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Detachment of glycolytic enzymes from cytoskeleton of melanoma cells induced by calmodulin antagonists

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Abstract

Glycolysis, which is the primary energy source in cancer cells, is known to be controlled by allosteric regulators, as well as by reversible binding of glycolytic enzymes to cytoskeleton. We have previously found that different calmodulin antagonists decrease the levels of allosteric activators of glycolysis, and reduce ATP content and cell viability in B16 melanoma cells. Here we report of a novel, additional, mechanism of action of calmodulin antagonists in melanoma cells. We show that these drugs cause a detachment of the glycolytic enzymes, phosphofructokinase (ATP: D-fructose-6-phosphate 1-phosphotransferase, EC 2.7.1.11) and aldolase (D-fructose-1,6-bisphosphate D-glyceraldehyde-3-phosphate-lyase, EC 4.1.2.13), from cytoskeleton of B16 melanoma cells. This effect was dose- and time-dependent, and preceded the decrease in cell viability. The detachment of glycolytic enzymes from cytoskeleton would reduce the provision of local ATP, in the vicinity of the cytoskeleton-membrane and would affect cytoskeleton structure. Since the cytoskeleton is being recognized as an important modulator of cell function, proliferation, differentiation and neoplasia, detachment of the glycolytic enzymes from cytoskeleton induced by calmodulin antagonists, as well as their reported inhibitory action on cell proliferation, make these drugs most promising agents in treatment of cancer.

Keywords: Calmodulin antagonist; Glycolysis; Cytoskeleton; Melanoma cell

1. Introduction

Glycolysis is the primary energy source in cancer cells, exceeding the capacity of mitochondrial oxidative energy metabolism (Eigenbrodt et al., 1985; Fiechter and Gmünder, 1989; Beckner et al., 1990; Greiner et al., 1994). Glycolysis is known to be controlled by allosteric regulators (for reviews, see Beitner, 1979, 1984, 1985, 1990), as well as by reversible binding of the glycolytic enzymes to cytoskeleton (Arnold and Pette, 1968; for reviews see Clarke et al., 1985; Beitner, 1993; Pagliaro, 1993). The latter mechanism has recently attracted much attention. It has been shown by many laboratories that glycolytic enzymes bind reversibly to the cytoskeletal elements, particularly to the actin filaments and also to tubulin/microtubules (Arnold and Pette, 1968; Clarke and Masters, 1975; Clarke et al., 1985; Pagliaro and Taylor, 1988, 1992; Walsh et al., 1989; Lilling and Beitner, 1990, 1991; Lilling et al., 1991; Beitner, 1993; Lehotzky et al., 1993). The binding of glycolytic enzymes to cytoskeleton was demonstrated both in vitro, using purified components, and in vivo, in whole tissues, as well as in different cultured cells (Minaschek et al., 1992).

All glycolytic enzymes bind to cytoskeleton (Clarke et al., 1985) except hexokinase, which binds reversibly to mitochondria, where it is linked to oxidative phosphorylation (Gots et al., 1972; Viitanen et al., 1984; Mohan et al., 1989; Adams et al., 1991). Cytoskeletal glycolysis provides local ATP in the vicinity of the cytoskeleton (Beitner, 1993), which is known to interact dynamically with plasma membrane upon membrane-induced events (Geiger, 1983). Binding of glycolytic enzymes to cytoskeleton also affects cell structure, as glycolytic enzymes were found to cross-link actin-containing filaments into ordered supramolecular structures (Clarke et al., 1985). Many factors and conditions control the binding of glycolytic enzymes to cytoskeleton (Beitner, 1993; Parra and Pette, 1995), which is being recognized as an important modulator of metabolic functions in the cell. The actin cytoskeletal network is involved in events regulating cell proliferation and differentiation, and alterations in actin state were reported during malignant transformation of cells in culture, and in naturally occurring tumors (for review, see

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Rao and Cohen, 1991). Cell cycle-related changes in F-actin distribution were shown to correlate with glycolytic activity (Bereiter-Hahn et al., 1995).

