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Clotrimazole inhibits and modulates heterologous association of the key glycolytic enzyme 6-phosphofructo-1-kinase

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ABSTRACT

Clotrimazole is an antifungal azole derivative recently recognized as a calmodulin antagonist with promising anticancer effects. This property has been correlated with the ability of the drug to decrease the viability of tumor cells by inhibiting their glycolytic flux and consequently decreasing the intracellular concentration of ATP. The effects of clotrimazole on cell glycolysis and ATP production are considered to be due to the detachment of the glycolytic enzymes from the cytoskeleton. Here, we show that clotrimazole directly inhibits the key glycolytic enzyme 6-phosphofructo-1-kinase (PFK). This property is independent of the anti-calmodulin activity of the drug, since it is not mimicked by the classical calmodulin antagonist compound 48/80. However, the clotrimazole-inhibited enzyme can be activated by calmodulin, even though calmodulin has no effect on PFK activity in the absence of the drug. Clotrimazole alone induces the dimerization of PFK reducing the population of tetramers, which is not observed when calmodulin is also present. Since PFK dimers are less active than PFK tetramers, this can explain the inhibitory effect of clotrimazole on the enzyme. Additionally, clotrimazole positively modulates the association of PFK with erythrocyte membranes. Altogether, our data support a hitherto unrecognized action of clotrimazole as a negative modulator of glycolytic flux through direct inhibition of the key enzyme PFK.

1. Introduction

Clotrimazole (CTZ; 1-[(2-chlorophenyl)-diphenyl-methyl]imidazole) is a therapeutically important imidazole-derived anti-mycotic agent that is clinically safe and readily tolerated by humans [1,2]. Its anti-mycotic effect is due to the inhibition of ergosterol synthesis, which makes the plasma membrane of fungi leaky [1]. It has a multiplicity of effects on a variety of cellular targets, including the sarcoplasmic reticulum Ca^{2+} pump [3], the capacitative Ca^{2+} channel [4] and normal and cancer cells in vitro and in vivo [5], where it interferes with cellular Ca^{2+} homeostasis [6]. Recently, CTZ was recognized as a calmodulin (CaM) antagonist [7,8]. The present work was undertaken in response to the discovery that CTZ has potent effects on the viability of human cancer cells because it alters cytoskeleton-associated glycolytic enzymes, inhibiting cell glycolysis and ATP production [9–13].

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Abbreviations: CaM, calmodulin; CTZ, clotrimazole; PFK, 6-phosphofructo-1-kinase; phosphofructokinase; 48/80, compound 48/80

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The mechanism of this anti-glycolytic activity of CTZ is considered to involve its action as a CaM antagonist [6,9–13]. Recently, we demonstrated that 6-phosphofructo-1-kinase (PFK; phosphofructokinase; ATP:D-fructose-6-phosphate-1-phosphotransferase; EC 2.7.1.11), the key regulatory enzyme of the glycolytic pathway and a CaM-binding protein, is regulated by CaM, which induces dimerization of PFK from tetramers without compromising catalytic activity of the purified enzyme [14].

The aim of the present study was to compare CTZ and compound 48/80, a classical CaM antagonist [15], for their ability to modify the activity and the oligomeric conformation of PFK, with a view to correlate these findings with the antineoplastic properties of CTZ.

2. Materials and methods

2.1. Materials

ATP, fructose-6-phosphate, CaM, CTZ and compound 48/80 were purchased from Sigma Chemical Co. (St. Louis, MO, USA). ^{32}Pi was purchased from Instituto de Pesquisas Energéticas e Nucleares (São Paulo, Brazil). [γ-^{32}P]ATP was prepared according to Maia et al. [16]. Purified PFK was obtained from rabbit skeletal muscle according to the method developed by Kemp [17], with modifications introduced by Kuo et al. [18]. Protein concentrations were measured as described by Lowry et al. [19].

2.2. Light scattering

Light-scattering (L.S.) measurements were performed in a Jasco spectrofluorimeter, in a medium containing 100 mM Tris–HCl (pH 8.2), 5 mM $MgCl_2$, 100 μM $CaCl_2$, and the additives indicated for each experiment. Appropriate reference spectra were subtracted from the data to correct for background interferences, which were always less than 5% of the total signal. Incident light wavelength was set at 510 nm, and scattering was recorded at 90° between 500 and 520 nm.

2.3. Intrinsic fluorescence measurements

Intrinsic fluorescence measurements were performed in a Jasco spectrofluorimeter, in a medium containing 100 mM Tris–HCl (pH 8.2), 5 mM $MgCl_2$, 100 μM $CaCl_2$, and the additives indicated for each experiment. Appropriate reference spectra were subtracted from the data to correct for background interferences, which were always less than 2% of the fluorescence signal. Excitation wavelength was set at 280 nm, and fluorescence emission was recorded at 90°, scanned from 300 to 400 nm.

2.4. PFK activity

PFK activity was measured by the method described by Sola-Penna et al. [20] with the modifications introduced by Zancan and Sola-Penna [21,22], in a reaction medium containing 50 mM Tris–HCl (pH 8.2), 5 mM $MgCl_2$, 100 μM $CaCl_2$, 5 mM $(NH_4)_2SO_4$, 1 mM [γ-^{32}P]ATP (4 μCi/nmol), 1 mM fructose-6-phosphate, and 5 μg/ml purified PFK. The reaction was stopped by addition of a suspension of activated charcoal in 0.1 M HCl and 0.5 M mannitol; after centrifugation, the supernatant containing [1-^{32}P]fructose-1,6-bisphosphate formed was analyzed in a liquid scintillation counter. Appropriate blanks in the absence of fructose-6-phosphate were subtracted from all measurements.

