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Effects of mercury on myosin ATPase in the ventricular myocardium of the rat

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Abstract

Mercury reduces twitch and tetanic force development in isolated rat papillary muscles, and a putative toxic effect on the contractile machinery has been suggested. Based on that, the actions of $HgCl_2$ on the myosin ATPase activity of the left ventricular myocardium were investigated. Samples for assay of myosin ATPase activity were obtained from rats' left ventricles. Increasing concentrations of $HgCl_2$ reduced dose-dependently the activity of the myosin ATPase. This reduction was observed even at very small concentrations, 50 nM $HgCl_2$. This effect was dependent on the presence of SH groups in the myosin molecule since DTT and glutathione protected the myosin ATPase against toxic effects of mercury; full activity being restored by using 500 nM DTT or 500 nM glutathione. Results also suggested that the metal acts as an uncompetitive inhibitor with a K_i of 200 nM $HgCl_2$. Our results suggest that mercury reduces the activity of the myosin ATPase by an uncompetitive mechanism at a very low dose that does not depress force. DTT and glutathione are effective for protection against the actions of mercury suggesting that SH groups might be the sites of action of the metal on the myosin molecule.

Keywords: Mercury; Dithiothreitol; Glutathione; Rat left ventricle; Myosin ATPase

1. Introduction

Mercury is a heavy metal that affects the activity of several enzymes, ion channels or receptors (Prabhu and Salama, 1990; Boraso and Williams, 1994; Hulme et al., 1990; Chiamvimonvat et al., 1995) by combining with SH groups (Clarkson, 1972; Halbach et al., 1981; Halbach, 1990) that are necessary for their normal function. These actions affect several tissue functions and several reports suggested toxic effects showing a force depression in papillary muscles (Halbach et al., 1981; Halbach, 1989; Oliveira et al., 1994a; Vassallo et al., 1999) and ventricular pressure in vivo and in vitro (Su and Chen, 1979; Rhee and Choi, 1989; Massaroni et al., 1992, 1995).

On the heart muscle these effects included a biphasic response. At 1 μM $HgCl_2$, an increase in force occurs, probably as a result of a digitalis-like effect inhibiting the activity of the plasma membrane Na^+,K^+-ATPase (Halbach et al., 1981; Magour et al., 1987; Anner et al., 1992). Second, at higher concentrations, contractile depression

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occurs probably resulting from an enhanced inhibition of the sarcolemmal Na^+,K^+-ATPase together with the inhibition of the sarcoplasmic reticulum Ca^{2+}-ATPase activity (Ahammad-Sahib et al., 1988; Halbach et al., 1981; Hechtenberg and Beyersmann, 1991; Temma et al., 1978) leading to a calcium overload. The rate of force development (dF/dt) also increased at lower concentrations of $HgCl_2$ (1 and 2.5 μM) returning to control values at larger concentrations. In the μM range a dose-dependent reduction of time to peak tension occurs because mercury triggers calcium release from sarcoplasmic reticulum (Abramson and Salama, 1989; Prabhu and Salama, 1990). Since the metal also reduces Ca^{2+} uptake by inhibiting the sarcoplasmic reticulum Ca^{2+}-ATPase (Ahammad-Sahib et al., 1988; Hechtenberg and Beyersmann, 1991), the release of Ca^{2+} upon activation reduces post-rest relative potentiation (Oliveira et al., 1994a). Besides, the reduction of tetanic tension development was also observed (Oliveira et al., 1994a; Vassallo et al., 1999).

We previously demonstrated that this action on the contractile proteins was related to a reduction of the activity of the myosin ATPase (Vassallo et al., 1999). Mercury (0.15 μM) reduced myosin ATPase activity by 50% and full activity was restored by 500 nM DTT suggesting that the metal affects SH groups. However, there are no reports on the actions of mercury on myosin ATPase, describing the type of inhibition caused by the metal and the role of SH groups in this inhibition. We then investigated the effects of mercury on the activity of the myosin ATPase obtained from the ventricular myocardium of rats and reanalyzed the role of SH groups in those effects by evaluating the protective action of DTT or glutathione.

2. Materials and methods

2.1. Rat tissue sampling

Male Wistar rats (200–250 g) were used. The care and use of laboratory animals were according to NIH guidelines. Rats were killed by cervical dislocation after a brief ether exposure and the hearts were rapidly removed. Tissue samples (left ventricles) were quickly harvested, rinsed, blotted and homogenized.

