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Original paper

Clotrimazole potentiates the inhibitory effects of ATP on the key glycolytic enzyme 6-phosphofructo-1-kinase

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ABSTRACT

Clotrimazole (CTZ) has been proposed as a potential anti-neoplastic agent, which inhibits glucose metabolism. The present work aimed to evaluate the effects of CTZ on the kinetic mechanism of 6-phosphofructo-1-kinase (PFK). We show that CTZ promotes a dose-dependent inhibition of PFK, presenting a K_i of 28 ± 2 μM. Inhibition occurs through the dissociation of the enzyme tetramers, as demonstrated through fluorescence spectroscopy and gel filtration chromatography. Moreover, the affinities of the enzyme for ATP and fructose-6-phosphate are reduced 50% and 30%, respectively. Furthermore, the affinity of PFK for ATP at the inhibitory site becomes 2-fold higher. Altogether, the results presented here suggest that PFK inhibition by CTZ involves a decrease in the affinity of PFK for its substrates at the catalytic site with the concomitant potentiation of the inhibitory properties of ATP.

Introduction

Clotrimazole (CTZ[1]) is an antifungal imidazole derivative, which has been described as a potential anti-neoplastic drug [1–6]. Its anti-neoplastic properties are associated with its ability to decrease glucose consumption and energy metabolism in tumor cells [1,2]. There are several proposed targets for the action of CTZ on cell metabolism, and many of them concern the glycolytic pathway [6,7]. We have previously described that this drug decreases the viability of breast cancer cells by inhibiting the glycolytic pathway [3], and that this inhibition is probably due to a direct effect of CTZ on the major glycolytic enzyme, 6-phosphofructo-1-kinase (PFK; phosphofructokinase-1; EC 2.7.1.11) [4].

PFK is the key enzyme regulating glycolysis; therefore, it undergoes a complex regulation by several metabolites and cellular signals [8]. Among the molecular mechanisms regulating PFK activity is the stabilization of PFK in distinct oligomeric conformations, where the transition between fully active tetramers and quite inactive dimers appears to be the major step [9,10]. This transition is involved in the regulation of PFK activity by several modulators, such as its substrates [11,12], allosteric ligands [13], hormones [14–16], other intracellular proteins [8,17–22] and drugs [4,23–26].

The aim of the present work was to understand the mechanism by which CTZ inhibits PFK and therefore to contribute to the elucidation of its property to decrease cell glucose consumption and energy metabolism.

Materials and methods

Materials

ATP, fructose-6-phosphate and CTZ 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. [27]. Purified PFK was obtained from rabbit skeletal muscle according to the method developed by Kemp [28]. All other reagents were of the highest quality available.

Radiometric assay for PFK activity

PFK activity was measured by the method described in [29] with the modifications introduced in [16,30], using a reaction medium containing 50 mM Tris–HCl (pH 7.5), 5 mM $MgCl_2$, 5 mM $(NH_4)_2SO_4$, 1 mM [γ-^{32}P]ATP (4 μCi/nmol), 1 mM fructose-6-phosphate (F6P) and 1 μg/ml purified PFK. Modifications to pH and the concentrations of ATP, F6P and PFK are specified for each experiment in the figure legends. The reaction was stopped by

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[1] *Abbreviations used:* CTZ, clotrimazole, 1-[(2-chlorophenyl)-diphenyl-methyl]imidazole; CM, center of mass of intrinsic fluorescence spectrum; F6P, fructose-6-phosphate; PFK, 6-phosphofructo-1-kinase, phosphofructokinase.

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addition of a suspension of activated charcoal in 0.1 M HCl and 0.5 M mannitol. After centrifugation, the supernatant, which contained [1-^{32}P]fructose-1,6-bisphosphate, was analyzed in a liquid scintillation counter. Appropriate controls in the absence of fructose-6-phosphate were performed and subtracted from all measurements to discount ATP hydrolysis. One mU was considered as the formation of 1 nmol fructose-1,6-bisphosphate per minute.

