Effects of vanadate on intracellular Ca^{2+} redistribution and hexose transport across plasma membrane in cultured mouse fibroblasts

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The effects of vanadate on intracellular Ca^{2+} sequestration and hexose transport were studied in Swiss 3T3 cells. Vanadate inhibited ATP-dependent Ca^{2+} uptake by saponin-permeabilized Swiss 3T3 cells at 10^{-5} and 10^{-7} M Ca^{2+} at which the Ca^{2+} uptake was sensitive and insensitive to oligomycin plus antimycin A, respectively. On the other hand, vanadate stimulated 2-deoxy-D-glucose (2DG) uptake in a dose- and time-dependent way. The stimulation of 2DG uptake by vanadate was inhibited by EGTA plus A23187 and the inhibition was reversed by Ca^{2+} restoration. These results suggest that an increase in cytosolic Ca^{2+} by inhibition of intracellular ATP-dependent Ca^{2+} sequestration by vanadate results in the stimulation of hexose transport in Swiss 3T3 cells.

Vanadate *Intracellular Ca^{2+}* *Ca^{2+} sequestration* *Hexose transport* *Membrane transport* *Cultured fibroblast*

1. INTRODUCTION

Hexose transport across plasma membrane is a primary regulatory site of cellular metabolism. In quiescent fibroblasts, hormones, growth factors and tumor promoters increase hexose transport prior to stimulation of DNA synthesis and cell proliferation [1]. We have proposed the possibility that Ca^{2+} regulates hexose transport, the activation of which by tumor promoter and epidermal growth factor (EGF) depends on Ca^{2+} [2].

Vanadate is known to have a wide range of biochemical and physiological activities. In vitro, vanadate inhibits a number of phosphate transfer reactions such as some ATPases, phosphatases and kinases [3]; in cultured human and mouse fibroblasts, vanadate stimulates DNA synthesis and it acts synergistically with EGF and insulin [4,5]. However, the effect of vanadate on hexose transport has not been studied in cultured fibroblasts. In isolated fat cells, vanadate, like insulin, increases hexose transport [6]. It has been suggested that this insulin-mimetic effect of vanadate was associated with a rise in the cytoplasmic Ca^{2+} level resulting from the inhibition of Ca^{2+}-ATPase of endoplasmic reticulum by vanadate [7].

Here, we examine the effect of vanadate on Ca^{2+} uptake by saponin-permeabilized Swiss 3T3 mouse fibroblasts and on hexose transport in intact Swiss 3T3 cells. From the results, we suggest that the vanadate-induced redistribution of intracellular Ca^{2+} results in the stimulation of hexose transport in Swiss 3T3 cells.

2. MATERIALS AND METHODS

2.1. *Cell culture*

Swiss 3T3 mouse fibroblasts [8] were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS), as in [9]. Cells were seeded at 3×10^5 cells/2 ml into plastic petri dishes 35 mm in diameter and grown at 37°C in a water-saturated atmosphere contain-

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ing 5% CO_2 in air. Three days later, dishes containing monolayer cells which had just become confluent were used for experiments.

2.2. *Measurement of Ca^{2+} uptake by saponin-permeabilized cells*

Swiss 3T3 cells were washed twice with 2 ml of 20 mM Tris–maleate buffer (pH 6.8) containing 130 mM KCl and 0.1% bovine serum albumin (BSA), followed by addition of 1 ml of this buffer containing 0.2 mg/ml saponin. After 10 min of incubation at room temperature (about 20°C), the saponin solution was removed and the cells were rinsed twice with 2 ml of 20 mM Tris–maleate buffer (pH 6.8) containing 130 mM KCl only. By this treatment, Swiss 3T3 cells became almost completely permeable to 0.3% trypan blue.

$^{45}Ca^{2+}$ uptake was started by addition of 1 ml assay solution composed of 20 mM Tris–maleate buffer (pH 6.8), 130 mM KCl, 5 mM $MgCl_2$ and free $^{45}Ca^{2+}$ (0.1 or 2 μCi/ml). After 10 min incubation at room temperature, 1 mM MgATP was added and the incubation was continued. After a designated period, the uptake was stopped by rapid washing (3 times) with ice-cold 20 mM Tris–maleate buffer (pH 6.8) containing 130 mM KCl and 10^{-5} M Ca^{2+}. Cells were solubilized with 0.1 N NaOH/0.1% SDS solution and aliquots of the lysate were taken for assay of radioactivity and for determination of protein concentration. As non-specific uptake of $^{45}Ca^{2+}$, we accounted the radioactivity adsorbed by the dish when the same procedures as above were carried out. Data are expressed as nmol Ca^{2+} taken up per mg cell protein after correction for non-specific uptake.

