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Ca^{2+} uptake and IP_3-induced Ca^{2+} release in permeabilized human lymphocytes

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The $^{45}Ca^{2+}$ uptake and $^{45}Ca^{2+}$ release in saponin-permeabilized human lymphocytes were studied. An ATP-dependent Ca^{2+} uptake into a nonmitochondrial, intracellular Ca^{2+} store is observed which is approx. 2 orders of magnitude greater than the ATP-independent Ca^{2+} uptake. The Ca^{2+} uptake is inhibited by vanadate, but it is insensitive to oligomycin and ruthenium red. IP_3 induces dose-dependent $^{45}Ca^{2+}$ release. For half-maximum Ca^{2+} release 0.25–0.5 μM IP_3 is required. The results of our studies suggest that $^{45}Ca^{2+}$ is predominantly stored within the endoplasmic reticulum of the lymphocytes.

Ca^{2+}; Inositol phosphate; Lymphocyte; (Human)

1. INTRODUCTION

In various mammalian cells inositol 1,4,5-trisphosphate (IP_3) functions as an intracellular messenger [1,2]. Upon stimulation of an extracellular receptor, membrane-associated phosphatidylinositol 4,5-bisphosphate is split into diacylglycerol and IP_3. The release of IP_3 initiates a cascade of events which finally results in the mobilization of intracellular Ca^{2+}. Polyphosphoinositides participate in the activation of lymphocytes. Recently, the activation of mouse B-lymphocytes by an anti-Ig-induced release of IP_3 and the concomitant increase in $[Ca^{2+}]_i$ have been reported [3,4]. The receptor-transduced mobilization of intracellular Ca^{2+} in T-cells also seems to be mediated by IP_3 [5]. This paper is concerned with the $^{45}Ca^{2+}$ uptake and IP_3-induced $^{45}Ca^{2+}$ release in saponin-permeabilized human lymphocytes.

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2. MATERIALS AND METHODS

Human lymphocytes from fresh blood of healthy adult donors were isolated by Ficoll-Paque density gradient centrifugation [6]. The isolated lymphocytes were washed twice with Hank's balanced salt solution [7]. In order to remove the contaminating monocytes, 1×10^7 lymphocytes were suspended in 1 ml of a modified RPMI-1640 solution and kept overnight in polycarbonate dishes (pH 7.4, 20°C). The RPMI-1640 solution was supplemented with 20 mM Hepes, 10% (v/v) fetal calf serum and 10 μg/ml gentamycin. The cell number was determined with a Coulter DN counter. The yield of lymphocytes ranges from 1×10^8 to 2×10^8 cells/500 ml blood. The cell population as assessed by morphological criteria consisted of pure, peripheral lymphocytes. The samples were entirely devoid of blood platelets, erythrocytes and lymphocytes. All experiments were executed with Saarstaedt polycarbonate centrifuge tubes.

Before experiments, the lymphocytes were washed once with about 10 ml buffer A (buffer A: 20 mM NaCl, 100 mM KCl, 5 mM $MgSO_4$, 0.96 mM Na_2PO_4, 25 mM Hepes, 1 mM EGTA,

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2% BSA). 1×10^7 cells/ml buffer A were preincubated for 60 min and permeabilized by the addition of 150 μg/ml saponin and 3 mM ATP (5 min, pH 7.2, 37°C). Under these conditions nearly all lymphocytes become permeable for trypan blue. Subsequently, 2×10^7 lymphocytes were incubated in 1 ml Ca^{2+}/EGTA solution and labeled with $^{45}Ca^{2+}$. The Ca^{2+}/EGTA solution consisted of buffer A plus 490 μM $^{45}Ca^{2+}$ (spec. act. 3 μCi/490 nmol), 5 μg/ml rotenone, 3 mM ATP, 5 mM creatine phosphate and 5 U/ml creatine phosphokinase which served as an ATP-regenerating system. $[Ca^{2+}]_f$ was adjusted to 180 nM according to Burgess et al. [8] if not indicated otherwise. Endogenous ATP production by the mitochondria was inhibited by rotenone.

