	^{45}Ca	0.25	(0.062)	46	41	11
L	^{45}Ca	0.20	(0.050)	136	138	0
L	^{45}Ca	0.20	(0.050)	95	90	5
L	Atomic absorption	0.17	(0.042)	271	245	10
L	Atomic absorption	0.14	(0.035)	267	244	9
L	Atomic absorption	0.06	(0.015)	133	124	7

[a] Estimated concentration of protein assuming 25 % contamination by mitochondria.
[b] Incubated 30 min with standard conditions except 6 mM sodium succinate added and Na_2ATP reduced from 5 to 1 mM.

systems or as a result of a more favorable inhibitor to mitochondria ratio in the lysosomal sample.

Succinate can replace ATP as the energy source for calcium uptake by mitochondria in the presence of oxygen but not under anaerobic conditions. Aliquots of secondary lysosomal and mitochondrial fractions were incubated either in air or under nitrogen (Table II). In the mitochondrial fractions, the reduced concentration of ATP (1 mM) was capable of supporting calcium uptake in the absence of O_2 up to a protein concentration of 0.05–0.06 mg/ml. Further increases in protein (mitochondrial) concentration caused a shift toward succinate as the source of ATP and anaerobiosis became inhibitory to further net calcium uptake. Calculation of calcium uptake by the lysosomal fraction as a function of total protein showed an apparent lack of dependence upon oxygen up to a protein concentration of 0.25–0.30 mg/ml. If one assumes, however, that all of the calcium accumulation in the secondary lysosomal fraction was by the contaminating mitochondria, oxygen dependence was

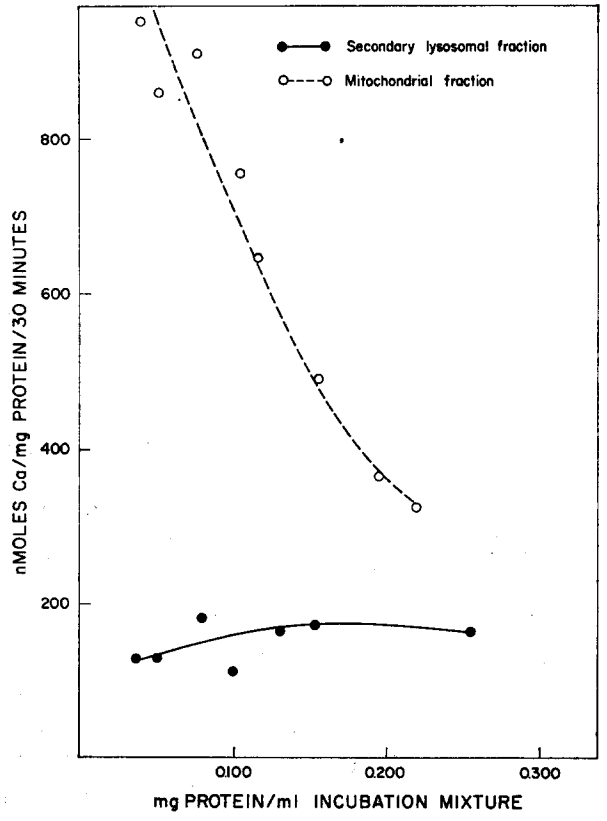

Fig. 1. Specific Ca^{2+} uptake by rat renal mitochondria and secondary lysosomal preparations. Ca^{2+} uptake was measured in the following medium: 56 mM Tris · HCl (pH 7.2), 40 mM KCl, 5 mM $MgCl_2$, 5 mM Na_2ATP, 5 mM KH_2PO_4, 100 μM $CaCl_2$, and 0.04–0.06 μCi of ^{45}Ca. Aliquots of each fraction containing varying amounts of protein were added at zero time. At the end of 30 min incubation, a 1-ml sample was withdrawn, filtered and ^{45}Ca determined as described in the text.