2.3. *Adjustment of free Ca^{2+} concentration*

Free Ca^{2+} concentration of the assay solution was adjusted with $CaCl_2$ and EGTA, at which 8.45×10^5 M^{-1} was used as the apparent binding constant of EGTA to Ca^{2+} at pH 6.8 [10] and the contaminated Ca^{2+} concentration was estimated to be approx. 10^{-5} M [11].

2.4. *Measurement of hexose uptake by intact cells*

The uptake of 2-deoxy-D-glucose (2DG) by Swiss 3T3 cells was measured as in [9]. Cells were rinsed with 2 ml Ca^{2+}-, Mg^{2+}-free phosphate-buffered saline (PBS) containing 1 mg/ml BSA and the uptake was initiated by addition of 0.75 ml PBS containing 4 μM [^{3}H]2DG (1 μCi/ml). After cells were incubated for the designated period at room temperature, the uptake was stopped by rapid washing (twice) with 2 ml PBS containing BSA, and finally once with PBS. Cells were dissolved in 1 ml of 0.1 N NaOH/0.1% SDS solution, and aliquots of the lysate were taken for assay of radioactivity and for determination of protein concentrations. Data are expressed as pmol 2DG taken up/mg cell protein per min after correction for non-specific uptake determined with L-[^{3}H]glucose. The linearity of 4 μM 2DG uptake was maintained for at least 5 min.

2.5. *Determination of protein concentrations*

Concentration of protein was assayed by a modified Lowry method [12].

2.6. *Materials*

$^{45}CaCl_2$ (16.78 mCi/mg) and 2-deoxy-D-[G-^{3}H]-glucose (5 Ci/mmol) and L-[1(n)-^{3}H]glucose (10.7 Ci/mmol) were purchased from New England Nuclear. Na_3VO_4, ATP, oligomycin and antimycin A were obtained from Sigma; A23187 was from Calbiochem. Epidermal growth factor (EGF) was a kind gift from Dr Matuo, Japan Chemical Research, Kobe. All other chemicals were obtained from commercial sources, and either reagent grade of the highest purity otherwise available.

3. RESULTS

As shown in fig.1A, 1 mM ATP markedly stimulated Ca^{2+} uptake by saponin-permeabilized Swiss 3T3 cells. However, an increase in the ATP-stimulated Ca^{2+} uptake was completely inhibited by addition of 1 mM vanadate (Na_3VO_4). When vanadate was present before addition of ATP, vanadate decreased ATP-stimulated Ca^{2+} uptake in a dose-dependent manner (fig.1B). At 10^{-5} M Ca^{2+}, ATP-stimulated Ca^{2+} uptake was sensitive to oligomycin plus antimycin A (fig.2A). At this Ca^{2+} concentration, therefore, mitochondria appeared to be the major uptake site of Ca^{2+}. Whether or not the mitochondrial inhibitors were present, vanadate inhibited the ATP-stimulated Ca^{2+} uptake at 10^{-5} M Ca^{2+}. On the other hand, at 10^{-7} M Ca^{2+}, oligomycin plus antimycin A could not inhibit ATP-stimulated Ca^{2+} uptake