The Ca^{2+} uptake and Ca^{2+} release in permeabilized lymphocytes were followed by withdrawing aliquots of 100 μl each from the samples at appropriate time intervals. The aliquots were added to 1 ml ice-cold buffer B and the cells were sedimented by centrifugation (buffer B: 130 mM KCl, 5 mM $MgCl_2$, 25 mM Hepes, 5 mM EGTA, pH 7.2). The pellet was washed once with 1 ml EGTA-free buffer B and the $^{45}Ca^{2+}$ was extracted with 100 μl of 0.1% Triton X-100/1 N NaOH (60 min, 56°C). Upon neutralization of the extracts, the $^{45}Ca^{2+}$ was counted. The protein content of the samples was determined according to Lowry et al. [9]. 2.5×10^7 cells correspond to 1 mg cell protein.

$^{45}Ca^{2+}$ and IP_3 were purchased from Amersham-Buchler (Braunschweig); rotenone, oligomycin, RPMI-1640, BSA, CP, CP kinase and Triton X-100 from Serva (Heidelberg); ruthenium red, Hepes, EGTA, Mg-ATP and vanadate from Sigma (Taufkirchen); and NaCl, KCl, $MgSO_4$, Na_2HPO_4, NaH_2PO_4, HCl and NaOH of p.a. grade were from Merck (Darmstadt).

3. RESULTS

Lymphocytes were isolated from fresh human blood by density gradient centrifugation with Ficoll-Paque [8] and stored overnight in modified RPMI-1640 solution. Before experiments, the lymphocytes were washed and permeabilized by saponin. The lymphocytes then were incubated in a Ca^{2+}/EGTA solution. The Ca^{2+} uptake and IP_3-induced Ca^{2+} release were assessed by using $^{45}Ca^{2+}$.

Table 1 shows the ATP-dependent Ca^{2+} uptake in saponin-permeabilized lymphocytes. Ca^{2+} uptake was initiated by adding 3 mM ATP to the cell suspensions. $^{45}Ca^{2+}$ within the cells was counted 45 min after the addition of ATP. Mitochondrial Ca^{2+}-ATPase and mitochondrial substrate consumption were inhibited by oligomycin and ruthenium red, respectively. At $[Ca^{2+}]_f$ 180 nM, the Ca^{2+} uptake is insensitive to oligomycin and ruthenium red, but on increasing $[Ca^{2+}]_f$ an increasing fraction of the ATP-dependent Ca^{2+} uptake is inhibited by oligomycin and ruthenium red. These results suggest that at a low $[Ca^{2+}]_f$ $^{45}Ca^{2+}$ is predominantly stored in a nonmitochondrial Ca^{2+} pool while at a high $[Ca^{2+}]_f$ a considerable fraction of $^{45}Ca^{2+}$ is taken up by the mitochondria.

The time course of the ATP-dependent and ATP-independent Ca^{2+} uptake in permeabilized lymphocytes is shown in fig.1. The ATP-dependent Ca^{2+} uptake exhibits a continuous increase in intracellular $^{45}Ca^{2+}$ over a period of approx. 60 min. Thereafter, a steady-state distribution of $^{45}Ca^{2+}$ is attained. The maximum ATP-dependent Ca^{2+} uptake, as assessed from the equilibrium distribution of $^{45}Ca^{2+}$, displays strong variations and ranges from 4 to 16 nmol/mg cell protein. This corresponds to 1.6–6.4 nmol/10^7

Table 1

Effect of mitochondrial Ca^{2+}-transport inhibitors on ATP-dependent Ca^{2+} uptake in permeabilized human lymphocytes

$[Ca^{2+}]_f$	Ca^{2+} content (nmol/mg cell protein)		Inhibition (%)
	Control	Oligomycin ruthenium red	
180 nM	3.8	3.8	0
234 nM	7.8	4.9	37
758 nM	208.9	60.1	71
1.32 μM	323.7	42.7	87
3.30 μM	216.6	27.9	87

2×10^7 cells/ml Ca^{2+}-EGTA solution. $^{45}Ca^{2+}$ within the cells was counted 30 min after the addition of ATP. Mitochondrial Ca^{2+} uptake was inhibited by 3 μg/ml oligomycin plus 1 mM ruthenium red. $[Ca^{2+}]_f$ as indicated. pH 7.2, 37°C