2.5. Preparation of calcium- and calmodulin-depleted erythrocyte membranes

Calcium- and calmodulin-free red blood cell ghosts were prepared according to Alves et al. [23] from outdated human blood. Briefly, the blood was centrifuged at 750 × *g* for 15 min at 0–4 °C. Plasma and buffy coat were aspirated to remove polynuclear and mononuclear cells and erythrocytes were resuspended to 50% hematocrit in ice-cold buffer containing 121 mM NaCl, 25.3 mM $NaHCO_3$, and 1.3 mM $CaCl_2$ (pH 7.8). The supernatant was removed after centrifugation at 750 × *g* for 15 min. The pellet was washed four times with the same buffer to remove lymphocytes or granulocytes and the cells were lysed in ice-cold distilled water containing 2 mM EDTA for 30 min with gentle shaking. Erythrocyte membranes were obtained by homogenization of the lysate in a Potter–Elvehjem glass homogenizer and were centrifuged at 30,000 × *g* for 30 min. The pellet was washed five times with 2 mM EDTA and stored at 4 °C.

2.6. Enzyme–membrane interactions

Association of PFK with erythrocyte membranes was assessed as described previously [22]. Purified rabbit skeletal muscle PFK (2.7 mg/ml) and erythrocyte membranes (0.1 mg protein/ml) were incubated in 100 μl of a medium containing different insulin concentrations (0–1000 nM) in 50 mM Tris–HCl (pH 8.2), 5 mM $(NH_4)_2SO_4$, 5 mM $MgCl_2$, and 1 mM ATP during 1 h at 37 °C. After incubation, mixtures were centrifuged at 20 psi for 20 min on a Beckman Airfuge (~90.000 × *g*, Beckman Instruments, CA, USA) for separation of erythrocyte membranes. The membrane pellets were diluted 10-times, which releases all PFK bound to the membrane [22], and PFK content was analyzed measuring its catalytic activity as described above.

2.7. Statistical analysis

Statistical analyses and non-linear regression were performed using the software SigmaPlot 9.0 integrated with SigmaStat 3.1 packages (Systat, CA, USA). $P < 0.05$ was defined as the limit for statistically different mean values, based on Student's *t*-test or ANOVA, as appropriate.

3. Results

3.1. Effects of CTZ and compound 48/80 on purified PFK activity

The effects of CTZ and compound 48/80 on the activity of PFK were evaluated in the absence and presence of 30 nM CaM. All experiments were performed in the presence of 100 μM Ca^{2+}, a concentration at which PFK high affinity site for CaM is

saturated with Ca_4CaM, but Ca^{2+} does not directly affect enzyme structure or activity [14]. Fig. 1 shows that 50 μM CTZ alone inhibits PFK activity by 49.2 ± 12.5%. However, when the effects of 50 μM CTZ on PFK activity are evaluated in the presence of 30 nM CaM, there is a 70.3 ± 8.6% stimulation of the enzyme when compared to control (Fig. 1, gray bars). Neither CaM nor the traditional CaM antagonist compound 48/80 alone had any effect on PFK activity (Fig. 1). Having previously demonstrated that CaM does not alter the catalytic activity of PFK [14], we in fact did not expect compound 48/80 to affect PFK activity. However, on analyzing the combined effects of CaM and CTZ, we found that CaM not only reverses the inhibitory effect of CTZ (compare black and gray bars in CTZ group of Fig. 1), but it also activates the enzyme when CTZ is present (compare gray bars in CTZ and control groups).

In order to better understand the effects of CTZ on PFK we examined the CTZ concentration dependence of PFK activity in the absence and presence of CaM. The results are shown in Fig. 2, where it can be seen that CTZ has a biphasic effect on PFK activity in the absence of CaM (Fig. 2, filled circles). On the other hand, in the presence of 30 nM CaM, CTZ promotes a dose-dependent activation of PFK that saturates at 100 μM CTZ (Fig. 2, open circles).

Our previous work demonstrated that CaM binds to PFK with a stoichiometry of 1 CaM per PFK protomer (achieved at 30 nM CaM), inducing the dimerization of the enzyme without altering its catalytic activity [14]. CaM was originally described as an inhibitor of PFK, since when it binds at a stoichiometry of 2 CaM per PFK protomer, the enzyme is inhibited [24–29]. However, CaM intracellular concentrations [30] are not sufficient to saturate the low-affinity CaM binding site of PFK [14,27,29]. More recently, it has been proposed that CaM activates the glycolytic flux, as well as key glycolytic enzymes, including PFK [31,32]. This apparent contradiction has not yet been resolved. It may be that the formation of active PFK dimers induced by CaM has a physiological significance that is not evident in our results with purified proteins. This may be reflected in the fact that, in the presence of CTZ, CaM promotes a significant activation of PFK in relation to the controls, not just a reversal of CTZ inhibition.