Intrinsic fluorescence measurements

Intrinsic fluorescence measurements of PFK were performed as described previously [17] using the same conditions described for the radioassay. Excitation wavelength was fixed at 280 nm, and fluorescence emission was scanned from 300 to 400 nm. The center of mass of the intrinsic fluorescence spectra (CM) was calculated using:

$$\mathrm{CM} = \frac{\sum \lambda \times I_\lambda}{\sum I_\lambda}, \tag{1}$$

where λ is the wavelength and I_λ is the fluorescence intensity at a given λ. Center of mass is used to evaluate the oligomeric state of PFK because the dissociated enzyme exposes its tryptophans to the aqueous milieu to a greater extent than the oligomer; thus, the fluorescence emitted by these tryptophans is of lower energy. Consequently, the center of mass of a population of tetramers is smaller than that of a population of dimers, as confirmed in many recent publications [8,11–13,17,26].

Sepharose 12 chromatography

Sepharose 12 chromatography was conducted as described previously [12]. PFK samples were pre-incubated for 1 h in a medium containing 50 mM Tris–HCl (pH 7.4), 5 mM $MgCl_2$, 5 mM $(NH_4)_2SO_4$, 1 mM F6P and 1 mM ATP in the absence or presence of 50 μM CTZ and then applied into a Superose-12 (HR 10/30) column linked to a HPLC system (Shimadzu, Tokyo, Japan). A buffer containing 20 mM Tris–HCl 20 (pH 7.4) was used to elute the column at a flow rate of 0.4 ml/min, and 0.5 ml fractions were collected and measured by automatically recording the absorbance at 280 nm. Dextran blue was used to determine the V_o (exclusion volume), and molecular weight standards were used to estimate the molecular weight of the peaks.

Statistics and calculations

Statistical analyses were performed using the software SigmaPlot 10.0 integrated with SigmaStat 3.51 (Systat, CA, USA). Student's *t*-test or one-tailed ANOVA were used to evaluate the significance of different numerical values. $P < 0.05$ was considered to be statistically significant.

Kinetic parameters for the effects of ATP on PFK were calculated considering the two components for PFK modulation by this metabolite. The first component is the stimulatory component for the substrate saturation curve, in which PFK exhibits an allosteric pattern that is described by the equation:

$$v = \frac{\mathrm{Vmax_{app}} \times [\mathrm{ATP}]^{n_s}}{K_{0.5}^{n_s} + [\mathrm{ATP}]^{n_s}}, \tag{2}$$

where v is the PFK activity at a given concentration of ATP ([ATP]), $\mathrm{Vmax_{app}}$ is the apparent maximal velocity calculated, $K_{0.5}$ is the affinity constant for this component and n_s is the cooperativity index for this component. The second component is the inhibitory component that can be adjusted by the equation:

$$v = \frac{\mathrm{Vsat} \times I_{0.5}^{n_i}}{I_{0.5}^{n_i} + [\mathrm{ATP}]^{n_i}}, \tag{3}$$

where v is the PFK activity at a given concentration of ATP ([ATP]), $I_{0.5}$ is the affinity constant for this component, n_i is the cooperativity index for this component and Vsat is the PFK activity when the first component is saturated. Assuming this statement, Vsat is a function of the first component of the curve and can be substituted by Eq. (2) to result in the following equation:

$$v = \frac{\frac{\mathrm{Vmax_{app}} \times [\mathrm{ATP}]^{n_s}}{K_{0.5}^{n_s} + [\mathrm{ATP}]^{n_s}} \times I_{0.5}^{n_i}}{I_{0.5}^{n_i} + [\mathrm{ATP}]^{n_i}}, \tag{4}$$

which was fitted to the experimental data through non-linear regression for the effects of ATP on PFK activity.

Kinetic parameters for the effects of F6P on PFK were calculated through non-linear regression using the experimental data to fit the parameters of the equation:

$$v = \frac{\mathrm{Vmax} \times [\mathrm{F6P}]^n}{K_{0.5} + [\mathrm{F6P}]^n}, \tag{5}$$

where v is the PFK activity calculated for a given concentration of F6P ([F6P]), Vmax is the maximal velocity calculated at saturating concentrations of F6P, $K_{0.5}$ is the affinity constant for F6P, which is equal to the concentration of F6P responsible for half-activation of the PFK by F6P, and n is the cooperativity index for this phenomenon.