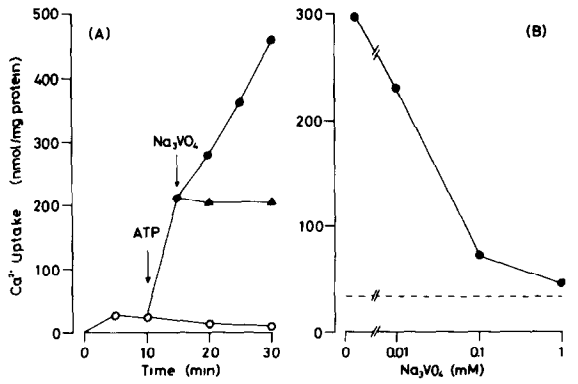

Fig.1. Effect of vanadate on ATP-stimulated Ca^{2+} uptake by saponin-permeabilized Swiss 3T3 cells. (A) Time course of the ATP-stimulated $^{45}Ca^{2+}$ uptake and the effect of vanadate. In saponin-treated Swiss 3T3 cells, $^{45}Ca^{2+}$ uptake at 10^{-5} M $^{45}Ca^{2+}$ (0.1 μCi/ml) was started at time zero. At 10 min, 1 mM MgATP (●) or 4 μl distilled water as control (○) and, at 15 min, 1 mM Na_3VO_4 (▲) was added. Each point represents the average value for a duplicate experiment. (B) Effect of various concentrations of vanadate on the ATP-stimulated $^{45}Ca^{2+}$ uptake. In saponin-treated Swiss 3T3 cells, $^{45}Ca^{2+}$ uptake at 10^{-5} M $^{45}Ca^{2+}$ (0.1 μCi/ml) was started in the presence of various concentrations of Na_3VO_4. After 10 min of incubation, 1 mM MgATP was added and the uptake was continued for additional 10 min. The broken line represents the level of Ca^{2+} uptake without MgATP for these experiments. Each point represents the average value for a duplicate experiment.

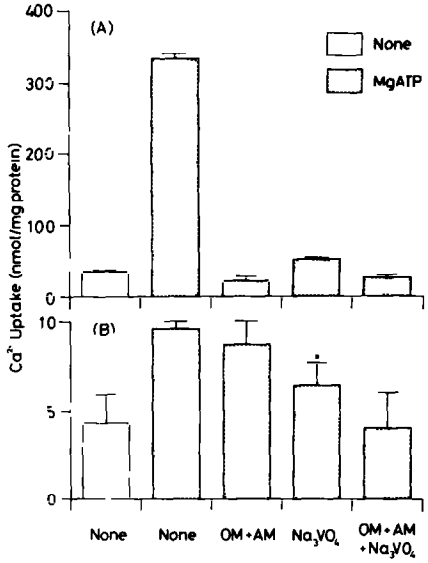

Fig.2. Effect of oligomycin plus antimycin A and vanadate on ATP-stimulated Ca^{2+} uptake at 10^{-5} or 10^{-7} M Ca^{2+} by saponin-treated Swiss 3T3 cells. In saponin-treated Swiss 3T3 cells, $^{45}Ca^{2+}$ uptake at 10^{-5} M $^{45}Ca^{2+}$ (0.1 μCi/ml) (A) or 10^{-7} M $^{45}Ca^{2+}$ (2 μCi/ml) (B) was started in the presence of 5 μg/ml oligomycin; OM, plus 1 μg/ml antimycin A; AM, and/or 1 mM vanadate; Na_3VO_4. After 10 min incubation, 1 mM MgATP was added and the uptake was continued for additional 10 min. Each value represents mean ± SE for triplicate dishes. * $p < 0.05$ (*vs* control + MgATP).

(fig.2B). Possibly, at 10^{-7} M Ca^{2+}, Ca^{2+} is taken up by a non-mitochondrial Ca^{2+} storage site such as the endoplasmic reticulum [13]. At this Ca^{2+} concentration, vanadate also suppressed the ATP-stimulated Ca^{2+} uptake.

Fig.3 shows the effect of vanadate on 2DG uptake by intact Swiss 3T3 cells. Vanadate at 2 mM stimulated 2DG uptake within 30 min and the effect increased with time (fig.3A). Vanadate stimulated 2DG uptake in a dose-dependent fashion (fig.3B); vanadate, at concentrations higher than 10 μM, significantly stimulated 2DG uptake. However, under the condition that intracellular Ca^{2+} was released by 5 μM Ca^{2+} ionophore A23187 plus 5 mM EGTA [2], vanadate-stimulated 2DG uptake was suppressed 70% (table 1). The inhibition of vanadate-stimulated 2DG uptake was completely reversed by restoration of 5 mM Ca^{2+} in the medium. As

