Ca\textsuperscript{2+}.induced changes in energy metabolism and viability of melanoma cells

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Summary
Cancer cells are characterized by a high rate of glycolysis, which is their primary energy source. We show here that a rise in intracellular-free calcium ion (Ca\textsuperscript{2+}), induced by Ca\textsuperscript{2+}-ionophore A23187, exerted a deleterious effect on glycolysis and viability of B16 melanoma cells. Ca\textsuperscript{2+}-ionophore caused a dose-dependent detachment of phosphofructokinase (EC 2.7.1.11), one of the key enzymes of glycolysis, from cytoskeleton. It also induced a decrease in the levels of glucose 1,6-bisphosphate and fructose 1,6-bisphosphate, the two stimulatory signal molecules of glycolysis. All these changes occurred at lower concentrations of the drug than those required to induce a reduction in viability of melanoma cells. We also found that low concentrations of Ca\textsuperscript{2+}-ionophore induced an increase in adenosine 5'-triphosphate (ATP), which most probably resulted from the increase in mitochondrial-bound hexokinase, which reflects a defence mechanism. This mechanism can no longer operate at high concentrations of the Ca\textsuperscript{2+}-ionophore, which causes a decrease in mitochondrial and cytosolic hexokinase, leading to a drastic fall in ATP and melanoma cell death. The present results suggest that drugs which are capable of inducing accumulation of intracellular-free Ca\textsuperscript{2+} in melanoma cells would cause a reduction in energy-producing systems, leading to melanoma cell death.

Keywords: Melanoma; Ca\textsuperscript{2+}; glycolysis; phosphofructokinase; hexokinase; glucose 1,6-bisphosphate

MATERIALS AND METHODS

Materials
Ca\textsuperscript{2+}-ionophore A23187 was obtained from Sigma Chemical Co. Other chemicals and enzymes were either from Sigma Chemical Co. or from Boehringer Mannheim GmbH. Tissue culture reagents were purchased from Biological Industries, Beit Haemek, Israel.

Cell culture
B16 F10 mouse melanoma cells were grown in RPMI-1640 medium supplemented with 10% fetal calf serum and antibiotics, at 37°C in humidified atmosphere at 5% carbon dioxide and 95% air. Cells were passaged two to three times weekly.

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Treatment of culture

Melanoma cells (8 x 10⁵ cell ml⁻¹) were seeded in tissue culture plates (10 cm). After 48 h, cells were washed twice with phosphate-buffered saline (PBS). Then the cells were incubated at 37°C in PBS containing 5 mM glucose and 1.8 mM calcium chloride in the absence and presence of Ca²⁺-ionophore A23187 for different concentrations. Ca²⁺-ionophore A23187 was dissolved in dimethyl sulphoxide (DMSO). DMSO was added to the controls.

Extraction and determination of glucose 1,6-bisphosphate, fructose 1,6-bisphosphate and ATP

Glucose 1,6-bisphosphate, fructose 1,6-bisphosphate and ATP were extracted from B16 melanoma cells, as described previously (Glass-Marmor et al, 1996). Glucose 1,6-bisphosphate was measured by the fluorometric method of Passonneau et al (1969); fructose 1,6-bisphosphate and ATP were measured by the method of Lowry et al (1964).

Separation and assay of bound and soluble enzymes

Separation of cytoskeleton-bound and soluble phosphofructokinase from B16 melanoma cells was described previously (Glass-Marmor and Beitner, 1997). Cytoskeleton-bound and soluble phosphofructokinase were assayed as described previously (Lilling and Beitner, 1990).

Separation and assay of mitochondrial-bound and soluble hexokinase from B16 melanoma cells was described previously (Penso and Beitner, 1998).

Measurement of intracellular free calcium

B16 melanoma cells were incubated with 4 μM Fura-2-AM for 40 min in balanced salt solution (BSS) (135 mM sodium chloride, 4.5 mM potassium chloride, 1.5 mM calcium chloride, 0.5 mM magnesium chloride, 5.6 mM glucose, 10 mM HEPES, pH 7.4, in double distilled water), at 37°C. Then the cells were harvested using trypsin (0.25%)-EDTA (0.05%) and precipitated by centrifugate at 270 g for 10 min. The cell suspension (0.5 x 10⁵ ml⁻¹), in BSS in 3-cm quartz cuvette, was excited at 335 nm with a slit 5-nm wide, and emissions collected at 500 nm at 30°C (Hill et al, 1989). Changes in fluorescence were recorded using a Perkin-Elmer LS-50 fluorescent spectrophotometer equipped with temperature-controlled stirred cuvette. To calibrate the fluorescence signal, we used 10 μM digitonin and 50 mM EGTA. Each sample was treated with 10 μM Ca²⁺-ionophore A23187.

