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# VITAMIN C DECREASES INTRACELLULAR CALCIUM LEVEL IN HUMAN LYMPHOID CELLS

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Human lymphocytes have low levels of many antioxidant enzymes however they are know to concentrate vitamin C. Cell injury, including oxidative stress effects, is associated with calcium influx so the influence of vitamin C on the maintenance of calcium levels in leukocytes was studied. Incubation of Molt-3 human lymphoblastoid cells with physiologically relevant concentrations of vitamin C and the calcium ionophore A23187 reversed the calcium influx and increased nuclear protein level associated with the ionophore alone. It is concluded that intracellular vitamin C can inhibit calcium influx into leukocytes so helping to minimise cell damage.

Key words: *Ascorbic acid, calcium ionophore, Molt-3*

## INTRODUCTION

Most species, with the notable exception of humans, other primates, fruit bats, guinea pigs and some birds species are capable of the synthesising vitamin C (ascorbic acid). Vitamin C has a number of functions such as its metabolic role as a redox co-enzyme for dopamine B-hydroxylase and peptidyl glycine hydroxylase. It is now recognised that vitamin C also functions as a major radial trapping antioxidant and is the first antioxidant to be consumed when plasma is subjected to an oxidative challenge *in vitro* (1). Also, accumulating research evidence suggests that vitamin C may co-operate with other antioxidants particularly vitamin E (alpha-tocopherol), by regenerating vitamin E from the tocopheroxyl radical formed during the oxidation of vitamin E (2).

In humans dietary vitamin C is readily incorporated into circulating leukocytes where its role has not, however, been precisely defined. This may be of particular relevance in lymphocytes where the relatively high nuclear to cytoplasmic ratio would lead to lower cellular levels of cytoplasmic antioxidant

enzymes such as catalase or superoxide dismutase. Vitamin C has been seen to plateau at a concentrations of about 3 mM in lympocytes (3).

Calcium ion influx into cells accompanies cell injury caused by a variety of stimuli, including oxidative stress and calcium also appears to be involved in the production of reactive oxygen species (ROS). Calcium ionophore A23187 has been shown to induce a burst of metabolic activity culminating in the release of ROS (4, 5). Metal-chelating agents such as ethylenediaminetetraacetic acid (EDTA) and oxalic acid tend to reduce this catalytic activity. Hydrogen peroxide (which can produce ROS) induces raised intracellular calcium levels $[Ca^{2+}]_i$, the response being suppressed by antioxidants (4). At non cytotoxic concentrations ROS- induced increase in $[Ca^{2+}]_i$ may be significant in the activation of the transcription factor NF-κB (6). ROS may also induce NF-κB activation by promoting phosphorylation (4). It has also been suggested that calcium is critical for cell cycle progression, since cell proliferation is inhibited by treatments that reduce ($[Ca^{2+}]_i$) or antagonise the calcium/calmodulin cascade (7).

In this report we present the results of an investigation into the effect of vitamin C on intracellular Ca levels in human lymphoblastoid cells (Molt-3) in the presence of A23187. We hypothesised that vitamin C could inhibit calcium influx under conditions where influx is promoted, i.e. mimicing the effects of a range of hazardous stimuli.

## MATERIALS AND METODS

### *Cell Culture*

The Molt-3 cells, an established line of human lymphoblastoid cells, were cultured in medium (RPMI-1640 medium supplemented with 10% fetal calf serum and antibiotics) at 37°C in a 5% humidified atmosphere. Cell viability was evaluated by the Trypan blue exclusion test. At the concentrations used none of the compounds affected cell viability.

### *Cell treatment with vitamin C*

The cells ($3 \times 10^5$/ml) were pretreated at 37°C with doses of vitamin C (ascorbic acid, Sigma, UK) between 10 μM and 100 μM in a total volume of 20 ml of culture medium for 24 h and then incubated with 5 μM A23187 for 5h. Controls were established with culture medium containing the same concentrations of ethanol diluent but without ascorbic acid.

### *Calcium Measurement*

Cytosolic free $[Ca^{2+}]$ was measured with the calcium sensitive fluorescent probe Fura-2/AM (Fura,-2; Sigma, UK), according to the method of Grynkiewicz et al (8). The buffer used for fluorescence measurements was modified Krebs-Ringer buffer with HEPES (KRH), pH 7.4 at 37°C, containing 145 mM $NaCl_2$, 5 mM KCl, 1mM $Na_2HPO_4$, 0.5 mM $MgSO_4$, 5 mM glucose and 20 mM HEPES. The pH of the buffer was adjusted to 7.4 at 37°C with NaOH. The A23187 was dissolved in ethanol as a 1 mM stock solution and stored at −20°C.