The kinetic parameters for the inhibition of PFK by CTZ were calculated, fitting the equation below to the PFK catalytic velocity measured in the presence of different concentrations of CTZ. The equation used was:

$$v = \frac{V_0 \times K_i^n}{K_i^n + [\mathrm{CTZ}]^n} + V_{\mathrm{rem}}, \tag{6}$$

where v is the PFK activity calculated at a given concentration of CTZ ([CTZ)], V_{rem} is the remaining PFK activity once maximal inhibition has been reached, Vo is the apparent PFK activity in the absence of CTZ, considering that at saturating concentrations of CTZ, the PFK activity is equal to V_{rem} (thus, the actual PFK activity in the absence of CTZ can be calculated by the sum of Vo and V_{rem}), K_i is the inhibition constant and n is the cooperativity index.

Kinetic parameters for changes in the center of mass of the intrinsic fluorescence emission spectra of PFK as a function of incubation time were calculated, fitting the equation below to the center of mass calculated from the fluorescence emission spectra obtained at the incubation times specified. The first-order kinetics equation used was:

$$\mathrm{CM} = \mathrm{CM_0} + (\mathrm{CM}_m \times (1 - e^{-k \cdot t})), \tag{7}$$

where CM is the predicted center of mass of the intrinsic fluorescence spectrum, $\mathrm{CM_0}$ is the center of mass of the intrinsic fluorescence spectrum predicted at time 0 of incubation, $\mathrm{CM_m}$ is the maximal change of the center of mass predicted for the infinite time, t is the time of incubation and k is the first-order kinetics constant rate.

Results

The effects of CTZ on PFK activity were evaluated after pre-incubation of the enzyme in the presence of increasing concentrations of CTZ for 1 h at 37 °C. Under these conditions, CTZ promotes a dose-dependent inhibition of PFK activity, presenting a K_i of 28 ± 2 μM and a maximal inhibition of 70% (Fig. 1A), as calculated using Eq. (6). This inhibitory effect is totally dependent on the pre-incubation of the enzyme with the drug, as no inhibition is ob-