Our previous experiments have revealed that growth-promoting hormones, insulin and growth factors, stimulate glycolysis by increasing the binding of glycolytic enzymes to cytoskeleton and by raising the level of glucose 1,6-bisphosphate, the signal molecule, which is a potent allosteric activator of glycolysis. We have also shown that all these effects of insulin and growth factors are prevented by treatment with calmodulin antagonists, which strongly suggest that Ca^{2+}/calmodulin is involved in their stimulatory action on glycolysis, which supplies energy for cell growth (Chen-Zion et al., 1992a,b, 1993; Beitner, 1993; Livnat et al., 1993, 1994, 1995). Calmodulin is a multifunctional Ca^{2+} binding protein that has been implicated in the regulation of numerous cellular events, including that of normal and abnormal cell proliferation (Veigl et al., 1984; Hait and Lazo, 1986; Rasmussen and Means, 1987; Reddy, 1994). Calmodulin antagonists were reported to inhibit cellular proliferation of various cells (Hait and Lee, 1985; Susuki et al., 1986; Ford et al., 1989; Mac Neil et al., 1993a; Hait et al., 1994), including melanoma (Ito and Hidaka, 1983; Mac Neil et al., 1984; Al-Ani et al., 1988).

Recent experiments from our laboratory have revealed that calmodulin antagonists decrease the levels of glucose 1,6-bisphosphate and fructose 1,6-bisphosphate, the two allosteric stimulatory signal molecules of glycolysis, and reduce ATP content and cell viability in B16 melanoma cells (Glass-Marmor et al., 1996). In the present research, we investigated whether calmodulin antagonists also exert an effect on the cytoskeleton-bound glycolytic enzymes, phosphofructokinase (ATP: D-fructose-6-phosphate 1-phosphotransferase, EC 2.7.1.11), the rate-limiting enzyme of glycolysis, and aldolase (D-fructose-1,6-bisphosphate D-glyceraldehyde-3-phosphate-lyase, EC 4.1.2.13), in B16 melanoma cells. We used the same calmodulin antagonists as in our previous research (Glass-Marmor et al., 1996), namely: thioridazine (10-[2-(1-methyl-2-piperidyl)ethyl]-2-methylthiophenothiazine), an antipsychotic phenothiazine, clotrimazole (1-(α-2-chlorotrityl)imidazole) and bifonazole (1-(α-biphenyl-4-ylbenzyl)imidazole), the antifungal azole derivatives, that were recently recognized as calmodulin antagonists (Hegemann et al., 1993; Mac Neil et al., 1993a), and CGS 9343B (1,3-dihydro-1-[1-[(4-methyl-4*H*,6*H*-pyrrolo[1,2-*a*][4,1]-benzoxazepin-4-yl)methyl]-4-piperidinyl]-2*H*-benzimidazol-2-one (1:1) maleate), a more selective inhibitor of calmodulin activity (Norman et al., 1987).

2. Materials and methods

2.1. Materials

Thioridazine hydrochloride was obtained from Taro Pharmaceutical (Haifa, Israel). Clotrimazole and bifonazole were purchased from Sigma (St. Louis, MO, USA). CGS 9343B was obtained from Ciba-Geigy (Summit, NJ, USA).

Other chemicals and enzymes were either from Sigma or from Boerhinger-Mannheim (Mannheim, Germany). Tissue culture reagents were purchased from Biological Industries (Beit Haemek, Israel).

2.2. 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% CO_2 and 95% air. Cells were passaged two to three times weekly.

2.3. Treatment of culture

Melanoma cells (9×10^5 cell ml^{-1}) were seeded in tissue culture plates (10 cm). After 48 h, cells were washed twice with phosphate-buffered saline (PBS). The cells were incubated at 37°C in PBS containing 5 mM glucose in the absence and presence of calmodulin antagonists for different times and concentrations. For longer incubation period (5 h and more), the cells were incubated in RPMI-1640 medium. Thioridazine was dissolved in water, clotrimazole and bifonazole were dissolved in water containing acidified 6% polyethylene glycol 400. CGS 9343B was dissolved in water containing 2% ethanol. The appropriate solvents were added to the controls.

2.4. Separation and assay of bound and soluble enzymes

After the incubation, the medium was collected in a centrifuge tube and the cells, harvested with trypsin (0.25%)-EDTA (0.05%), were added. Then the cells were pelleted by centrifugation for 10 min at 270 g.