Cells were loaded with 1 μM Fura-2/AM for 30 min at 37°C in RPMI-1640 with 20 mM HEPES. After centrifugation, the cells were left for another 20 min at room temperature to allow them to re-equilibrate with respect to Fura-2. The cells were then washed twice with RPMI-1640 with 20 mM HEPES, and once with the buffer (KRH). Cells resuspended in KRH buffer were used for the fluorescence measurement. The sample volume was adjusted to 2 ml with KRB buffer. Fluorescence was measured in a cuvette at an excitation wavelength of 340/380 nm and emission wavelength of 510 nm (Perkin-Elmer spectrophotometer with fluorescence measurement). Parallel measurement were made using untreated cells. A23187 (5 μM), a range of concentrations of vitamin C (10 μM to 20 mM) and 1 μM fura-2 were added directly to cells in a cuvette and intracellular calcium was measured.

Calibration of the fluorescence signal was performed basically according to the method described by Grynkiewicz *et al.* (8). The maximal fluorescence F (max) was obtained after adding 0.2% Triton X-100 to the sample and the minumum fluoresscence (Fmin) was obtained by adding 25 mM EGTA (pH 8.6). Intracellular free calcium concentration were calculated from the equation: $[Ca^{2+}]$ nM = Kd $(F-F_{min})/(F_{max}-F)$, (where Kd (224 nmol/L) is the dissociation constant of the Fura-2-$Ca^{2+}$ complex. F is the fluorescence of the intact cell suspension. All measurements were performed five times.

## *Protein Assay*

Nuclear extract were prepared from cells treated with vitamin C and then A23187 for protein assay as described (9, 10). Briefly, cells were added to ice cold phosphate-buffered saline and centrifuged for 10 min at 150 × g. Cells were resuspended in 1 ml of buffer A [10 mM HEES, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 10 mM phenymethysufonyl fluoride (PMSF), 2.5 mM dithiothreitol (DTT)] and centrifuged at 10.000 × g at 4°C for 10 min then treated with buffer A + 0.1% Nonidet P40 on ice for 10 min. After further centrifugation at 10.000 × g for 10 min the pellet was treated with buffer C [10 mM HEPES, pH 7.9, 420 nM NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, 25% glycerol, 0.5 mM PMSF] on ice for 20 min and centrifuged at 10.000 × g for 10 min. The supernatant was added to 75 μl of buffer D [10 mM HEPES, pH 7.9, 50 mM KCl, 0.2 mM EDTA, 20% glycerol, 0.5 mM PMSF, 0.5 mM DTT] per sample. The protein concentration of this crude nuclear extract was then measured by the Bradford method (11) with bovine serum albumin as a standard. All measurements were performed five times.

## *Statistical Analyses*

Statistical significance was evaluated by Student's t-test for unpaired data.

## RESULTS

For this series experiments Molt-3 cells were pretreated for 24 h with a range of vitamin C concentrations, then incubated with 5 μM A23187 for 5 h. Intracellular calcium concentrations were measured in cells following loading with Fura-2 and were found to be decreased in the cells exposed to vitamin C relative to the levels found in the cells which had been incubated without vitamin C. These results are summarized in *Table 1.*

*Table 1.* The Effect of Vitamin C on $[Ca^{2+}]_i$ in Molt-3 Cells

| Pre-treatment: | Control | A23187 | A23187 plus 10 μM Vitamin C | A23187 plus 50 μM Vitamin C | A23187 plus 100 μM Vitamin C |
|---|---|---|---|---|---|
| $[Ca^{2+}]_i$ (nM) | 135 ± 37.4 * | 187.1 ± 14.6 | 119.2 ± 50.0 * | 115.9 ± 43.6 ** | 116.7 ± 42.5 ** |

Molt-3 cells were pretreated with vitamin C, or control, for 24 h and incubated A23187 (5 μM) for 5 h. Values represent the mean ± SD of five measurements

* Significantly different from the A23187 (no vitamin C) treatment group at $p<0.05$.