2.2. Preparation of tissue

Myosin was prepared from minced and homogenized left ventricles ($N=10$), extracted briefly with KCl-phosphate buffer (0.3 M KCl, 0.2 M phosphate buffer, pH 6.5) (Bremel and Weber, 1975). After precipitation of myosin by 15-fold dilution with water, the muscle residue was separated by filtration using cheesecloth. This procedure precipitates fragments of cells including membranes. The filtrate containing myosin was centrifuged at $33\,000\times g$ for 30 min. After decanting the supernatant the precipitate was re-dissolved in 0.6 M KCl for elution of myosin under high ionic strength and 1 ml of water was added for each g of tissue to produce a new precipitate. The material was again centrifuged at $33\,000\times g$ for 30 min and the muscle residue was separated by filtration. The material was re-dissolved again in 14 ml of water per g of tissue, centrifuged and filtered as before. The precipitate was dissolved in 50 mM HEPES, pH 7.0 and 0.6 M KCl plus 50%, v/v, glycerol and stored at −20 °C. To use the stored myosin, it was diluted in water (1:12) and centrifuged at $600\times g$ for 15 min. The precipitate was re-suspended in 50 mM HEPES, pH 7 and 0.6 M KCl, and centrifuged at $600\times g$ again. The supernatant was used for enzyme assays.

2.3. Assay of myosin ATPase activity

Myosin ATPase activity was assayed according to previous reports (Claude and Swynghedauw, 1975; Cappelli et al., 1989) by measuring Pi liberation from 1 mM ATP in the presence of 50 mM HEPES, pH 7, 0.6 M KCl, 5 mM $CaCl_2$ or 10 mM EGTA in a final volume of 200 μl. Under this high ionic strength and no addition of Mg^{2+} to the incubation medium, only myosin activity is measured and there is no significant activity for Mg^{2+}-ATPase, which is an actin-contaminated myosin. These assay conditions also excluded the activity of Ca^{2+}-ATPase from the sarcoplasmic reticulum membranes that require high Mg^{2+} and low Ca^{2+} concentrations. ATP was added to the reaction mixture and pre-incubated for 5 min at 30 °C. The reaction was initiated by adding the enzyme fraction (3–5 μg protein) to the reaction mixture. Incubation times and protein concentration were chosen in order to ensure the linearity of the reaction. Samples were assayed in duplicate or triplicate and corrected for non-enzymatic hydrolysis by using controls assayed in the same conditions, except that the protein sample was added after the interruption of the reaction by using 200 μl of 10% trichloroacetic acid. The

reaction was initiated by addition of the protein sample to avoid the inactivation at 30 °C caused by the lack of substrate. The enzyme activity was calculated as the difference between the activities observed in the presence of Ca^{2+} and that in the presence of 10 mM EGTA. Inorganic phosphate was determined by the method of Chan et al. (1986). The specific activity was reported as nanomolar Pi released per minute per milligram of protein. To evaluate if mercury was capable to affect the myosin ATPase activity protein samples were incubated with increasing concentrations of $HgCl_2$ (50–2000 nM). For determination of the K_m value increasing concentrations of ATP (50, 100, 150 and 200 μM) were used. The constant of dissociation of the enzyme-substrate-inhibitor complex (K_i) was determined by plotting the concentration of the substrate divided by the V_{max} vs. the concentration of the inhibitor (Cornish-Bowden, 1974). To investigate the dependence of $HgCl_2$ actions on SH groups, the myosin ATPase activity was determined in the presence of 500 nM of dithiothreitol (DTT) or 500 nM of reduced glutathione, two well-known protectors of SH groups.

2.4. Protein determination

Protein was measured by the Coomassie blue method according to Bradford (1976), using bovine serum albumin as standard.

2.5. Drugs and reagents

ATP, dithiothreitol (DTT), reduced glutathione (GSH), mercuric chloride ($HgCl_2$), ethylene glycol-bis (β-amino ethyl ether)-*N*,*N*,*N*′,*N*′-tetra acetic acid (EGTA) and *N*-(2-Hydroxyethylpiperazine-*N*′-[2-ethanesulfonic acid]) sodium salt (HEPES) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). KCl, $CaCl_2$, KH_2PO_4 was purchased from Merck. All other reagents were of analytical grade.

2.6. Statistical analysis

Data are reported as mean ± S.D. Comparisons between means were made using Student's *t* test or ANOVA plus the Tukey test when significance was attained ($P < 0.05$).