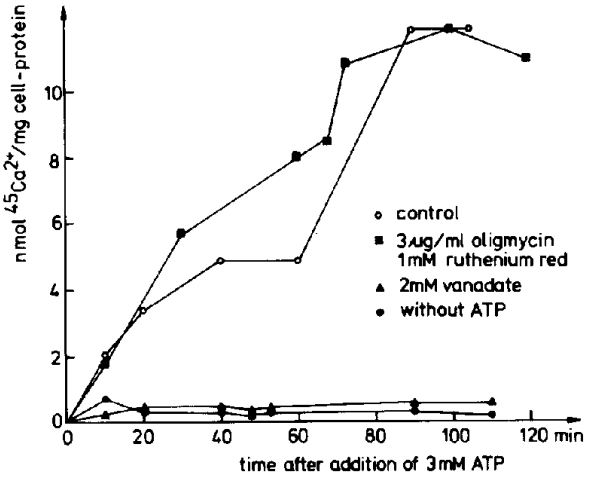

Fig.1. ATP-dependent and ATP-independent $^{45}Ca^{2+}$ uptake in permeabilized lymphocytes. 2×10^7 cells/ml Ca^{2+}-EGTA solution. $[Ca^{2+}]_f$ = 180 nM. pH 7.2, 37°C.

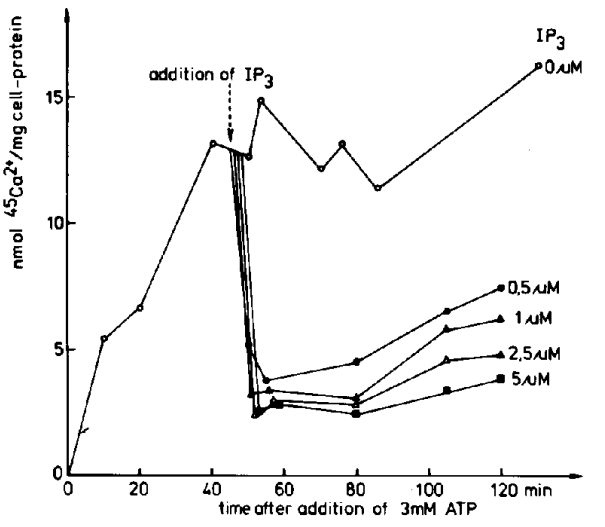

Fig.2. IP_3-induced $^{45}Ca^{2+}$ release in $^{45}Ca^{2+}$-labelled, permeabilized lymphocytes. 2×10^7 cells/ml Ca^{2+}-EGTA solution. $[Ca^{2+}]_f$ = 180 nM. IP_3 was added as indicated. pH 7.2, 37°C.

cells. The initial Ca^{2+} uptake exhibits similar variations and ranges from 110 to 300 pmol/min per mg cell protein. The ATP-independent Ca^{2+} uptake is approx. 2 orders of magnitude lower than the ATP-dependent Ca^{2+} uptake and displays the features of nonspecific Ca^{2+} binding to cell constituents.

The ATP-dependent uptake of $^{45}Ca^{2+}$ is insensitive to oligomycin and ruthenium red, but is completely inhibited by vanadate (fig.1). Vanadate reduces both the initial $^{45}Ca^{2+}$ uptake and maximum $^{45}Ca^{2+}$ uptake of the cells at steady state. The vanadate concentrations for half-maximum inhibition of the ATP-dependent Ca^{2+} uptake as obtained from the dose-response curves amount to about 0.25 mM. 2 mM vanadate causes almost complete inhibition of the $^{45}Ca^{2+}$ uptake in permeabilized lymphocytes.

The IP_3-sensitive Ca^{2+} release was assessed by labeling the permeabilized lymphocytes for approx. 60 min with $^{45}Ca^{2+}$ until a steady-state distribution of $^{45}Ca^{2+}$ was reached. As shown in fig.2, IP_3 induces instantaneous $^{45}Ca^{2+}$ release in permeabilized lymphocytes. 0.5 and 1.0 µM IP_3 cause Ca^{2+} release of about 60 and 72%, respectively. 2.5 µM IP_3 elicits maximum Ca^{2+} release which amounts to about 80% of the ATP-dependent, intracellularly stored $^{45}Ca^{2+}$. For half-maximum release of $^{45}Ca^{2+}$ from the IP_3-sensitive, intracellular Ca^{2+} pool, 250–500 nM IP_3 is required. The IP_3-induced release of $^{45}Ca^{2+}$ is followed by $^{45}Ca^{2+}$ reuptake. The exposure of lymphocytes to high doses of IP_3 decreases the rate of $^{45}Ca^{2+}$ reuptake. This effect most probably should be attributed to the slow breakdown of IP_3 in our assay.