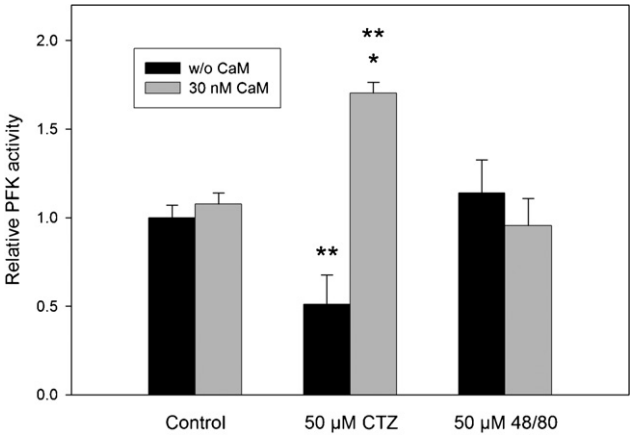

Fig. 1 – Effects of CTZ and 48/80 on the catalytic activity of purified PFK. Enzyme activity was measured as described under Section 2. Catalytic activity was calculated by linear regression to the formation of product as a function of time. Relative PFK activity was calculated by dividing the rate obtained for each condition by that obtained in the absence of CTZ, 48/80 and CaM. Plotted values represent mean ± standard errors of 5 independent experiments ($n = 5$). $^{*}P < 0.05$ in comparison with the same group in the absence of CaM. $^{**}P < 0.05$ in comparison with the control (±CaM, as appropriate).

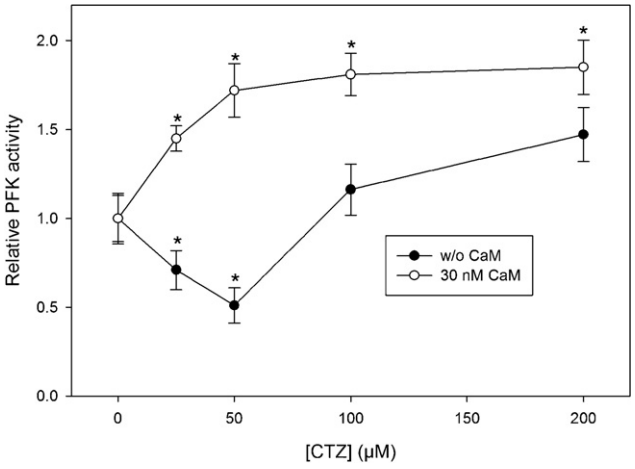

Fig. 2 – Titration of the catalytic activity of purified PFK by CTZ. Catalytic activity was calculated by linear regression to the formation of product as a function of time. Relative PFK activity was calculated by dividing the rate obtained by that obtained in the absence of CTZ either in the absence (filled circles) or in the presence of 30 nM CaM (open circles). Plotted values represent mean ± standard errors of 5 independent experiments ($n = 5$). $^{*}P < 0.05$ vs. control in the absence of CTZ (Student's t-test).

3.2. *Effects of CTZ and compound 48/80 on oligomeric structure of purified PFK*

In order to check whether the effects of CTZ on PFK activity are due to alterations in the equilibria among the enzyme oligomeric forms, we performed light-scattering measurements on PFK under the same conditions tested in Fig. 1. Light-scattering measurements have been used to evaluate the transitions between dimeric and tetrameric conformations of PFK [14,27,28,33,34]. We have shown that CaM induces a decrease in light scattered by PFK that is directly proportional to the formation of PFK dimers, also induced by CaM [14]. Here, we confirm that CaM induces the formation of PFK dimers, causing a decrease in light scattering (Fig. 3). CTZ has a similar effect (Fig. 3). However, the dimers formed in the presence of 50 μM CTZ have a lower catalytic activity and thus are different from those formed in the presence of 30 nM CaM (see Fig. 1). Actually, the dimerization can account for the inhibition of the enzyme promoted by 50 μM CTZ observed in Fig. 1. Comparing these results with those obtained in the presence of 50 μM compound 48/80, it is clear that the latter affects neither the catalytic activity (Fig. 1) nor the light scattered by PFK (Fig. 3).

In order to investigate whether the effect of CTZ on PFK oligomeric structure is associated with the effect on enzyme

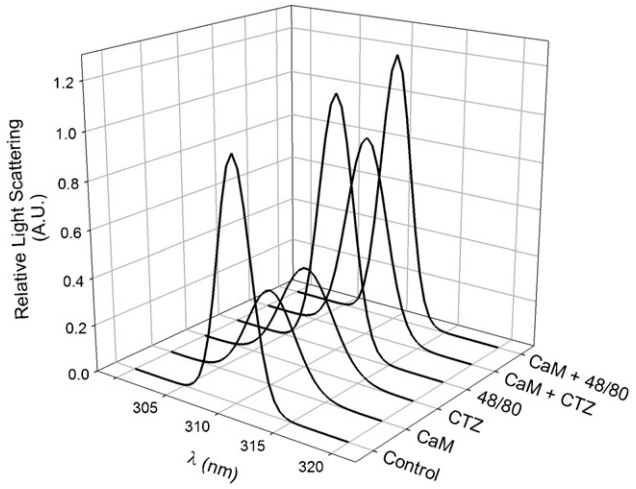

Fig. 3 – Effects of CTZ, CaM and 48/80 on light scattering of purified PFK. Light-scattering experiments were performed as described under Section 2. The plotted spectra are representative of a series of three independent experiments, all presenting the same results. When present, CTZ, CaM and 48/80 concentrations were 50 μM, 30 nM and 50 μM, respectively.