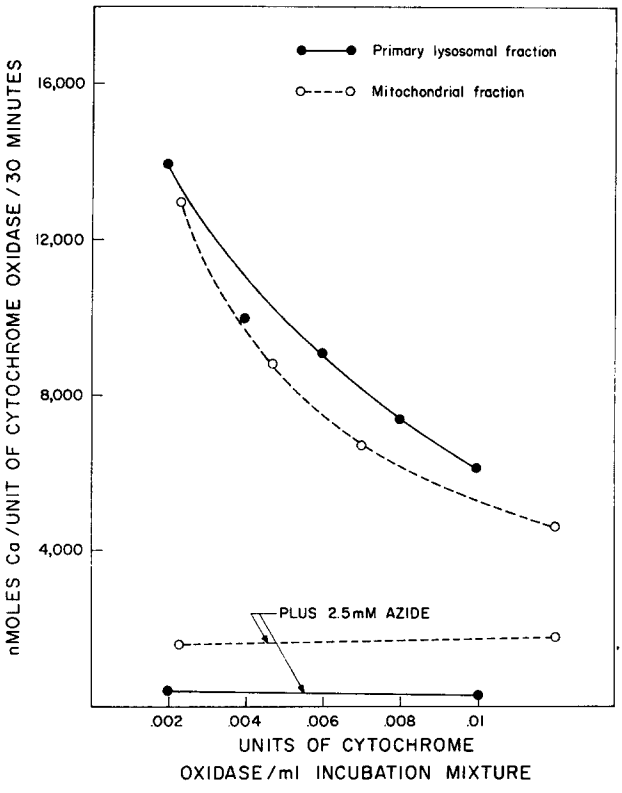

Fig. 2. Specific Ca^{2+} uptake as a function of cytochrome oxidase activities in rat renal mitochondria and primary lysosomal preparations with or without NaN_3. The incubation medium is that described in the legend of Fig. 1.

seen as the estimated mitochondrial protein concentration approached 0.065–0.075 mg/ml.

The effect of protein concentration on net calcium uptake in mitochondrial and secondary lysosomal fractions with ATP maintained at 5 mM is shown in Fig. 1. Net calcium uptake per mg protein increased in the mitochondrial fraction as the protein concentration was reduced. An examination of previous articles on calcium uptake by rat kidney mitochondria revealed a wide range of uptake values which also appeared to be correlated with protein concentration or calcium to protein ratios [1, 2]. In the secondary lysosomal fraction, net calcium uptake per mg protein declined slowly as the total protein concentration in the incubation mixture was reduced. These data suggest an inhibitory effect which prevented the mitochondria in the secondary lysosomal fraction from responding as did those in the mitochondrial fraction.

The primary lysosomal fraction from rat kidney appeared to possess neither an inhibitor effect nor its own calcium uptake system (Fig. 2). The net calcium uptake curves (expressed as a function of cytochrome oxidase activity) of the mitochondrial fraction and a primary lysosomal fraction possessed an almost identical shape and

both showed steep increase in net calcium uptake in response to dilution of the protein concentration of the incubation mixture. NaN_3 at a concentration of 2.5 mM substantially reduced uptake in both fractions at both low and high concentrations of protein.

DISCUSSION

Renal lysosomes have not been examined for their calcium capture potential although there are several reasons to suggest that they might be more suitable than mitochondria as temporary storage sites for Ca^{2+}. Calcium uptake by mitochondria is at the expense of ATP production and thus diverts them from this vital task. Secondary lysosomes, on the other hand, offer a compartment of sufficient size which serves to isolate a wide variety of unwanted or harmful materials including the minerals copper [9], iron [10], uranium [11], and crystalline deposits of calcium phosphate [12]. Moreover, Stauber and Schottelius [13] have described net calcium uptake as occurring in a primary lysosomal fraction from chick muscle.

We have found that both primary and secondary lysosomal fractions from rat renal cortex show net accumulation of calcium in the presence of ATP. These fractions are, however, contaminated with mitochondria and since azide and other inhibitors of mitochondrial calcium uptake are also effective in reducing this uptake, we conclude that neither possesses an independent uptake capability for Ca^{2+}. It is possible that the inhibitors are effective against calcium transport enzymes in both organelles but azide does not inhibit calcium uptake by either plasma membrane or microsomes from rat renal cortex [2]. It may be significant that Stauber and Schottelius [13] did not test these inhibitors in their lysosomal fraction from muscle.

The large increase in calcium uptake which accompanied the reduction in protein concentration in the mitochondrial fraction suggests a shift toward optimal conditions. A similar shift was not observed with the secondary lysosomal fraction. If all of the uptake in the lysosomal fraction was achieved by the contaminating mitochondria, it would follow that the presence of the secondary lysosomes or associated substances prevented the full expression of the calcium uptake system. The nature of this inhibitory effect may merit further study.

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