** Significantly different from the A23187 (no vitamin C) treatment at $p<0.01$.

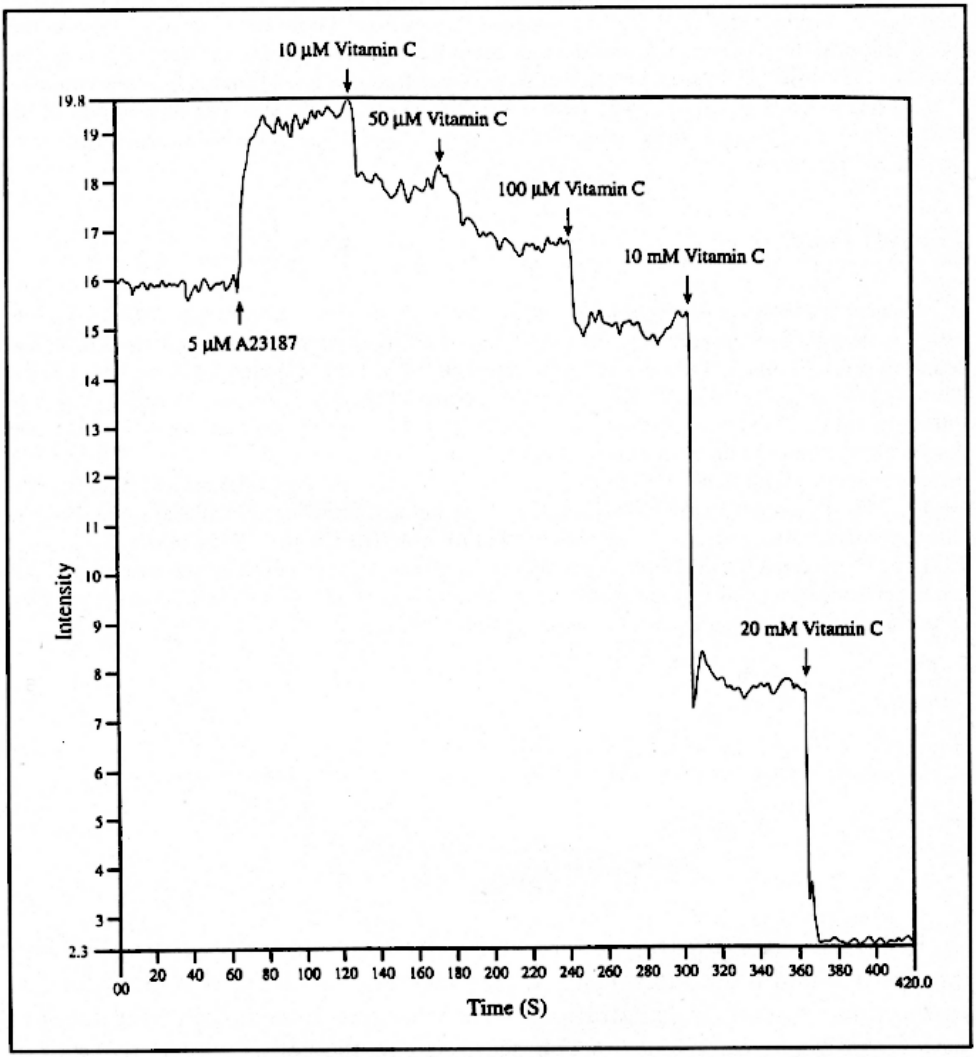


*Fig. 1.* Sample spectrophotometer tracing of fluorescence intensity due to intracellular calcium and Fura-2 on addition of vitamin C to A23187 — treated Molt-3 cells. The experiment was repeated five times with similar results.

A23187 was added to cells loaded with 1 μM Fura-2 in a cuvette after establishing a baseline of $[Ca^{2+}]_i$, and calcium level increased after A23187 addition. Different doses (10 μM—20 mM) vitamin C were added to the cells in the cuvette. Intracellular calcium concentrations decreased almost immediately following vitamin C addition. A sample spectrophotometer tracing is shown in *Fig. 1.*

Total nuclear protein levels were found to be decreased in the vitamin C pretreated cells relative to the untreated cells. Nuclear protein concentrations are shown in *Fig. 2.*

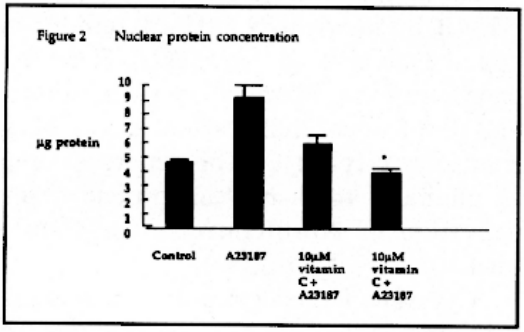


*Fig. 2.* Nuclear protein concentrations in Molt-3 cells pretreated with vitamin C, or control, for 24 and incubated with A23187 (5 μM) for 5 h. Values represent the mean ± SD of five experiments.
*Significantly different from the A23187 treatment group at $p < 0.05$.