The particulate (cytoskeleton-bound) and soluble phosphofructokinase or aldolase were separated by the method described previously (Lilling and Beitner, 1990), as follows. The precipitated cells were homogenized in Potter homogenizer for 90 s, in 0.4 ml of ice cold 0.25 M sucrose containing 1 mM dithiotreitol and 20 mM NaF, pH 7.5. Samples (2 μl) were removed for protein determination. The homogenate was centrifuged at 4°C for 15 min at $27\,000 \times g$. The pellet, which was redissolved in 0.2 ml homogenizing solution, is referred to as 'bound fraction', and the supernatant is referred to as the 'soluble fraction'.

Cytoskeleton-bound and soluble phosphofructokinase and aldolase were assayed as described previously (Lilling and Beitner, 1990). Phosphofructokinase was assayed under maximal (optimal) conditions (pH 8.2), by coupling with aldolase, triose-phosphate isomerase and α-glycerophosphate dehydrogenase. After the addition of fructose 6-phosphate, the rate of disappearance of NADH was measured spectrophotometrically at 340 nm and at 25°C.

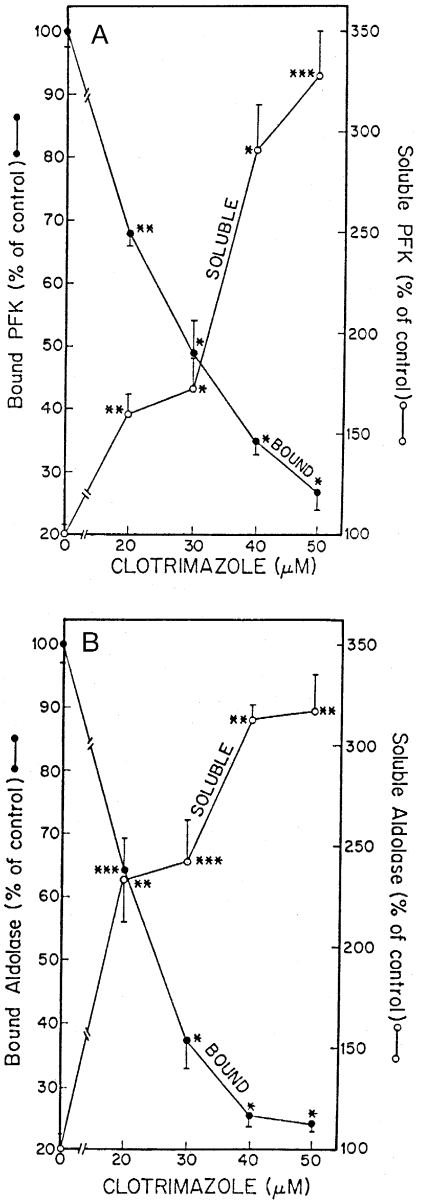

Fig. 1. Dose–response curves of the effect of clotrimazole on cytoskeleton-bound and soluble (a) phosphofructokinase (PFK) and (b) aldolase, in B16 melanoma cells. Cells were incubated for 1 h in absence and presence of different concentrations of clotrimazole. 100% activity of bound and soluble phosphofructokinase and aldolase was 87 ± 6, 52 ± 4 and 60 ± 4, 23 ± 1 (mU/mg protein), respectively. Each point is the mean $\pm$ S.E.M. of 2–3 separate experiments which were performed in triplicate. * $P < 0.005$, ** $P < 0.05$, *** $P < 0.01$.

Aldolase activity was assayed after the addition of fructose 1,6-bisphosphate by coupling with triose-phosphate isomerase and α-glycerophosphate dehydrogenase.