Figure 1 Effect of Ca²⁺-ionophore A23187 (10 μM) on intracellular free calcium, using Fura 2, in B16 melanoma cells. The resting level of intracellular free calcium was 240 ± 24 nM (n = 4). The results shown are a single representative experiment from six separate experiments.

Figure 2 Dose–response curves of the effect of Ca²⁺-ionophore A23187 on glucose 1,6-bisphosphate (Glc-1,6-P₂) (A) and fructose 1,6-bisphosphate (Fru-1,6-P₂) (B) levels in B16 melanoma cells. Cells were incubated for 1 h in the absence and presence of different concentrations of Ca²⁺-ionophore A23187. One hundred per cent Glc-1,6-P₂ and Fru-1,6-P₂ refers to 1.65 ± 0.09 and 14.5 ± 0.3 (nmol mg⁻¹ protein) respectively. Each point is the mean ± s.e.m. of 2–3 separate experiments which were performed in triplicate. *P < 0.005.
Cell viability determination

After incubation in absence and presence of Ca²⁺-ionophore A23187, the cells were harvested with trypsin (0.25%)-EDTA (0.05%) and centrifuged for 10 min at 270 g. The precipitated cells were suspended in PBS and counted in a haemocytometer (Neubauer). Cell viability was determined by trypan blue dye exclusion.

Protein measurement

Protein was measured by the method of Bradford (1976) with crystalline bovine serum albumin as a standard.

RESULTS

The results presented in Figure 1 show that Ca²⁺-ionophore A23187 induced an increase in intracellular concentration of free
Ca\(^{2+}\) in B16 melanoma cells. Figure 2 shows that Ca\(^{2+}\)-ionophore A23187 exerted a concentration-dependent decrease in the levels of Glc-1,6-P\(_2\) (Figure 2A) and Fru-1,6-P\(_2\) (Figure 2B) in melanoma cells.

The results presented in Figure 3 show that Ca\(^{2+}\)-ionophore A23187 exerted a dose-dependent decrease in cytoskeleton-bound phosphofructokinase in B16 melanoma cells, with a corresponding increase in soluble activity. Phosphofructokinase activity was assayed under maximal (optimal) conditions (pH 8.2), in which the enzyme is not sensitive to allosteric effectors (Beitner et al, 1978). Therefore, changes in the levels of allosteric regulators would not be expressed in its activity, and the results reveal solubilization of the cytoskeleton-bound enzyme.

The results presented in Figure 4 show that Ca\(^{2+}\)-ionophore A23187 induced a concentration-dependent reduction in viable melanoma cells. As shown in Figure 5, the decrease in Glc-1,6-P\(_2\), Fru-1,6-P\(_2\), and cytoskeleton-bound phosphofructokinase, occurred at lower concentrations of the drug than those required to decrease cell viability.

As shown in Figure 6, the level of ATP was elevated by low concentrations of Ca\(^{2+}\)-ionophore A23187, reaching maximum at 5 μM, and thereafter it was markedly reduced. The elevation in ATP resulted, most probably, from the concomitant increase in the activity of mitochondrial-bound hexokinase (Figure 7), which is linked to oxidative phosphorylation. A parallel increase in soluble hexokinase was also found (Figure 7). Hexokinase activity was assayed in these experiments under regulatory (suboptimal) conditions, in which it is sensitive to allosteric inhibition by Glc-1,6-P\(_2\) (Beitner et al, 1979). As shown in Figure 8, the mitochondrial-bound and soluble hexokinase from B16 melanoma cells exhibited similar sensitivity to inhibition by Glc-1,6-P\(_2\). When hexokinase activity was assayed under maximal (optimal) conditions, in which it is not sensitive to inhibition by Glc-1,6-P\(_2\) (Beitner et al, 1979), no significant changes occurred in the activity of hexokinase from both the mitochondrial and soluble fractions of the cell; hexokinase maximal activity in the mitochondrial and soluble fractions of controls was 125.4 ± 1.8 and 472.4 ± 13.6 (U mg\(^{-1}\) protein), respectively, and after treatment with 5 μM Ca\(^{2+}\)-ionophore A23187, it was 147.2 ± 4.4 and 466.8 ± 9.1 respectively.

