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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-