2.5. Cell viability determination

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

2.6. Protein measurement

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

3. Results

Fig. 1a shows that clotrimazole exerted a dose-dependent decrease in cytoskeleton-bound phosphofructokinase

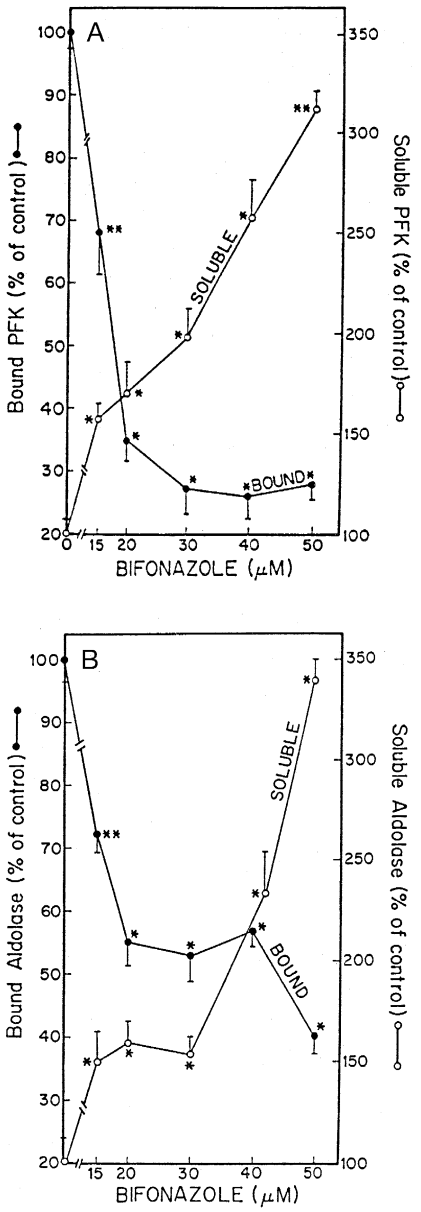

Fig. 2. Dose—response curves of the effect of bifonazole on cytoskeleton-bound and soluble (a) phosphofructokinase (PFK) and (b) aldolase, in B16 melanoma cells. Conditions as in Fig. 1. Each point is the mean $\pm$ S.E.M. of 2–3 separate experiments which were performed in triplicate. * $P < 0.05$, ** $P < 0.005$.

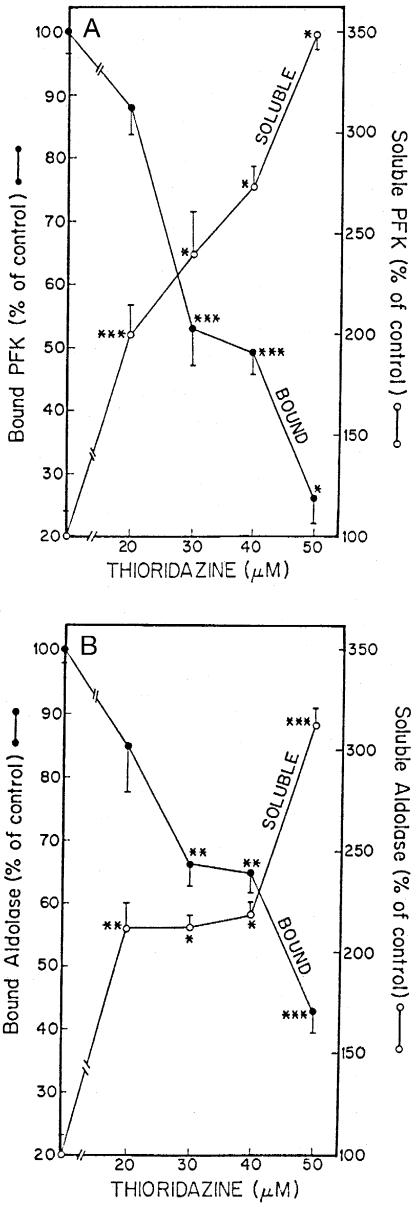

Fig. 3. Dose–response curves of the effect of thioridazine on cytoskeleton-bound and soluble (a) phosphofructokinase (PFK) and (b) aldolase, in B16 melanoma cells. Conditions as in Fig. 1. Each point is the mean ± S.E.M. of 2–3 separate experiments which were performed in triplicate. * $P < 0.005$, ** $P < 0.05$, *** $P < 0.01$.

