ENERGY REQUIREMENT FOR CALCIUM UPTAKE BY THYMUS LYMPHOCYTES

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Received 10 February 1978

1. Introduction

A central role of Ca^{2+} in growth and division of lymphocytes has been assessed using lectins and ionophore to promote Ca^{2+} influx in these cells [1–7]. However the energy required for Ca^{2+} transport into lymphocytes has not been determined in the absence of such compounds. Reports on other cells [8–12] showed that calcium uptake by intact isolated cells implies mitochondrial energy from two kinds of energetic substrates, ATP and respiratory substrates. Addition of these substrates in the incubation medium might be used as a device to investigate, in situ, the role of mitochondria in cellular Ca^{2+} uptake.

In this paper we report that none of the respiratory substrates could support by themselves Ca^{2+} uptake in thymus lymphocytes as they did in other cells [8–12]. In the presence of ATP, substantial accumulation of $^{45}Ca^{2+}$ occurred to a similar extent and with similar specificity as in other cells. By the use of ATP, the role of mitochondria in cellular calcium uptake could be shown in situ. Indeed the integrity of the mitochondrial oxidative and phosphorylative mechanisms was required for the ATP-supported Ca^{2+} uptake. The oxidation of NAD-linked substrates stimulated this transport whereas an opposite effect has been shown in cancer cells [12].

2. Experimental

Thymus glands were obtained from month 4–6 male rabbits killed by air injection into the marginal vein of the ear. The glands were immersed in cold medium 199 (Institut Pasteur, Paris) and dissected to remove fat and capillaries. The cells were separated from one another using a Dounce homogeniser. The resulting medium was filtered through a coarse gauze and the filtrate was centrifuged at 400 rev/min for 10 min at 4°C. The cells were washed twice in 10 vol. 'saline medium' – 130 mM NaCl, 5 mM KCl and 10 mM Hepes buffer [2-(*N*-2-hydroxyethyl-piperazin-*N'*-yl) ethanesulfonic acid (sodium salt)], pH 7.4, at 4°C, and gently suspended in saline medium. Cell count showed that 10^6 cells contained 18 μg protein and had dry wt 25 μg. The stock cell suspension was kept at 0°C until used, usually within 3 h. All batches of cells were checked with the Trypan blue exclusion test as discussed [12]: 2–6% were dye-permeable. The incubations were carried out in plastic tubes immersed in a 25°C water bath. 30×10^6 cells were incubated in final vol. 1 ml with the other components and the mixture equilibrated for 2 min at 25°C with gentle magnetic stirring. Ca^{2+} was then added at time zero. Generally 2.5 mM $CaCl_2$ was used labelled with 5 μCi $^{45}Ca^{2+}$/tube. Samples were taken periodically and $^{45}Ca^{2+}$ measured as in [12].

3. Results

3.1. *The ATP-supported Ca^{2+} uptake*

Figure 1a shows typical uptake of Ca^{2+} by thymus lymphocytes. Without additions, in saline medium only, the cells accumulated little or no Ca^{2+}. However addition of ATP produced a significant rise in $^{45}Ca^{2+}$ uptake, equivalent to 22 μg ions/mg protein. Maximum uptake was obtained by 1.5 mM $CaCl_2$ and