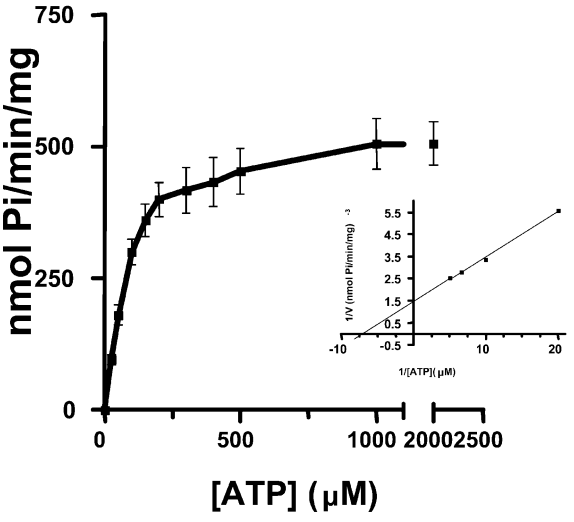

Fig. 1. Effects of increasing concentrations of ATP, used as the substrate on the velocity of hydrolysis of the myosin Ca^{2+}-ATPase. Data represent a typical experiment performed that was performed with 4 different preparations, in duplicate. Each point represents mean ± S.D. The inset represents the Lineweaver-Burk plot of myosin Ca^{2+}-ATPase activity. ATP hydrolysis was measured as described in Materials and Methods in the range of 50–200 μM. The K_m values and the maximal velocity (V_{max}) were calculated from the *x* and *y* intercepts, respectively. Each point represents mean ± S.D. S.D. values are not represented because they are smaller than the symbols. The equation for the regression curve is $y = 1.4 + 0.21x$, $R^2 = 0.996$.

3. Results

Fig. 1 shows the dependence of the velocity of hydrolysis of the myosin Ca^{2+}-ATPase on the concentration of ATP used as substrate. The enzyme exhibits the expected hyperbolic kinetic as the concentration of the substrate increases. Using a Lineweaver–Burk plot, the values for (K_m, app) and (V_{max}, app) were obtained (Figure 1, see inset). The apparent K_m obtained was 125 ± 10.8 μM and the apparent V_{max} obtained was 435 ± 41.8 nmol Pi/min/mg of protein (means of 4 experiments ± S.D.).

Fig. 2 shows the actions of $HgCl_2$ on the activity of the myosin ATPase. It can be seen that the inhibitory effect of the metal is dose-dependent and occurs at very low concentrations since inhibition is already observed at 50 nM $HgCl_2$.

The kinetic of the interaction between mercury and left ventricular myosin ATPase was determined. The Lineweaver–Burk double-reciprocal plot was obtained over a range of ATP concentrations between 100 and 300 μM in the absence and

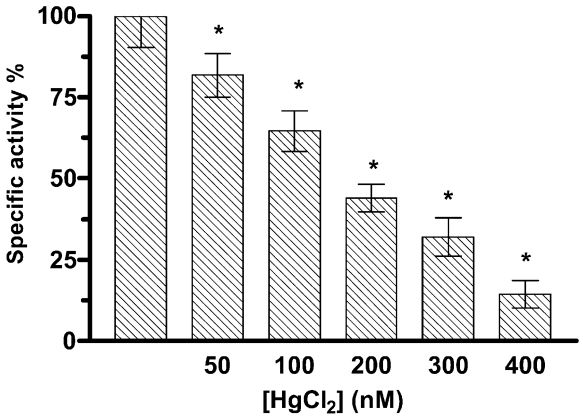

Fig. 2. Effects of increasing concentrations of $HgCl_2$ on the activity of the myosin Ca^{2+}-ATPase. 100% activity was 462 ± 45.4 nmol of Pi/min/mg. The reaction media are described in Section 2. Results are means $\pm$ S.D. for 4 different experiments, in duplicate. $P < 0.05$, ANOVA comparisons with 0 $HgCl_2$.

in the presence of mercury (100–250 nM). Results indicated that the inhibition of ATP hydrolysis by mercury is clearly uncompetitive (Fig. 3). K_i value (the dissociation constant of the enzyme–substrate-inhibitor complex) was calculated by the Cornish-Bowden method (1974), which provides