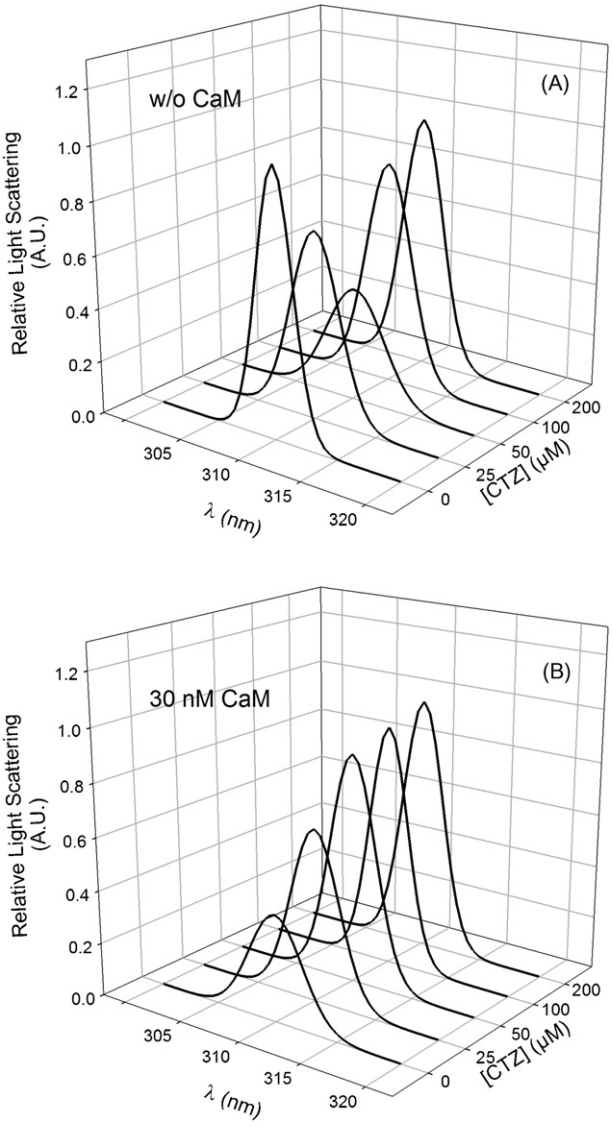

Fig. 4 – Light scattering of purified PFK at different CTZ concentrations. Light-scattering experiments were performed as described under Section 2, either without CaM (A), or in the presence of 30 nM CaM (B). The plotted spectra are representative of a series of three independent experiments, all presenting the same results. In the absence of CaM (A), results obtained in the presence of 25 or 50 μM CTZ were statistically different from controls in the absence of CTZ ($P < 0.05$, two-tailed ANOVA). In the presence of 30 nM CaM (B), results obtained in the presence of any concentration of CTZ tested were different from control in the absence of CTZ ($P < 0.05$, two-tailed ANOVA).

kinetics, we performed a CTZ titration of PFK using light-scattering measurements in the absence and presence of 30 nM CaM. Fig. 4A shows that, in the absence of CaM, CTZ induces PFK dimerization up to 50 μM, an effect that is lost at higher concentrations of the drug. In the presence of 30 nM CaM, CTZ reverses the dimerization promoted by CaM (Fig. 4B). To confirm these results, we evaluated the effects of CTZ on the PFK intrinsic fluorescence emission spectrum in the absence and presence of 30 nM CaM. We have previously demonstrated that PFK dimerization reduces its intrinsic fluorescence [14]. Fig. 5A shows that, in the absence of CaM, the fluorescence is reduced as CTZ increases to 50 μM, whereas higher concentrations of the drug reverse the effect. In the presence of 30 nM CaM, CTZ promotes a dose-dependent tetramerization of the enzyme (Fig. 5B). Curiously, although CTZ induces the dimerization of the enzyme, it counteracts the dimerization of PFK induced by CaM (Figs. 3 and 4). This counteracting effect of CTZ is also seen when the dimerization of PFK is promoted by CTZ itself (25–50 μM, Fig. 2).

CTZ has been identified as a CaM antagonist [7,8], and this property is confirmed by the data in Fig. 3, where CTZ clearly antagonizes the CaM effects on oligomerization of PFK, similarly to the classical CaM antagonist, compound 48/80. However, CTZ can be also considered a partial CaM agonist, since CTZ alone, like CaM, induces the dimerization of PFK. This proposal is corroborated by the fact that this property is dependent on Ca^{2+}: neither CaM nor CTZ has any effect on catalytic activity or light scattering of PFK in the absence of Ca^{2+} (data not shown; for CaM effects see [14]). In addition, the dual effects of CTZ in the absence of CaM indicate that it may act as a partial agonist or as an antagonist depending on its concentration and on the presence or not of another agonist, i.e. CaM. This may mean that CTZ binds to two distinct sites on PFK: the first, with higher affinity, induces dimerization (partial agonist effects), and the second, with lower affinity, induces the tetramerization of the enzyme (antagonist effects).