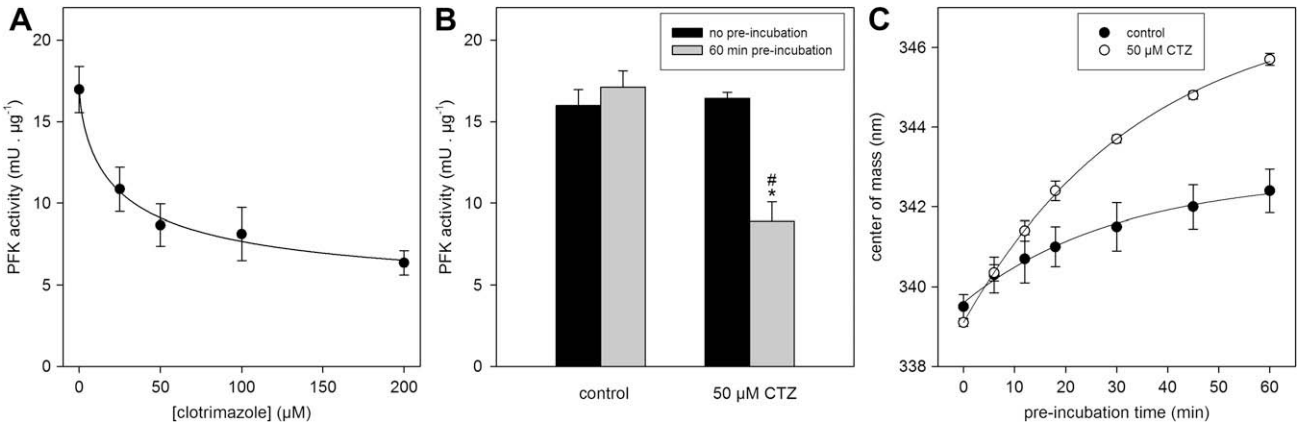

Fig. 1. Effects of CTZ on PFK activity and oligomeric conformation. PFK activity and oligomeric conformation were assessed as described under Material and Methods in the basic reaction medium. (A) Dose–response curve of CTZ on PFK activity. The enzyme was pre-incubated in the presence of CTZ, at the concentrations indicated on the abscissa, for 1 h prior to starting the activity assay. Plotted points represent mean ± standard error of three independent experiments ($n = 3$). A solid line was obtained by fitting Eq. (6) to the experimental data. (B) Effects of pre-incubation on the inhibition of PFK activity by CTZ. Black bars represent mean ± standard error of PFK activity measured before pre-incubating the enzyme. Gray bars represent mean ± standard error of PFK activity measured after pre-incubating the enzyme for 1 h in the absence or presence of 50 μM CTZ. $^{*}P < 0.05$, comparing control to pre-incubation (Student's t-test). $^{\#}P < 0.05$, comparing control to the activity measured after 1 h pre-incubation with 50 μM CTZ (Student's t-test). (C) Time-course of the effects of CTZ on the center of mass of PFK intrinsic fluorescence spectra. The center of mass was calculated by applying Eq. (1) to the experimental data of the intrinsic fluorescence spectra of PFK in the absence or the presence of 50 μM CTZ. Plotted values represent means ± standard error of three independent measurements ($n = 3$). Solid lines were obtained by fitting Eq. (7) to the plotted values.

served from 50 μM CTZ without pre-incubation (Fig. 1B). We have previously shown that CTZ promotes the dissociation of active PFK tetramers into dimers, suggesting that this mechanism is involved in the inhibitory effects of the drug [4]. Therefore, we decided to evaluate the kinetics of the dissociation of PFK tetramers in the presence of CTZ. The oligomeric conformation of the enzyme was assessed through calculating the center of mass of the intrinsic fluorescence emission spectrum of the enzyme. We have extensively characterized the intrinsic fluorescence spectra of PFK and found that the dimeric conformation of PFK displays a center of mass that is shifted to the red-region (higher wavelengths) as compared to that for the tetrameric conformation, validating this technique for assessment of the oligomeric state of PFK [8,11–13,17,26]. The time-course of PFK tetramer dissociation in the absence and presence of CTZ is presented in Fig. 1C. It can be observed that, upon dilution in the reaction medium, the center of mass of the intrinsic fluorescence spectra of PFK increases with first-order kinetics in both the absence or the presence of CTZ (Fig. 1C). This result corroborates that PFK oligomers dissociate upon dilution [17,31]. Therefore, when the concentrated enzyme stock (10 mg/ml) is diluted into the reaction medium to reach a final concentration of 1 μg/ml PFK, the complex oligomers of PFK dissociate to form a less complex oligomeric mixture, which is mainly composed of tetramers and dimers. Although this phenomenon occurs both in the absence and presence of CTZ, the dissociation is faster when the drug is present in the medium (constant rates of $0.033 \pm 0.002\ min^{-1}$ and $0.078 \pm 0.005\ min^{-1}$, for control and 50 μM CTZ, respectively, as calculated with Eq. (7)). Moreover, in the presence of the drug, the shift in the center of mass of the intrinsic fluorescence spectra is more pronounced than in the control, which is consistent with the fact that dimmers are the major conformation in the presence of the drug. Furthermore, the disso-