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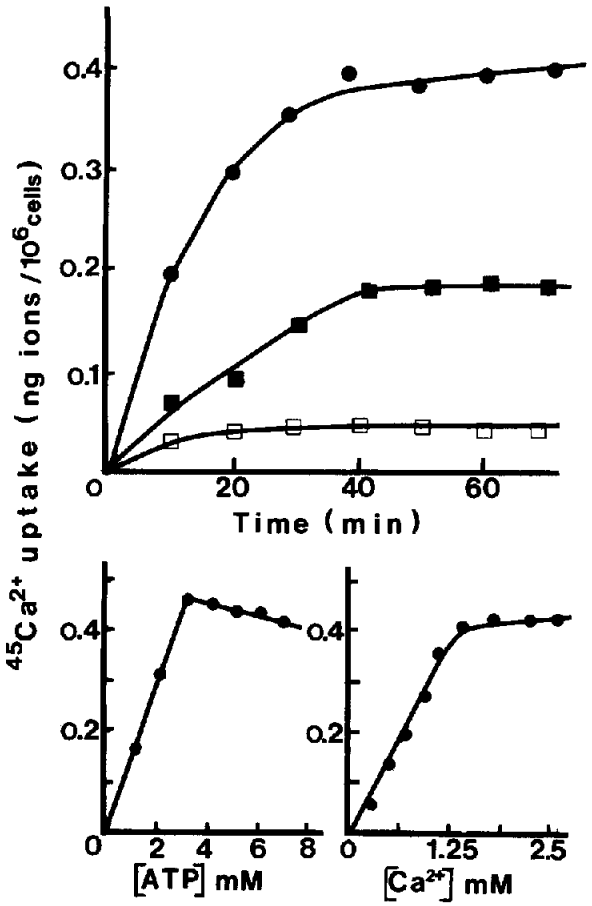

Fig.1. Characterisation of ATP-supported Ca^{2+} uptake by thymus lymphocytes. 32 × 10^6 cells were incubated in saline medium with 2.5 mM $CaCl_2$ (□), supplemented with 3 mM ATP (●) or 3 mM ATP + 20 μM Ruthenium Red (■), added in parallel experiments. Similar experiments were performed with increasing amounts of ATP or Ca^{2+}. $^{45}C^{2+}$ uptake was measured after 50 min incubation.

3 mM ATP. 20 μM Ruthenium Red, an inhibitor of Ca^{2+} transport in mitochondria and plasma membranes [13,14], inhibited 50% ATP-supported Ca^{2+} uptake. Higher concentrations were not more effective. ATP was completely specific in the support of Ca^{2+} uptake: GTP, UTP, ADP, GDP, UDP, AMP, GMP and UMP were completely inactive in supporting Ca^{2+} transport. Tri- and di-phosphonucleotides inhibited the ATP-supported Ca^{2+} uptake (table 1).

Various compounds acting on the plasma membrane and mitochondria were tested for their ability

Table 1
Effect of nucleotides on Ca^{2+} uptake by thymus lymphocytes

Nucleotides (3 mM)	$^{45}Ca^{2+}$ uptake (ng-ions/10^6 cells)	
	with 3 mM ATP	without ATP
–	0.63	0.07
ATP	0.52	0.63
GTP	0.37	0.09
UTP	0.38	0.12
CTP	0.39	0.10
ADP	0.41	0.09
GDP	0.27	0.08
UDP	0.26	0.08
AMP	0.64	0.10
GMP	0.64	0.12
UMP	0.59	0.10

30 × 10^6 cells were incubated in saline medium with 2.5 mM $CaCl_2$ for 50 min in parallel experiments

to interfere with the ATP-supported Ca^{2+} uptake. Both ouabaine, an inhibitor of Na, K-ATPase, and Verapamil (10^{-9}–10^{-4} M), which blocks the permeation of Ca^{2+} through the plasma membrane of different types of cells [15–17], were totally inactive. Table 2 shows that both oligomycin, an inhibitor of mitochondrial ATPase [18], and atractyloside, an antagonist of the ATP/ADP translocase of mitochondria [19], inhibited the ATP-supported Ca^{2+} influx.

Table 2
Effect of inhibitors on ATP-supported Ca^{2+} uptake

Inhibitors		$^{45}Ca^{2+}$ uptake (ng-ions/10^6 cells)
	–	0.59
Atractyloside	2.5 μM	0.44
	5 μM	0.22
	50 μM	0.20
Oligomycin	2.5 μg/ml	0.28
	5 μg/ml	0.18
	10 μg/ml	0.17

34 × 10^6 cells were incubated in saline medium with 3 mM ATP and 2.5 mM $CaCl_2$ for 50 min in parallel experiments. The uptake of $^{45}Ca^{2+}$ in the absence of ATP was 0.09 ng-ions/10^6 cells

These data suggested that an oligomycin-sensitive ATPase, probably located in the mitochondria, as shown by the atractyloside sensitivity, could be involved in cellular Ca^{2+} uptake.