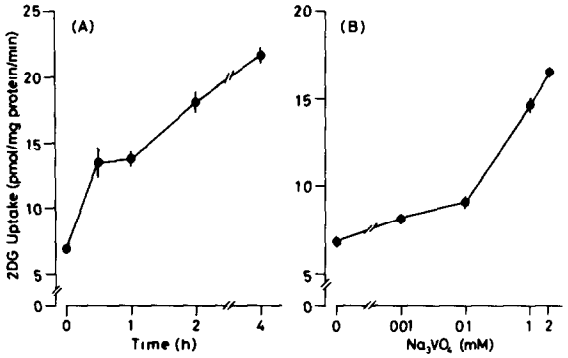

Fig.3. Effect of vanadate on 2DG uptake by Swiss 3T3 cells. (A) Time course of the effect of vanadate on 2DG uptake. Cells were treated with 2 mM vanadate for the indicated period of time, and then 2DG uptake during 5 min was determined as described in section 2. (B) Effect of various concentrations of vanadate on 2DG uptake. Cells were incubated with various concentrations of vanadate for 2 h, and then 2DG uptake during 5 min was determined as described in section 2. Each point represents mean ± SE for triplicate dishes.

Table 1

Effect of EGTA, A23187 and Ca^{2+} on vanadate-stimulated 2DG uptake by Swiss 3T3 cells

	Treatment	2DG uptake (pmol/mg protein per min)
5 mM EGTA	control	3.10 ± 0.15
	Na_3VO_4 (2 mM)	8.95 ± 0.77
	A23187 (5 μM) + Na_3VO_4 (2 mM)	5.54 ± 0.40
	A23187 (5 μM)	3.58 ± 0.24
5 mM EGTA + 5 mM Ca^{2+}	control	2.61 ± 0.53
	Na_3VO_4 (2 mM)	10.4 ± 0.53
	A23187 (5 μM) + Na_3VO_4 (2 mM)	12.0 ± 1.41
	A23187 (5 μM)	4.35 ± 0.15

Medium was replaced with DMEM supplemented with 0.25% dialyzed FCS containing 5 mM EGTA or 5 mM EGTA plus 5 mM $CaCl_2$ and the indicated agents were added. After 1 h of incubation at 37°C, 2DG uptake during 5 min was determined as described in section 2

Table 2

Effect of vanadate and EGF on 2DG uptake by Swiss 3T3 cells

Treatment	2DG uptake (pmol/mg protein per min)
Control	8.32 ± 0.54
Na_3VO_4 (2 mM)	16.9 ± 0.38
EGF (10 ng/ml)	16.4 ± 1.48
EGF (10 ng/ml) + Na_3VO_4 (2 mM)	18.6 ± 0.71

The indicated agents were added simultaneously and after 2 h incubation at 37°C, 2DG uptake during 5 min was determined as described in section 2

shown in table 2, simultaneous addition of vanadate and EGF did not show an additive effect.

4. DISCUSSION

Vanadate has been considered to be an inhibitor of non-mitochondrial Ca^{2+} sequestration [14]. However, in this study, we found that vanadate inhibits ATP-dependent intracellular Ca^{2+} sequestration within both mitochondrial and non-mitochondrial Ca^{2+} storage sites (fig.1,2). Thus, inhibitory effects of vanadate on the intracellular Ca^{2+} sequestration may result in an increase in cytosolic free Ca^{2+} in Swiss 3T3 cells.

It was also found that vanadate stimulates hexose transport in Swiss 3T3 cells (fig.3). The effective concentration of vanadate on hexose transport in Swiss 3T3 cells is comparable to that in isolated rat adipocytes [6]. Our previous results suggested that intracellular Ca^{2+} can control hexose transport system in Swiss 3T3 cells [2]. Probably, intracellular Ca^{2+} plays an important role in the stimulation of hexose transport by vanadate, because vanadate-stimulated hexose transport was clearly suppressed under the condition that intracellular Ca^{2+} was washed out, and was recovered by Ca^{2+} restoration (table 1). Vanadate may stimulate hexose transport system through a similar mechanism to EGF, because EGF-stimulated hexose transport depends on intracellular Ca^{2+} [2] and a simultaneous addition of vanadate with EGF did not cause an additive effect on hexose transport (table 2).

In conclusion, we suggest that vanadate inhibits intracellular ATP-dependent Ca^{2+} sequestration within mitochondrial and non-mitochondrial Ca^{2+} storage sites, followed by an increase in cytosolic free Ca^{2+} level and thereby stimulates hexose transport in Swiss 3T3 cells.

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