**DISCUSSION**

Ca\(^{2+}\)-ionophore A23187 induced an increase in intracellular-free Ca\(^{2+}\) concentration in B16 melanoma cells (Figure 1). These results are compatible with the results of Hill et al (1989). The increase in intracellular-free Ca\(^{2+}\) in melanoma cells induced by Ca\(^{2+}\)-ionophore A23187 caused solubilization of cytoskeleton-bound phosphofructokinase (Figure 3). The detachment of phosphofructokinase from cytoskeleton, would reduce cytoskeletal glycolysis and, thereby, the provision of local ATP, in the vicinity of the cytoskeleton membrane, and would affect cytoskeleton structure (Clarke et al, 1985).

The present results also reveal that Ca\(^{2+}\)-ionophore A23187 decreased the levels of Glc-1,6-P\(_2\) (Figure 2A) and Fru-1,6-P\(_2\) (Figure 2B), the two stimulatory signal molecules of glycolysis and ATP levels (Beitner, 1993). However, as shown in Figure 6, the levels of ATP were not reduced by low concentrations of Ca\(^{2+}\)-ionophore A23187, but rather elevated, reaching a maximum increase at a concentration of 5 μM, and thereafter decreased. The source of the increased ATP at low concentrations of the ionophore, is either mitochondrial or due to glycolysis. Mitochondrial-bound hexokinase was activated at low
concentrations of the ionophore (Figure 7), which correlated with the increase in ATP (Figure 6), reaching a maximum at a concentration of 5 μM, and thereafter declined. Hexokinase was shown to bind to porin at the contact sites between the mitochondrial inner and outer membranes (Kottke et al, 1988; Adams et al, 1991; Brdiczka, 1991). The mitochondrial-bound hexokinase preferentially utilizes mitochondrially-generated ATP (Gots et al, 1972; Gots and Bessman, 1974; Viitanen et al, 1984). In addition, the contacts were shown to have a higher Ca2+-binding capacity compared to the outer and inner mitochondrial membrane. The mitochondrial-bound hexokinase enhances the uptake of Ca2+ by the mitochondria (Kottke et al, 1988), and a rise in mitochondrial Ca2+ stimulates intramitochondrial oxidative metabolism and ATP production (Denton and McCormack, 1990; McCormack and Denton, 1990).

The increase in mitochondrial-bound hexokinase was most probably not a result of translocation from the cytosol, as activity of the cytosolic (soluble) enzyme was also increased (Figure 7). The activation of both the mitochondrial-bound and soluble hexokinase resulted most probably from the decrease in Glc-1,6-P2 levels (Figure 2A), which is a potent inhibitor of hexokinase from both the mitochondrial and soluble fractions of the melanoma cells (Figure 8). The findings that the activity of hexokinase was not changed under maximal (optimal) conditions, in which the enzyme is not subject to inhibition by Glc-1,6-P2, strengthens this postulation. The increase in ATP induced by low concentrations of Ca2+-ionophore, which most probably results from the increase in the mitochondrial-bound hexokinase activity, reflects a defence mechanism to prevent cell death. This mechanism can no longer operate at high concentrations of the Ca2+-ionophore, which causes a decrease in mitochondrial-bound hexokinase (Figure 7), leading to a fall in ATP (Figure 6) and cell death (Figure 4). These experiments fit with the general schemes of apoptosis following accumulation of intracellular free calcium, as described recently by Ichas and Mazat (1998). The increase in cytosolic calcium can induce an increase in mitochondrial calcium, leading to an opening of the permeability transition pore, accompanied by a drop in mitochondrial transmembrane potential.

In contrast to hexokinase, the activity of cytoskeleton-bound phosphofructokinase was not increased by low concentrations of Ca2+-ionophore but rather markedly reduced (Figure 3). This differs from normal tissues, in which we found a dual effect of Ca2+ on cytoskeleton-bound glycolytic enzymes (Beitner, 1993, 1998). This may be due to the changes in cytoskeleton structure and function in cancer cells (Rao and Cohen, 1991).

The Ca2+-induced decrease in the levels of Glc-1,6-P2 and Fru-1,6-P2 and the detachment of phosphofructokinase from cytoskeleton, occurred at lower concentrations of the drug than the reduction in cell viability (Figure 5), which indicates that these are primary changes which lead to cell death.

In summary, the present results reveal that accumulation of high concentrations of intracellular free Ca2+ in melanoma cells, induces a reduction in the energy-producing systems in different cellular compartments, leading to melanoma cell death. This may be the mechanism of action of certain drugs which are already used for melanoma treatment, and may also serve to evaluate the therapeutic action of new drugs.

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