## DISCUSSION

The hypothesis underlying the investigation reported here is that the accumulation of vitamin C by lymphocytes may protect these cells by preventing calcium influx. We have investigated the effect of vitamin C on $[Ca^{2+}]_i$ in Molt-3 human lymphoblastoid cells on exposure to the calcium ionophore A23187. A23187 is known to permeabilize the plasma membrane of cells and to induce the leakage of $Ca^{2+}$ (12), thereby mimicing, to some extent, the effects of many types of cell injury including lipid peroxidation following oxidative stress (13). The first aim of the study presented here was to provide direct evidence of the effect of vitamin C on $[Ca^{2+}]_i$. We believe that the results obtained would confirm our hypothesis, at least with regard to one human lymphoblastoid cell line. The ability of ascorbic acid to inhibit calcium uptake into cells or membrane vesicles has been reported previously (14). Those authors showed that the effect was specific as it could be blocked by the addition of ascorbate oxidase. Interestingly, ascorbate/$Fe^{2+}$-induced oxidative stress has been used in many studies on the relationship between calcium influx and cell injury. Agostinho *et al.* examined the effect of ascorbate/$Fe^{2+}$-induced oxidative stress on $[Ca^{2+}]_i$ concentrations and on

the voltage-sensitive $Ca^{2+}$ channels (VSCC) in chick retinal cells. The resting $[Ca^{2+}]_i$ was not affected by oxidation, but the $[Ca^{2+}]_i$ response to $K^+$-depolarization was significantly inhibited under oxidative stress conditions. The major route of $Ca^{2+}$ influx during depolarization is L-type channels in cultured retinal cells. The L-type VSCC are the most affected by oxidative stress (15). The effect of vitamin C on $Ca^{2+}$ uptake in cultured rat astrocytes was investigated. When astrocytes were cultured with vitamin C, the content of vitamin C in the cells was increased and conversely Na(+)-dependent $Ca^{2+}$ uptake was decreased (16).

Bowie and O'Neil examined the effect of vitamin C on the activation of NF-κB by interleukin-1 (IL-1), tumour necrosis factor (TNFα) and $H_2O_2$ in endothelial cells *in vitro* (17). They found that vitamin C, at millimolar concentrations, inhibited cytokine-stimulated NF-κB activation in primary and transformed endhothelial cells and $H_2O_2$ — mediated NF-κB activation in transformed cells (17). This report is compatible with our finding that vitamin C inhibited total nuclear protein levels in pretreated cells, because the activation of NF-κB involves its translocation from the cytoplasm to the nucleus.

Cytosolic free calcium ions play a major role in many cellular functions, including DNA and protein synthesis, cell proliferation and differentiation and gene expression (18, 19). Characteristics of Alzheimer's disease include loss of brain neurons associated with the deposit of beta-amyloid protein (A beta) which is thought to be toxic to neurons possibly *via* induction of intracellular calcium and generation of free radicals. Vitamin C completely abolished A beta-induced calcium increase and cell death in PC12 cells, indicating that calcium elevation and cell death are associated phenomena induced by A beta that can be rescued by antioxidants (20). An increase in cytosolic free calcium appears to activate a variety of biochemical reactions and thus may contribute to the many cellular changes inducible by ROS. A recent report by Bielefeldt *et al.* (21), using cultured human intestinal smooth muscle cells, demonstrated that catalase and superoxide dismutase (both antioxidant enzymes) blunted or completely abolished changes in $[Ca^{2+}]_i$ elicited by $H_2O_2$ and xanthine/xanthine oxidase (X/XO). Transcription of a number of eukaryotic genes is activated in response to an increase in $[Ca^{2+}]_i$. Reduced $[Ca^{2+}]_i$ on exposure to vitamin C may inhibit protein synthesis. Vitamin C was found to reduce $[Ca^{2+}]_i$ in A23187 — stimulated cells in a dose-dependent manner. Our findings would indicate that intracellular vitamin C can prevent changes in $[Ca^{2+}]_i$ that would otherwise influence a wide range of biochemical processes or cell damage. Thus, lymphocytes that can achieve mM concentrations of vitamin C derived from dietary sources can, perhaps, utilize this antioxidant instead of cytoplasmic antioxidant enzymes.

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