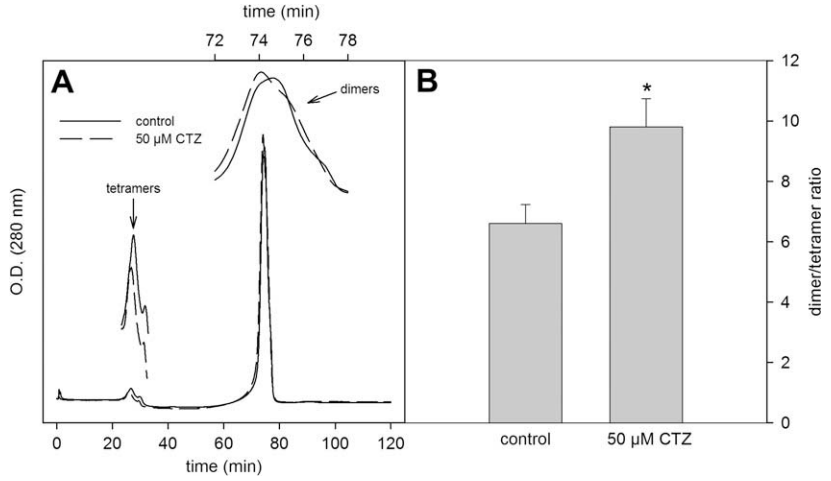

Fig. 2. Sepharose 12 chromatography of PFK with or without CTZ. The chromatographic conditions are described under Materials and methods. (A) Chromatographic pattern of PFK pre-incubated for 1 h in the absence (solid line) or presence of 50 μM CTZ (dashed line). The abscissa represents the time of the chromatographic run, and the ordinate represents the optical density of the eluate. The peak corresponding to the elution containing tetramers was magnified relative to the optical density, while the peak corresponding to the elution containing dimers was magnified relative to the retention time. The new abscissa values for this magnification are presented in the upper portion of (A). (B) Dimer/tetramer ratio obtained by dividing the peak area corresponding to dimers and tetramers from each chromatographic run. Bars represent mean ± standard error of three independent experiments ($n = 3$). $^{*}P < 0.05$, comparing to control (Student's t-test).

ciation of PFK tetramers is more pronounced in the presence of CTZ only after 20 min pre-incubation (Fig. 1C). This observation explains the requirement of pre-incubation for the inhibition of PFK activity by CTZ.

In order to understand the effects of CTZ on the oligomeric conformation of PFK, we performed Sepharose 12 chromatography on PFK pre-incubated or not with 50 μM CTZ for 1 h. The results are presented in Fig. 2A and show that two peaks eluted from the column. The first peak eluted from 23 to 30 min, which corresponds to a 340 kDa protein, and a second and major peak eluted from 72 to 78 min, which corresponds to a 170 kDa protein). This chromatographic pattern of PFK was previously demonstrated to result from the separation of PFK tetramers and dimers in the first and second peaks, respectively [12]. Magnification of these two peaks are presented and reveal that 50 μM CTZ promotes a decrease in the tetrameric population concomitantly with an increase in the dimeric conformation of the enzyme (Fig. 2A). This result is clearly observed by calculating the dimer/tetramer ratio; a significant increase in this ratio is observed in the presence of 50 μM CTZ (Fig. 2B). It is important to note that even under control conditions, the amount of PFK dimers was 6-fold higher than that of tetramers. However, this finding should be expected because during the chromatographic run, the enzyme is diluted in the column, increasing the proportion of the dissociated conformation [12,32].

The mechanism of CTZ-induced inhibition of PFK was assessed by evaluating the effects of the drug on the modulation of the enzyme by its substrates, ATP and fructose-6-phosphate (F6P). ATP exhibits a dual effect on PFK activity, acting as a substrate when bound to the high-affinity catalytic site on the enzyme and as an allosteric inhibitor when bound to the low-affinity inhibitory allosteric site. This effect is observed in Fig. 3A (filled circles) where the two components (stimulatory and inhibitory) for the ATP effects on PFK are presented. When the enzyme is pre-incubated for 1 h with 50 μM CTZ, PFK activity decreases at all ATP concentrations tested. Assessing the kinetic parameters for ATP modulation of PFK activity, we found that CTZ decreases the affinity of PFK for ATP at the catalytic site, increasing the $K_{0.5}$ from 0.36 ± 0.03 to 0.77 ± 0.08 mM ($P < 0.05$, Student's t-test). This effect is concomitant with the increase in the cooperativity index for the stimulatory component (n_s, Table 1), suggesting that CTZ intensifies the interdependence between the catalytic sites among different subunits of PFK. Because binding of ATP to the catalytic site stabilizes the tetrameric conformation of the enzyme [11,12], an increase of n_s is expected in the presence of CTZ, which stabilizes PFK dimers.

Table 1
Kinetic parameters for PFK modulation by its substrates. The parameters were calculated as described under Materials and methods, using Eq. (4) to calculate the parameter for ATP curve and Eq. (5) for F6P curve.

	Control	50 μM CTZ
ATP		
$K_{0.5}$ (mM)	0.36 ± 0.03	0.77 ± 0.08*
n_s	1.3 ± 0.2	2.1 ± 0.3*
$Vmax_{app}$ (mU/μg)	19.2 ± 1.7	14.6 ± 1.3*
$I_{0.5}$ (mM)	3.3 ± 0.3	1.7 ± 0.2*
n_i	1.6 ± 0.2	1.2 ± 0.2
F6P		
Vmax (mU/μg)	18.6 ± 1.4	16.2 ± 1.3*
$K_{0.5}$ (mM)	0.32 ± 0.02	0.48 ± 0.03*
n	1.2 ± 0.1	2.1 ± 0.2*

* $P < 0.05$ compared to control (Student's t-test).