in B16 melanoma cells, with a corresponding increase in soluble activity. Phosphofructokinase activity was assayed in all the experiments presented here, 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. As shown in Fig. 1b, clotrimazole also induced a dose-dependent decrease in cytoskeleton-bound aldolase, with a corresponding increase in soluble activity. The results presented in Fig. 2 show that bifonazole induced a similar dose-dependent solubilization of cytoskeleton-bound phosphofructokinase (Fig. 2a) and aldolase (Fig. 2b). Similarly to the effects of the azole-derivatives and under the same conditions (1 h incubation), thioridazine induced a marked dose-dependent decrease in cytoskeleton-bound phosphofructokinase (Fig. 3a) and aldolase (Fig. 3b), with a corresponding increase in their soluble activities. The results in Fig. 4 show that CGS 9343B also induced a decrease in cytoskeleton-bound phosphofructokinase and aldolase, however with this compound, longer incubation (5 h) was required to induce these effects.

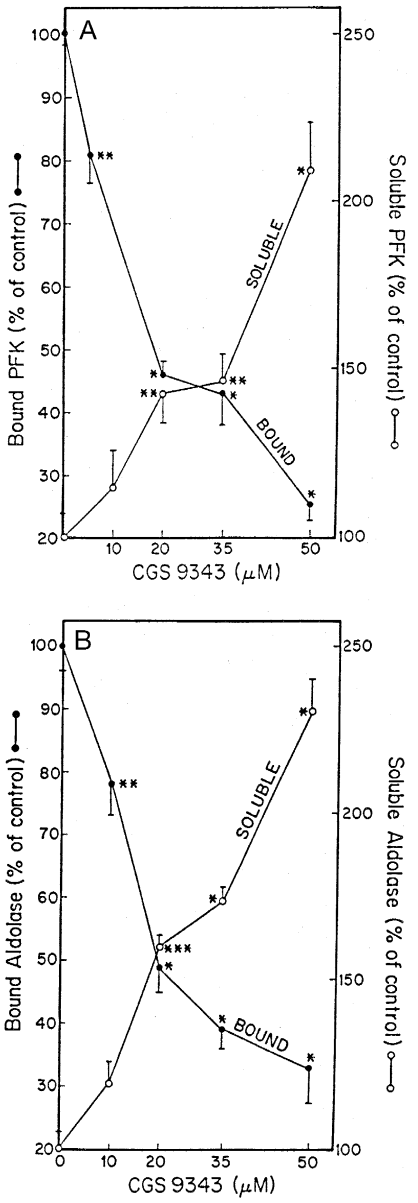

Fig. 4. Dose–response curves of the effect of CGS 9343B on cytoskeleton-bound and soluble (a) phosphofructokinase (PFK) and (b) aldolase in B16 melanoma cells. Cells were incubated for 5 h in absence and presence of different concentrations of CGS 9343B. 100% activity of bound and soluble phosphofructokinase and aldolase was 67 ± 5, 40 ± 2 and 70 ± 6, 37 ± 3 (mU/mg protein), respectively. Each point is the mean ± S.E.M. of 2–3 separate experiments which were performed in triplicate. * $P < 0.005$, ** $P < 0.05$, *** $P < 0.01$.