3.2. *Regulatory effects of mitochondrial respiration on the ATP-supported Ca^{2+} uptake*

Extensive research performed on isolated mitochondria showed that the uptake of Ca^{2+} is energetically coupled to electron transport [22,23]. Similar conclusions can be drawn from experiments performed with carefully isolated hepatocytes [10] or Ehrlich cancer cells [12]. With thymus lymphocytes, when the saline medium was supplemented with respiratory substrates (malate, succinate or α-glycerophosphate), no Ca^{2+} uptake occurred in the absence of ATP, whereas the typical respiratory tracing shown in fig.2 substantiated the normal respiratory properties of these cells. However mitochondrial electron transport played a regulatory role in the cellular ATP-supported Ca^{2+} uptake. Figure 2 shows that rotenone inhibited the major part of this uptake and abolished completely the cellular oxygen consumption in the absence of added substrate. Consequently it may be concluded that the ATP-supported Ca^{2+} uptake is ineffective in the absence of electron transport. Succinate stimulated this uptake (fig.2) with a maximum effect at 5 mM. Rotenone, which inhibits the electron transport merging from NAD-linked substrates, inhibited partly the ATP-succinate-supported Ca^{2+} uptake. In contrast, antimycin, which impaired electron transport from both NAD-linked substrates and succinate, abolished completely the ATP-succinate-supported Ca^{2+} influx.