Nevertheless, when analyzing the effects of CTZ on the inhibitory effect of ATP on PFK, it is clear that CTZ augments the affinity of the enzyme for ATP at the inhibitory site without compromising the cooperativity index for this component (n_i, Table 1). Recently, we demonstrated that the inhibitory effects of ATP on PFK activity are due to the dimerization of the enzyme that is induced when ATP is bound to the allosteric inhibitory site [11,12]. Therefore, the observed increase in the affinity of PFK for ATP at the inhibitory site in the presence of CTZ could be explained by the stabilization of PFK dimers by CTZ. Notwithstanding, the lack of an effect of CTZ on n_i suggests that CTZ does not interfere with the interdependence between the inhibitory sites, supporting the fact that the drug does not bind at this site. The effects of CTZ on the kinetic parameters for PFK activation by F6P are similar to those described above for the stimulatory component of the ATP effects on PFK. Briefly, CTZ inhibited PFK at all F6P concentrations tested (Fig. 3B), reducing the Vmax and the affinity of PFK for F6P, with a concomitant increase of the cooperativity index for this site (Table 1). The interpretation of these results could follow the same line of thought as discussed for ATP because binding of F6P to the catalytic site also stabilizes the tetrameric conformation of the enzyme [11,12]. Hence, CTZ counteracts the stabilization of PFK tetramers by its substrates without competing with them for binding at the catalytic site.

The oligomeric conformation of PFK is also modulated by pH. In acidic conditions, the dimeric conformation of the enzyme is stabilized, and in alkaline conditions, tetramers are preferentially

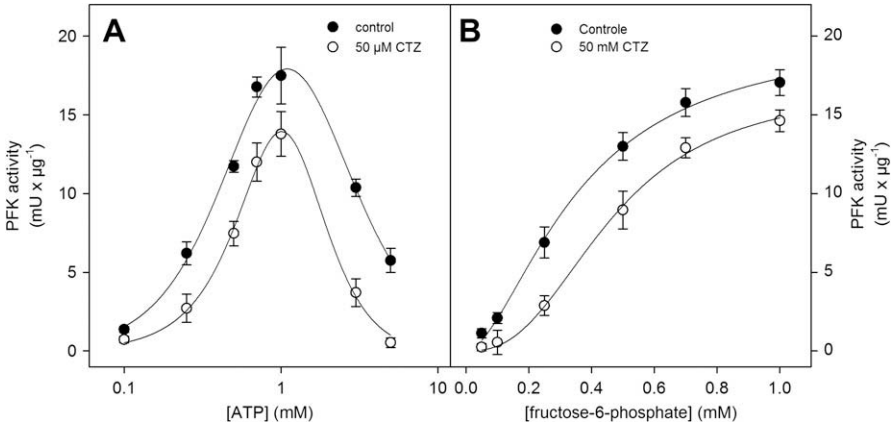

Fig. 3. The effects of CTZ on the modulation of PFK by its substrates. PFK activity was assessed as described under Materials and methods in the presence of ATP or F6P, at the concentrations indicated on the abscissa of (A) and (B), respectively. For (A), the concentration of F6P was 1 mM, and for (B), the concentration of ATP was 1 mM. Plotted values represent means ± standard error of three independent experiments ($n = 3$). Solid lines were obtained by fitting Eqs. (4) and (5) to the experimental data presented in (A) and (B), respectively.