3.3. *Effects of CTZ and compound 48/80 on PFK activity in the presence of erythrocyte membranes*

PFK is a multiregulated enzyme that interacts with several cytoskeletal elements, depending on the oligomeric

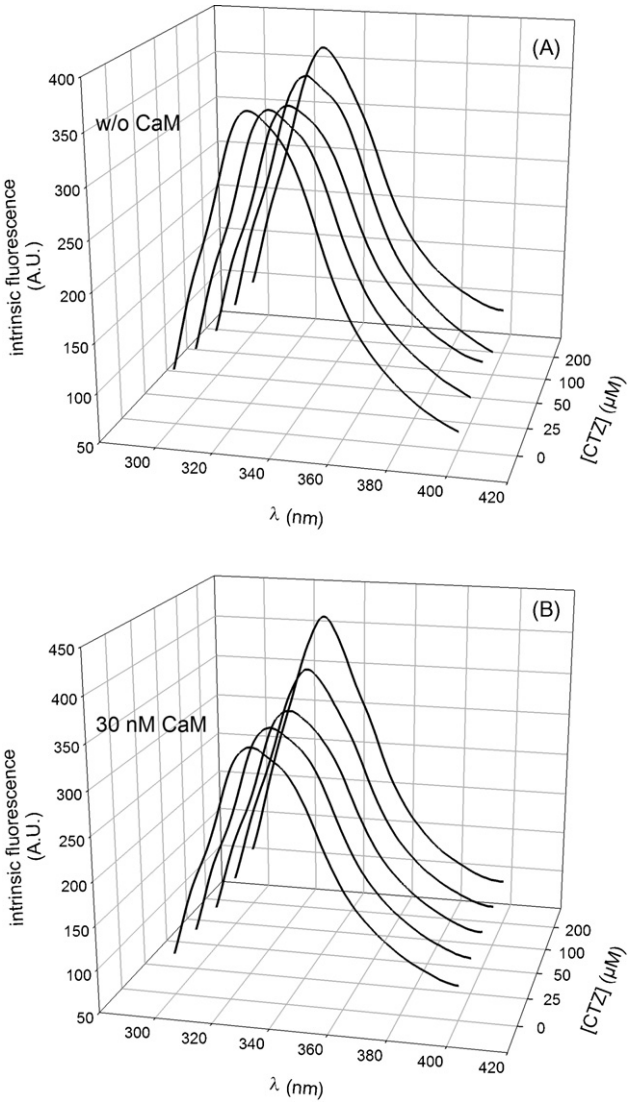

Fig. 5 – Intrinsic fluorescence spectra of purified PFK at different CTZ concentrations, either without CaM (A) or in the presence of 30 nM CaM (B). The plotted spectra are representative of a series of three independent experiments, all presenting the same results. In the absence of CaM (A), spectra obtained in the presence of 25 or 50 μM CTZ were statistically different from controls in the absence of CTZ ($P < 0.05$, two-tailed ANOVA). In the presence of 30 nM CaM (B), spectra obtained in the presence of any concentration of CTZ tested were different from control in the absence of CTZ ($P < 0.05$, two-tailed ANOVA).

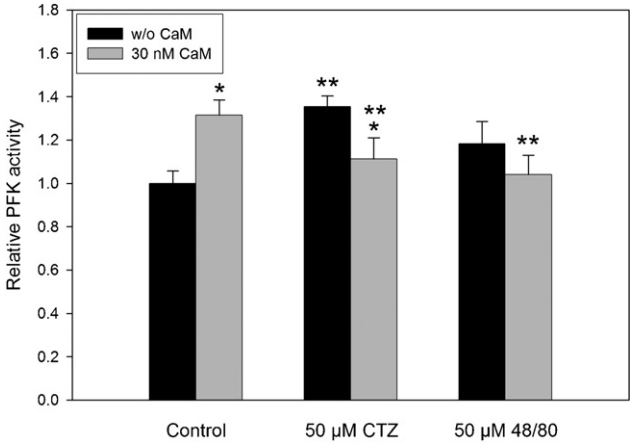

Fig. 6 – Effects of CTZ and 48/80 on catalytic activity of purified PFK in the presence of erythrocyte membranes. Catalytic activity was calculated by linear regression to the formation of product as a function of time. Relative PFK activity was calculated by dividing the rate obtained for each condition by that obtained in the absence of CTZ, 48/80 and CaM. Plotted values represent mean ± standard errors of 5 independent experiments ($n = 5$). $^{*}P < 0.05$ in comparison with the same group in the absence of CaM. $^{**}P < 0.05$ in comparison with the control group (±CaM, as appropriate).

conformation of the enzyme [35,36]. For instance, tetrameric forms of the enzyme have a higher affinity for actin, and the complex with actin stabilizes the tetrameric conformation, activating the PFK [37,38]. On the other hand, PFK dimers bind to tubulin and microtubules, forming an inactive complex [39,40]. PFK dimers also bind to erythrocyte membranes, in a complex that decreases both the enzyme activity and the glycolytic flux within these cells [41,42]. We have previously demonstrated that CaM directly interferes in the association of PFK with erythrocyte membranes [14,22], mediating some of the effects of insulin on these cells [21,22]. Here, we show that CaM stimulates PFK activity when the experiments are performed in the presence of erythrocyte membranes (Fig. 6, control). A similar effect is observed when CTZ is used instead of CaM (Fig. 6, compare black bars in CTZ and control groups). Similarly to the experiments performed without erythrocyte membranes, compound 48/80 did not significantly alter PFK activity in these experiments (Fig. 6). However, both CTZ and compound 48/80 antagonize CaM's stimulation of PFK activity in the presence of erythrocyte membranes (Fig. 6).