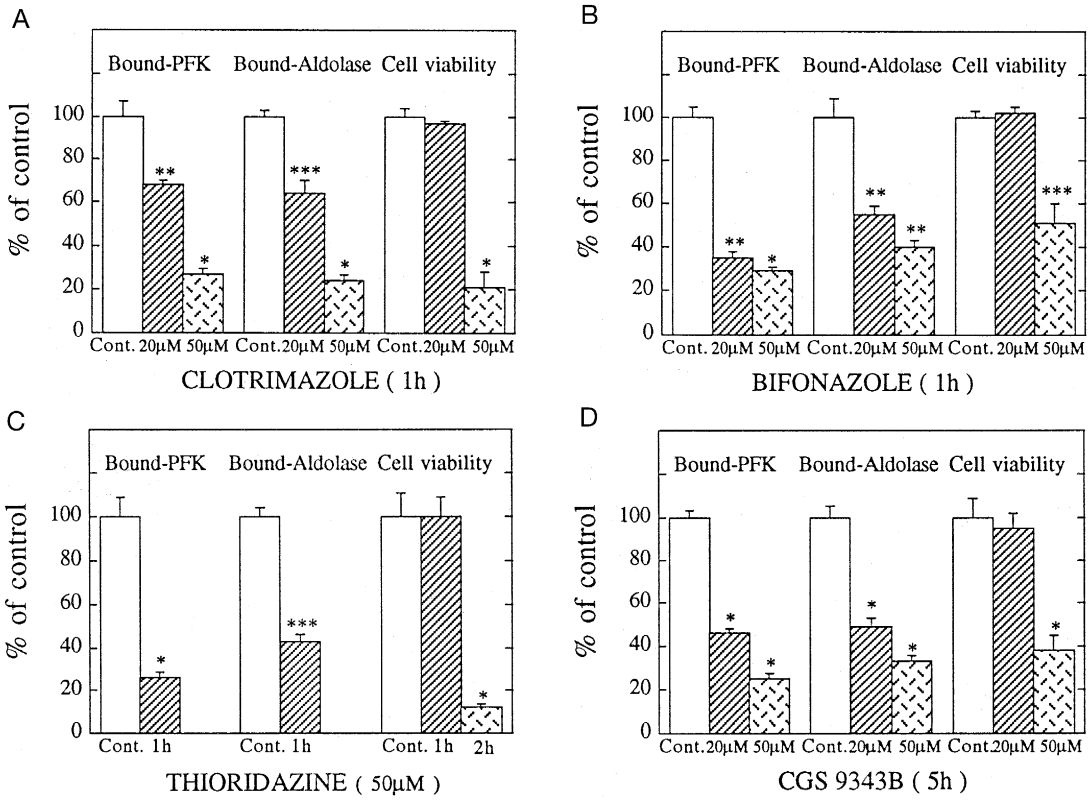

Fig. 5. Effect of calmodulin antagonists on cell viability and its relation to the binding of phosphofructokinase and aldolase to cytoskeleton in B16 melanoma cells. Cells were incubated with and without: (a) 20 μM and 50 μM clotrimazole for 1 h; (b) 20 μM and 50 μM bifonazole for 1 h; (c) 50 μM thioridazine for 1 h and 2 h; (d) 20 μM and 50 μM CGS 9343B for 5 h. 100% cell viability refers to 5×10^6 cell ml^{-1}; 100% activity of bound phosphofructokinase (PFK) and aldolase refers to 79 ± 6 and 63 ± 5 (mU/mg protein), respectively. Values are the mean ± S.E.M. of 2–3 separate experiments which were performed in triplicate. * $P < 0.005$, ** $P < 0.05$, *** $P < 0.01$.

The results presented in Fig. 5a–d show the effect of the calmodulin antagonists on cell viability and its relation to their reducing action on cytoskeleton-bound glycolytic enzymes. At high concentrations all four calmodulin antagonists markedly reduced viability of melanoma cells. The azole derivatives were more cytotoxic than thioridazine and CGS 9343B; 1 h incubation at high concentration (50 μM) of clotrimazole or bifonazole caused a significant reduction in viable cells, that resulted in marked cell detachment from culture plates. For thioridazine and CGS 9343B, longer incubation time was required to induce a decrease in cell viability. The results in Fig. 5 also clearly show that the decrease in cytoskeleton-bound phosphofructokinase and aldolase induced by all four compounds, preceded the decrease in cell viability.

4. Discussion

The results presented here reveal that all four calmodulin antagonists induced a significant dose-dependent detachment of the glycolytic enzymes, phosphofructokinase and aldolase, from cytoskeleton in B16 melanoma cells. The relative potency of the antifungal imidazole derivatives, clotrimazole and bifonazole (Figs. 1 and 2), which were recently reported to display calmodulin antagonistic activity (Hegemann et al., 1993; Mac Neil et al., 1993a), was similar to that of thioridazine and CGS 9343B (Figs. 3 and 4). Their IC_{50} in detaching cytoskeletal phosphofructokinase and aldolase was approximately 10–25 μM, which is close to their reported IC_{50} for calmodulin inhibition. (The reported IC_{50} for clotrimazole, bifonazole, CGS 9343B and thioridazine is 18.4, 14, 3.3 and 18 μM, respectively). One hour incubation with the azole derivatives or thioridazine was sufficient to cause a marked decrease in the cytoskeleton-bound glycolytic enzymes. On the other hand, CGS 9343B, the more selective inhibitor of calmodulin activity, which does not inhibit protein kinase C activity (Norman et al., 1987), required longer incubation time (5 h) to exert a similar action (Fig. 4). These findings suggest that in addition to calmodulin inhibition, protein kinase C inhibition may potentiate the effects of thioridazine and azole derivatives on cytoskeletal glycolytic enzymes. Thioridazine, as well as azole derivatives (Hegemann et al., 1996), are dual calmodulin/protein kinase C antagonists. However, the IC_{50} for protein kinase C inhibition for clotrimazole was reported to be 1 mM and bifonazole was even less potent (Hegemann et al., 1996). Since the effective concentrations of clotrimazole (Fig. 1) or bifonazole (Fig. 2) in decreasing cytoskeleton-bound