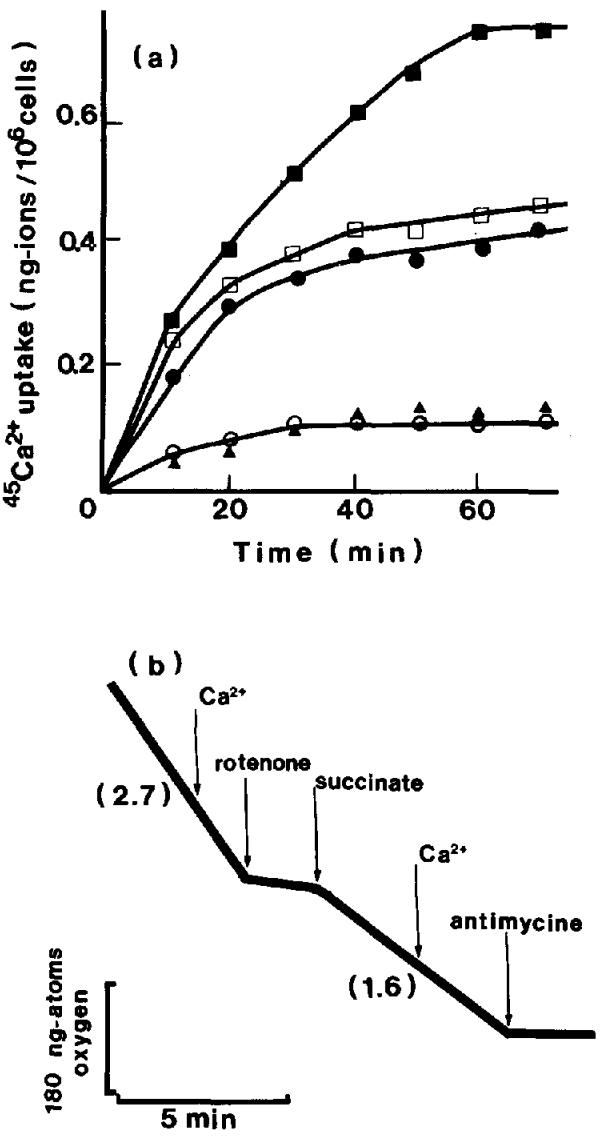

Fig.2. Dependence of the ATP-supported Ca^{2+} uptake on electron transport energy. (a) 34 × 10^6 cells were incubated with 2.5 mM $CaCl_2$ and 3 mM ATP (•) supplemented with 4 μM rotenone (▲); 5 mM succinate (■); 5 mM succinate, 4 μM rotenone (□); 5 mM succinate, 5 μM antimycin (○). (b) Oxygen consumption was followed with a Clark-type oxygen electrode in a water-jacketed 3 ml chamber at 25°C. 530 × 10^6 cells were incubated in the saline medium described for Ca^{2+} uptake. The additions indicated in the tracing were Ca^{2+} (2.5 mM), rotenone (4 μM), succinate (10 mM), and antimycin (5 μM). The numbers on the trace represent the rate of oxygen consumption in ng-atoms oxygen/min/mg protein.

4. Discussion

Our observations show that ATP was able to promote Ca^{2+} uptake in rabbit thymus lymphocytes. The specificity of ATP in promoting Ca^{2+} uptake, in contrast to other nucleotides, was shown in cultured chick-embryo fibroblasts [11], rat mast cells [9] and Ehrlich cancer cells [12] as presently in lymphocytes. Consequently, ATP might be considered as a general device to promote cellular Ca^{2+} uptake. Two effects of extracellular ATP have been proposed [12]:

1. To increase membrane permeability of the cell to both Ca^{2+} and ATP.
2. To supply energy during its oligomycin-sensitive hydrolysis in the mitochondria for the transport of cytosolic Ca^{2+} into the mitochondrial matrix.

As no transport process for nucleotides has been shown at the level of the plasma membrane, we may suppose that the inhibitory effect of atractyloside (table 2) was due to its well-documented antagonism with ATP/ADP translocation in the inner mitochondrial membrane.

The observation of an ATP-induced Ca^{2+} uptake raises the question of the localisation of the Ca^{2+} transport process(es) involved in this phenomenon. Considerable evidence indicates that the homeostasis of cell Ca^{2+} is maintained by 3 active processes located in the plasma membrane, the mitochondria and in other reservoirs, such as the sarcoplasmic reticulum in muscle cells. The Ca^{2+}, Mg^{2+}-ATPase of the plasma membrane is oligomycin-insensitive [20,21] and the Ca^{2+} flux promoted by this enzyme is outward directed. Consequently, the oligomycin sensitivity of cellular Ca^{2+} uptake should be related to an intramitochondrial hydrolysis of ATP. In our experimental conditions, Ca^{2+} did not cross the plasma membrane through Verapamil sensitive pores like those shown in cardiac cells [15], smooth vascular muscle cells [16] and pancreatic islet cells [17]. The observed cellular Ca^{2+} influx appeared to be highly dependent on a mitochondrial ATP- and electron transport-linked Ca^{2+} transport process.

The intervention of the mitochondrial electron transport in Ca^{2+} uptake was intensively studied with isolated mitochondria [22,23]. However, results obtained with intact cells showed that mitochondria in situ, may display a slightly different behaviour, possibly related to regulatory cytosolic components [12]. It was shown previously that NAD-linked substrates, i.e., site-1-linked, did not support Ca^{2+} uptake by intact cells [12]. Our results show that in thymus lymphocytes, both site-1- and site-2-linked substrates, i.e., NAD-linked substrates, succinate and α-glycerophosphate were unable to support cellular Ca^{2+} uptake in the absence of added ATP. Moreover, it has been suggested that NAD-linked substrates may stimulate Ca^{2+} release from mitochondria in intact cells, possibly in association with specific cytosol components [12]. This hypothesis, based on the observation of a stimulatory effect of rotenone on the rapid succinate-induced cellular uptake, cannot be applied to lymphocytes as no effect of rotenone was observed in the absence of ATP. Consequently it appears that, in thymus lymphocytes, cellular Ca^{2+} uptake requires both ATP and mitochondrial electron transport, each factor being ineffective in the absence of the other, whereas they can be dissociated in other tissues.

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