3.4. *Effects of CTZ and compound 48/80 on the association of PFK with erythrocyte membranes*

To determine whether the effects of CaM, CTZ and compound 48/80 observed in Fig. 6 were due to the association of the enzyme with erythrocyte membranes, we separated the membrane-bound enzyme from the soluble fraction. For this purpose, we incubated the PFK with the membrane preparation, in the absence and presence of the additives, and after a 90,000 × *g* centrifugation, measured PFK activity in the pellets containing only membrane-bound enzyme (see Section 2) [22]. Fig. 7 shows that CaM promotes the association of PFK with erythrocyte membranes to the same extent that it increases PFK activity in the presence of these membranes (compare control groups in Figs. 6 and 7). CTZ has similar effects, increasing both the association and the activation of enzyme activity in the presence of membranes. In addition, CTZ counteracts the effects of CaM on association of PFK with

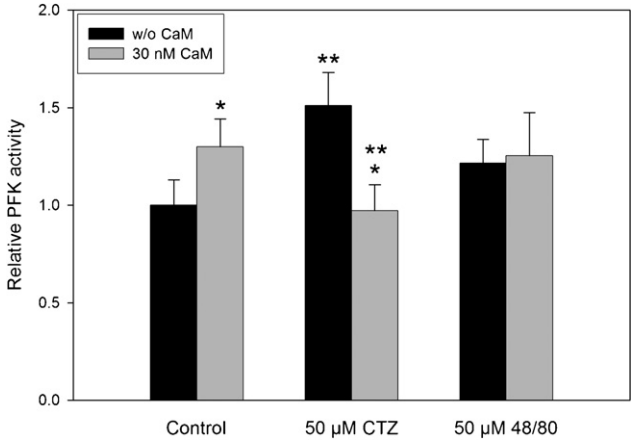

Fig. 7 – Effects of CTZ and 48/80 on the interaction of PFK with erythrocyte membranes. Interaction of PFK and erythrocyte membranes were assessed as described under Section 2. Plotted values represent mean ± standard errors of five independent experiments ($n = 5$). $^{*}P < 0.05$ in comparison with the same group in the absence of CaM. $^{**}P < 0.05$ in comparison with the control group (±CaM, as appropriate).

membranes (Fig. 7, compare gray bars in CTZ and control group), decreasing it to control levels in the absence of CaM (Fig. 7, compare gray bar in CTZ group with black bar in control group). When these experiments were performed in the presence of compound 48/80, we did not observe any significant effect either in the absence or in the presence of CaM (Fig. 7). In this case, compound 48/80 did not counteract CaM-induced PFK association with erythrocyte membranes, revealing that its ability to antagonize the activation of PFK by CaM in the presence of membranes does not involve this mechanism. The association of PFK with erythrocyte membranes is described as inhibitory for the enzyme [41,42]. However, we find some conditions, such as the stimulation of these cells with insulin and the presence of Ca_4CaM, where the associated enzyme is active due to its binding to a different site of the membrane (Zancan and Sola-Penna, unpublished results).

4. Discussion

PFK associates with the cytoskeleton in response to several signals [43–45]. Association of PFK and the cytoskeleton is directly correlated with the ability of tumor cells to generate ATP rapidly and in large amounts, guaranteeing cell survival and division. Indeed, there is a positive correlation between the aggressiveness of cancer cells and the interaction of PFK with cytoskeleton [45]. CTZ has been proposed as a promising antineoplastic agent against diverse types of cancer [6,9–13,46,47]. The novel therapeutic property of this drug is due to its ability to inhibit glycolysis [9–13,47], which is upregulated in cancer cells [48,49]; it also decreases drug resistance [48]. The effects of CTZ on glycolysis are due to its ability to lower the concentrations of the glucose activators glucose 1,6-bisphosphate and fructose 1,6-bisphosphate [11,47] and to decrease hexokinase activity by detaching it from the mitochondria [10], thereby decreasing cellular levels of ATP [47], detaching PFK from the cytoskeleton [11–13], and inducing apoptosis [13]. All of these mechanisms of action have been related to its activity as a CaM antagonist [9–13]. Data presented here reveal that this property may not be the only one that confers an antineoplastic action on CTZ. CTZ directly inhibits PFK, a phenomenon that would decrease cellular levels of fructose 1,6-bisphosphate and ATP, explaining some of the previously reported actions of this drug. In addition, we show that CTZ is also able to induce the dimerization of PFK, which has been reported to decrease the affinity of the enzyme for actin filaments [37,38]. This effect of CTZ explains its ability to detach PFK from the cytoskeleton. None of these properties is related to the CaM antagonist activity of CTZ, since compound 48/80 does not present these same actions. Altogether, our results reveal that CTZ inhibits cellular glycolysis by direct inhibition of PFK. It appears to stabilize the inactive dimeric conformation of the enzyme.