glycolytic enzymes are in the μM range, it is doubtful if protein kinase C inhibition is involved in their effects.

The results presented in Fig. 5 show that at high concentration, all four compounds markedly reduced viability of the melanoma cells. We have previously found (Glass-Marmor et al., 1996), that the reduction in viability was closely related to the decrease in ATP content induced by these drugs. Thioridazine and CGS 9343B required longer incubation to decrease cell viability. The greater cytotoxicity of the azole derivatives may result from their additional actions, e.g., inhibition of cytochrome P-450.

The detachment of glycolytic enzymes from cytoskeleton, induced by all four calmodulin antagonists, preceded the decrease in cell viability (Fig. 5a–d). We have recently found (Glass-Marmor et al., 1996) that the same four calmodulin antagonists in B16 melanoma cells decrease the levels of glucose 1,6-bisphosphate and fructose 1,6-bisphosphate, the two allosteric stimulatory signal molecules of glycolysis. These changes also preceded the reduction in cell viability. Here we show a novel mechanism of action of the calmodulin antagonists. The detachment of the glycolytic enzymes from cytoskeleton, induced by these drugs, will lead to a reduction in cytoskeletal glycolysis and thereby to a decrease in the provision of local ATP, in the vicinity of the cytoskeleton-membrane, which is required for the dynamic changes of the cell (Beitner, 1993). Local ATP is required for the numerous phosphorylation reactions of cytoskeletal proteins, which are known to affect their interactions with each other, as well as with plasma membrane. Other energy-dependent processes (e.g., ion movement, various translocations, etc.) also require local, glycolytically generated ATP. The detachment of glycolytic enzymes from the cytoskeleton, induced by the calmodulin antagonists, will also directly affect cytoskeleton structure, as glycolytic enzymes were found to cross-link actin-containing filaments into ordered supramolecular structures (for review, see Clarke et al., 1985). Calmodulin antagonists were also to cause cell detachment (Mac Neil et al., 1992, 1993b, 1994; Wagner et al., 1995). The calmodulin antagonistic activity on cytoskeleton-bound glycolytic enzymes may precede cell detachment; both may be early indicators of a decrease in cell viability.

Calmodulin antagonists are a new generation of drugs with broad therapeutic applications (for review, see Mannhold and Timmerman, 1992). Experiments in our laboratory have revealed that these drugs are most effective in treatment of skin burns and frostbite (Beitner, 1987; Beitner et al., 1989a,b, 1991). They also attenuate skin changes induced by uv radiation (Beitner, 1987; Anthony et al., 1994).

The present results reveal a novel mechanism of action of calmodulin antagonists, as detachers of cytoskeleton-bound glycolytic enzymes in B16 melanoma cells. This action is additional to their inhibitory action on glycolysis, by reducing the levels of allosteric activators which we found previously (Glass-Marmor et al., 1996). The importance of the role of actin cytoskeletal network in regulation of cell function, proliferation, differentiation and neoplasia, has been recognized in the last decade (for review, see Rao and Cohen, 1991). Since Ca^{2+}/calmodulin plays a critical role in both cell proliferation and glycolysis, the primary energy source in cancer cells, calmodulin antagonists are most promising agents for treatment of cancer. In addition, the results reported here suggest that detachment of glycolytic enzymes from cytoskeleton could be employed to serve as a marker for cancer cell dysfunction, to evaluate therapeutic action of anti-cancer drugs.

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