Table 2

Effect of vanadate on IP_3-induced Ca^{2+} release and Ca^{2+} reuptake in permeabilized lymphocytes

	$[^{45}Ca^{2+}]_{IP_3}/[^{45}Ca^{2+}]_{IP_3=0}$ (%)			
	$[IP_3]$: 0.5 µM	1.0 µM	2.5 µM	5.0 µM
5 min after IP_3	59	75	79	80
80 min after IP_3	42	51	62	69
80 min after IP_3 plus 2 mM vanadate	77	75	82	82

2×10^7 cells/ml incubation medium. $[Ca^{2+}]_f$ = 180 nM, pH 7.2, 37°C. Samples were incubated for 45 min. Subsequently, IP_3 or IP_3 + vanadate were added to the respective samples. Intracellular $^{45}Ca^{2+}$ was counted before ($[^{45}Ca^{2+}]_{IP_3=0}$) and after ($[^{45}Ca^{2+}]_{IP_3}$) the addition of IP_3. The Ca^{2+} stored within the IP_3-sensitive Ca^{2+} pool of the cells amounts to 4.2 nmol/mg protein

Vanadate augments the IP_3-induced Ca^{2+} release in permeabilized, $^{45}Ca^{2+}$-labeled lym-

phocytes (table 2), whereas oligomycin and ruthenium red have no effect on IP_3-induced Ca^{2+} release. 2.5–5.0 μM IP_3 elicits maximum Ca^{2+} release from the IP_3-sensitive Ca^{2+} pool, while in the presence of 2 mM vanadate, 0.5 μM IP_3 suffices for maximum Ca^{2+} release. The addition of vanadate has no direct, stimulating effect upon the Ca^{2+} release in permeabilized lymphocytes, but does inhibit the Ca^{2+} reuptake upon an IP_3 stimulus.

4. DISCUSSION

According to our results, most of the ATP-dependent, intracellularly stored $^{45}Ca^{2+}$ appears to be stored within the endoplasmic reticulum (ER) of the lymphocytes which has a higher affinity for Ca^{2+} than mitochondria. This corresponds to the Ca^{2+} uptake in liver cells [8] and pancreatic acinar cells [10]. In addition, the ATP-dependent Ca^{2+} uptake in permeabilized lymphocytes is inhibited by vanadate which is known to inhibit Ca^{2+} uptake by the ER [11], but it is not susceptible to inhibitors of mitochondrial Ca^{2+} transport [12,13]. The variations in Ca^{2+} uptake in permeabilized lymphocytes could result from the permeabilization procedure or from differences in the Ca^{2+}-transport capacity of the cells. We also have some evidence for individual differences in Ca^{2+} uptake, but no systematic studies concerning this point have been performed. The maximum Ca^{2+} release from the intracellular, IP_3-sensitive Ca^{2+} pool amounts to about 80% of the ATP-dependent, intracellularly stored $^{45}Ca^{2+}$. The remaining 20% of the accumulated $^{45}Ca^{2+}$ is insensitive to IP_3 and could be stored either in an IP_3-insensitive part of the ER or within the mitochondria.

The significance of the IP_3-mediated Ca^{2+} release from an intracellular Ca^{2+} store for the activation of lymphocytes is difficult to assess. For signal transduction, the Ca^{2+} release from intracellular Ca^{2+} stores appears to be negligible as compared to Ca^{2+} influx across the lymphocyte plasma membrane [14]. On the other hand, intracellularly stored Ca^{2+} is more rapidly available for the cell and possibly can be stored in close vicinity to the Ca^{2+} trigger for a biochemical reaction. Assuming a cell volume of 120 μm^3 and a cytosolic, intracellular $[Ca^{2+}]_f$ of 100–200 nM under resting conditions, cytosolic Ca^{2+} is 1.2×10^{-20}–2.4×10^{-20} mol/cell, while the Ca^{2+} content of the intracellular, IP_3-sensitive Ca^{2+} pool is approx. 1.6×10^{-16}–6.4×10^{-16} mol/cell. Thus a single maximal IP_3 pulse could induce Ca^{2+} release of 1.28×10^{-16}–5.12×10^{-16} mol/cell which could result in a transient increase of $[Ca^{2+}]_i$ by approx. 4 orders of magnitude.

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