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REFERENCES

[1] Burgess MA, Bodey GP. Clotrimazole (Bay b 5097): in vitro and clinical pharmacological studies. Antimicrob Agents Chemother 1972;2:423–6.
[2] Brugnara C, Armsby CC, Sakamoto M, Rifai N, Alper SL, Platt O. Oral-administration of clotrimazole and blockade of human erythrocyte Ca^{++}-activated K^+ channel—the imidazole ring is not required for inhibitory activity. J Pharm Exp Ther 1995;273:266–72.
[3] Snajdrova L, Xu AD, Narayanan N. Clotrimazole, an antimycotic drug, inhibits the sarcoplasmic reticulum calcium pump and contractile function in heart muscle. J Biol Chem 1998;273:28032–9.
[4] Alvarez J, Montero M, Garcia-Sancho J. Cytochrome P-450 may link intracellular Ca^{2+} stores with plasma-membrane Ca^{2+} influx. Biochem J 1991;274:193–7.
[5] Benzaquen LR, Brugnara C, Byers HR, Gattonicelli S, Halperin JA. Clotrimazole inhibits cell-proliferation in vitro and in vivo. Nat Med 1995;1:534–40.
[6] Aktas H, Fluckiger R, Acosta JA, Savage JM, Palakurthi SS, Halperin JA. Depletion of intracellular Ca^{2+} stores, phosphorylation of eIF2alpha, and sustained inhibition of translation initiation mediate the anticancer effects of clotrimazole. Proc Natl Acad Sci USA 1998;95:8280–5.
[7] Mac Neil S, Dawson RA, Crocker G, Tucker WFG, Bittiner B, Singleton JG, et al. Antiproliferative effects on keratinocytes of a range of clinically used drugs with calmodulin antagonist activity. Br J Dermatol 1993;128:143–50.