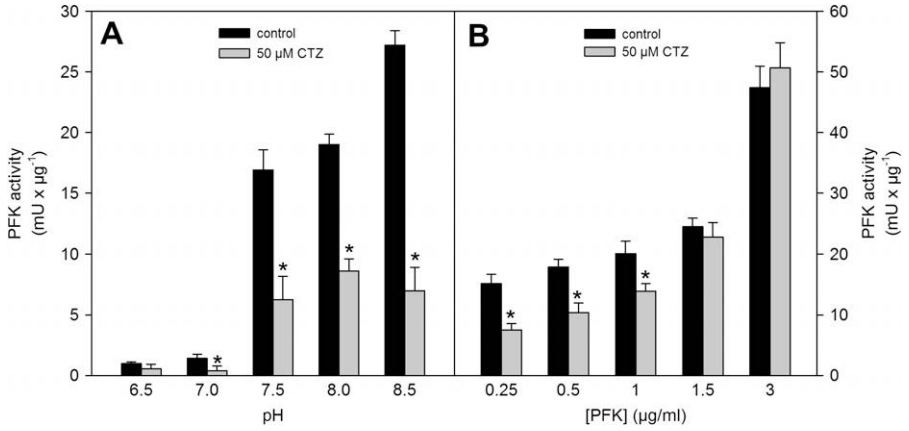

Fig. 4. The effects of pH and PFK concentration on the inhibition of PFK activity by CTZ. PFK activity was assessed as described under Materials and methods, varying the pH (A) or the PFK concentration (B) in the reaction medium, as represented on the abscissas. Bars represent means ± standard error of three independent experiments ($n = 3$). *$P < 0.05$, comparing to control (Student's t-test).

formed. This observation is clearly translated to the markedly low activity of the enzyme at acidic pH, in contrast to the high activity assessed at pH higher than 7.0 (Fig. 4A). However, CTZ significantly inhibited PFK activity at all the other pHs tested, except at pH 6.5, where the standard error was high due to the poor precision of PFK activity measurements (Fig. 4A). On the other hand, increasing the concentration of PFK in the conditions prevented CTZ-induced inhibition of the enzyme (Fig. 4B). Indeed, the inhibitory effects of CTZ decrease as the concentration of PFK increases. For example, at 1.5 μg/ml PFK or higher, no differences are observed for PFK activity assessed with or without CTZ. This decrease in CTZ-induced PFK inhibition is explained by the fact that complex oligomeric conformations of PFK, such as tetramers, are stabilized at high enzyme concentrations [13,17,31]. Furthermore, this decrease in inhibition cannot be attributed to an excess of PFK because 1.5 μg/ml is approximately 18 nM PFK monomers; thus, CTZ is in a large molar excess as compared to PFK in all of the conditions tested.

Discussion

Glycolysis is the major energetic metabolic pathway for growing cells, such as cancer cells [6,33,34]. Hence, the inhibition of the glycolytic rate of cancer cells reduces ATP generation and cell viability [1,2,25,35–38]. CTZ has been reported to decrease the viability of cancer cells affecting glycolysis through inhibition of PFK and its detachment from the cytoskeleton [1–3]. PFK has been proposed as an important target for the inhibition of glycolysis because this enzyme is considered to determine the rate of the glycolytic flux [3,26,39]. However, the lack of a specific inhibitor of this enzyme inspired many research groups to search for this molecule [4,13,26,36,40–43]. The results presented here reinforce that CTZ is a potent reversible inhibitor of PFK, decreasing its affinity for its substrates and interfering with the oligomeric state of the enzyme. Moreover, CT also increases the affinity of PFK for ATP at the inhibitory site. This event is particularly of interest because the intracellular concentrations of this metabolite vary in the range of the affinity constant of the inhibitory site [12]. Thus, the increase of affinity of this site for ATP might represent a significant inhibition of the enzyme at physiologic conditions, reflecting the decrease in the glycolytic rate observed in cells treated with CTZ [2,3]. Furthermore, the oligomeric conformation of PFK directly affects its association to the cytoskeleton, which is an important mechanism of enzyme regulation and is associated with cell survival [1,3,24,35,43,44]. Therefore, by interfering with the oligomeric conformation of PFK, CTZ affects the regulation of the enzyme by its substrates [11,12], hormones [14–16,30] and allosteric modulators [8,13], compromising the function of the enzyme and, consequently, the glucose metabolism in the cell. Recently, CTZ has been cited as a potential anti-neoplastic drug due to its ability to decrease glucose consumption and ATP production in cancer cells [6]. However, this drug was able to decrease the growth of gliomas in rats, augmenting the survival of the animals [5]. In conclusion, we believe that the present work will contribute to the elucidation of the molecular mechanisms by which CTZ promotes its anti-neoplastic effects, supporting its use in clinical trials.

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