[8] Hegemann L, Toso SM, Lahijani KI, Webster GF, Uitto J. Direct interaction of antifungal azole-derivatives with calmodulin: a possible mechanism for their therapeutic activity. J Invest Dermatol 1993;100:343–6.
[9] Glass-Marmor L, Beitner R. Detachment of glycolytic enzymes from cytoskeleton of melanoma cells induced by calmodulin antagonists. Eur J Pharmacol 1997;328:241–8.
[10] Penso J, Beitner R. Clotrimazole and bifonazole detach hexokinase from mitochondria of melanoma cells. Eur J Pharmacol 1998;342:113–7.
[11] Penso J, Beitner R. Clotrimazole decreases glycolysis and the viability of lung carcinoma and colon adenocarcinoma cells. Eur J Pharmacol 2002;451:227–35.
[12] Penso J, Beitner R. Detachment of glycolytic enzymes from cytoskeleton of Lewis lung carcinoma and colon adenocarcinoma cells induced by clotrimazole and its correlation to cell viability and morphology. Mol Genet Metab 2002;76:181–8.
[13] Meira DD, Marinho-Carvalho MM, Teixeira CA, Veiga VF, Da Poian AT, Holandino C, et al. Clotrimazole decreases human breast cancer cells viability through alterations in cytoskeleton-associated glycolytic enzymes. Mol Genet Metab 2005;84:354–62.
[14] Marinho-Carvalho MM, Zancan P, Sola-Penna M. Modulation of 6-phosphofructo-1-kinase oligomeric equilibrium by calmodulin: Formation of active dimmers. Mol Genet Metab 2006;87:253–61.
[15] Suzuki-Nishimura T, Oku N, Nango M, Uchida MK. PEI6, a new basic secretagogue in rat peritoneal mast cells: characteristics of polyethylenimine PEI6 resemble those of compound 48/80. Gen Pharmacol 1995;26:1171–8.
[16] Maia JCC, Gomes SL, Juliani MH. Preparation of [α-^{32}P] and [γ-^{32}P] nucleoside triphosphate, with high specific activity. In: Morel CM, editor. Genes and antigenes of parasites, a laboratory manual. Rio de Janeiro, RJ, Brazil: Fundação Oswaldo Cruz; 1983. p. 146–57.
[17] Kemp RG. Phosphofructokinase. Methods Enzymol 1975;42:72–7.
[18] Kuo H-J, Malencik DA, Liou R-S, Anderson SR. Factors affecting the activation of rabbit muscle phosphofructokinase by actin. Biochemistry 1986;25:1278–86.
[19] Lowry OH, Rosenbrough NL, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. J Biol Chem 1951;193:265–75.
[20] Sola-Penna M, Santos AC, Serejo FC, Alves GG, El-Bacha T, Faber-Barata J, et al. A radioassay for phosphofructokinase-1 activity in cell extracts and purified enzyme. J Biochem Biophys Methods 2002;50:129–40.
[21] Zancan P, Sola-Penna M. Regulation of human erythrocyte metabolism by insulin: cellular distribution of 6-phosphofructo-1- kinase its implication for red blood cell function. Mol Genet Metab 2005;86:401–11.
[22] Zancan P, Sola-Penna M. Calcium influx: a possible role for insulin modulation of intracellular distribution and activity of 6-phosphofructo- 1-kinase in human erythrocytes. Mol Genet Metab 2005;86:392–400.
[23] Alves GG, Lima LMTR, Favero-Retto MP, Lemos AP, Peres-Sampaio CE, Meyer-Fernandes JR, et al. *p*-Nitrophenylphosphatase activity catalyzed by plasma membrane (Ca^{2+}+Mg^{2+})ATPase: correlation with structural changes modulated by glycerol and Ca^{2+}. Biosci Rep 2001;21:25–32.
[24] Mayr GW, Heilmeyer Jr LM. Phosphofructokinase is a calmodulin binding protein. FEBS Lett 1983;159:51–7.
[25] Mayr GW. Interaction of calmodulin with muscle phosphofructokinase. Changes of the aggregation state, conformation and catalytic activity of the enzyme. Eur J Biochem 1984;143:513–20.
[26] Mayr GW. Interaction of calmodulin with muscle phosphofructokinase. Interplay with metabolic effectors of the enzyme under physiological conditions. Eur J Biochem 1984;143:521–9.
[27] Buschmeier B, Meyer HE, Mayr GW. Characterization of the calmodulin-binding sites of muscle phosphofructokinase and comparison with known calmodulin-binding domains. J Biol Chem 1987;262:9454–62.
[28] Lan JQ, Steiner RF. The interaction of troponin C with phosphofructokinase. Comparison with calmodulin. Biochem J 1991;274:445–51.
[29] Martin SR, Biekofsky RR, Skinner MA, Guerrini R, Salvadori S, Feeney J, et al. Interaction of calmodulin with the phosphofructokinase target sequence. FEBS Lett 2004;577:284–8.
[30] Ozturk Y, Aydin S, Ozcelikay AT, Altan VM, Yildizoglu-Ari N. Calmodulin content and in vitro contractility of duodenum from streptozotocin-induced diabetic rats: effects of insulin therapy and calmodulin antagonism. Eur J Pharmacol 1997;321:59–65.
[31] Beitner R. Calmodulin antagonists and cell energy metabolism in health and disease. Mol Genet Metab 1998;64:161–8.
[32] Berridge MJ, Lipp P, Bootman MD. The versatility and universality of calcium signalling. Nat Rev Mol Cell Biol 2000;1:11–21.
[33] Carpenter JF, Hand SC. Comparison of pH-dependent allostery and dissociation for phosphofructokinases from Artemia embryos and rabbit muscle: nature of the enzymes acylated with diethylpyrocarbonate. Arch Biochem Biophys 1986;248:1–9.
[34] Hand SC, Carpenter JF. pH-induced hysteretic properties of phosphofructokinase purified from rat myocardium. Am J Physiol 1986;250:R505–11.
[35] Ovadi J, Orosz F, Hollan S. Functional aspects of cellular microcompartmentation in the development of neurodegeneration: mutation induced aberrant protein–protein associations. Mol Cell Biochem 2004;256-7: 83–93.
[36] Ovadi J, Saks V. On the origin of intracellular compartmentation and organized metabolic systems. Mol Cell Biochem 2004;256–7:5–12.
[37] Liou R-S, Anderson S. Activation of rabbit muscle phosphofructokinase by F-actin and reconstituted thin filaments. Biochemistry 1980;19:2684–8.
[38] Clarke F, Stephan P, Morton DJ, Wiedemann J. The role of actin and associated structural proteins in the organization of glycolytic enzymes. In: Barden J, Dos Remedios C, editors. Actin: Structure and Function in Muscle and Non-Muscle Cells. Sydney: Academic Press; 1983. p. 249–57.
[39] Lehotzky A, Telegdi M, Liliom K, Ovádi J. Interaction of phosphofructokinase with tubulin and microtubule: quantitative evaluation of the mutual effects. J Biol Chem 1993;268:10888–94.
[40] Vertessy BG, Kovacs J, Low P, Lehotzky A, Molnar A, Orosz F, et al. Characterization of microtubule-phosphofructokinase complex: specific effects of MgATP and vinblastine. Biochemistry 1997;36:2051–62.
[41] Higashi T, Richards CS, Uyeda K. The interaction of phosphofructokinase with erythrocyte membranes. J Biol Chem 1979;254:9542–50.
[42] Campanella ME, Chu H, Low PS. Assembly and regulation of a glycolytic enzyme complex on the human erythrocyte membrane. Proc Natl Acad Sci USA 2005;105:2402–7.
[43] Alves GG, Sola-Penna M. Epinephrine modulates cellular distribution of muscle phosphofructokinase. Mol Genet Metab 2003;78:302–6.
[44] Silva APP, Alves GG, Araújo AHB, Sola-Penna M. Effects of insulin and actin on phosphofructokinase activity and

cellular distribution in skeletal muscle. Ann Acad Bras Cienc 2004;76:541–8.
[45] El-Bacha TP, Freitas MS, Sola-Penna M. Cellular distribution of phosphofructokinase activity and implications to metabolic regulation in human breast cancer. Mol Genet Metab 2003;79:294–9.
[46] Glass-Marmor L, Morgenstern H, Beitner R. Calmodulin antagonists decrease glucose 1,6-bisphosphate, fructose 1,6-bisphosphate, ATP and viability of melanoma cells. Eur J Pharmacol 1996;313:265–71.
[47] Klokouzas A, Barrand MA, Hladky SB. Effects of clotrimazole on transport mediated by multidrug resistance associated protein 1 (MRP1) in human erythrocytes and tumour cells. Eur J Biochem 2001;268:6569–77.
[48] Ashrafian H. Cancer's sweet tooth: the Janus effect of glucose metabolism in tumorigenesis. Lancet 2006;367: 618–21.
[49] Gatenby RA, Gillies RJ. Why do cancers have high aerobic glycolysis? Nat Rev Cancer 2004;4:891–9.