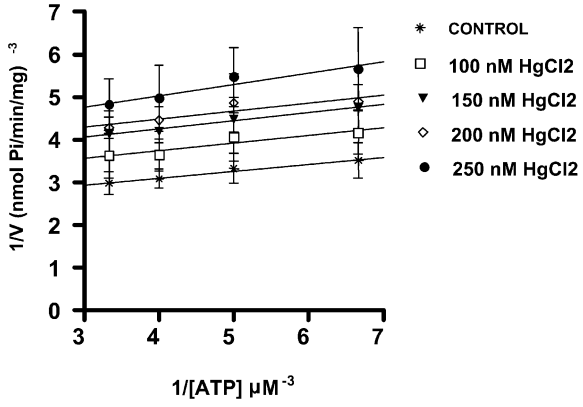

Fig. 3. Kinetic analysis of the inhibition of myosin Ca^{2+}-ATPase from rat left ventricles by $HgCl_2$. The graph shows double-reciprocal plot. ATP concentrations ranging for 150–300 µM in absence (control) and in the presence of ranging from 100–250 nM $HgCl_2$. Results here presented are mean $\pm$ S.D. for 5 experiments performed with different preparations. The equations for the regression curves are: for 100 nM $HgCl_2$, $y = 3.03 + 0.18x$, $R^2 = 0.867$; for 150 nM $HgCl_2$, $y = 3.47 + 0.19x$, $R^2 = 0.98$; for 200 nM $HgCl_2$, $y = 3.73 + 0.19x$, $R^2 = 0.81$; for 200 ATP/V, $y = 0.64 + 0.0018x$, $R^2 = 0.997$; for 250 nM $HgCl_2$, $y = 3.98 + 0.26x$, $R^2 = 0.916$.

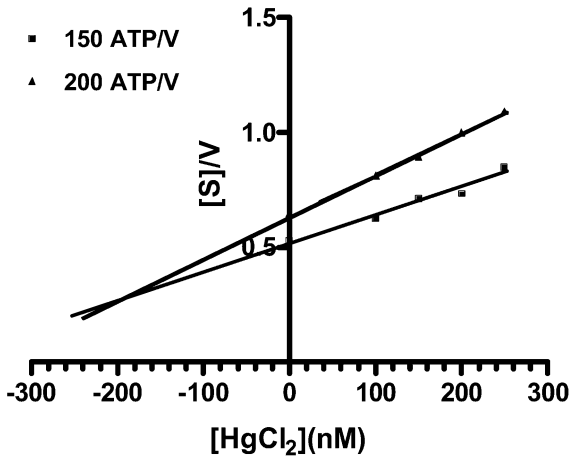

Fig. 4. Representation of K_i determination for myosin ATPase by Cornish-Bowden Plot. ATP hydrolysis was measured as described in Section 2. ATP concentration (150 and 250 µM) in the absence and in the presence of 100–250 nM $HgCl_2$. Results are means of 5 experiments, performed with different preparations. The equations for the regression curves are: for 150 ATP/V, $y = 0.52 + 0.00124x$, $R^2 = 0.967$; for 200 ATP/V, $y = 0.64 + 0.0018x$, $R^2 = 0.997$.

a simple way for determination of the inhibition constant (K_i). The K_i value calculated was 200 nM (Fig. 4).

Since mercury is known to affect SH groups, we investigated the protective effects of DTT (500 nM) and reduced glutathione (500 nM) on the

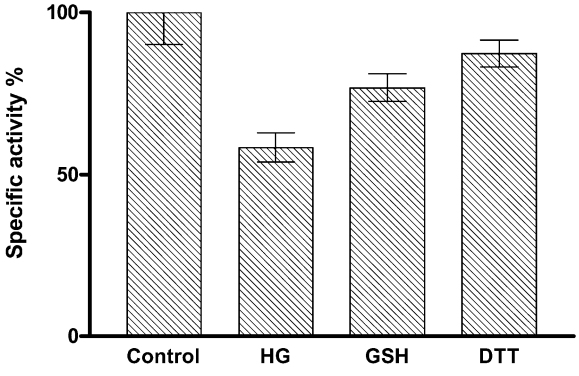

Fig. 5. Effects of the SH groups protectors on myosin ATPase inhibition produced by 250 nM $HgCl_2$. 100% correspond to 443.3 ± 43.9 nmol Pi/min/mg (mean $\pm$ S.D.; $n = 5$) for control (without mercury). Hg is the % of the activity obtained in the presence of 250 nM $HgCl_2$. GSH is the same that in Hg with addition of 500 nM glutathione and DTT is the same as in Hg with addition of 500 nM dithiothreitol. Results are mean $\pm$ S.D. for 5 experiments performed with different preparations. $P <$ 0.05, ANOVA comparisons with control, GSH and DTT.

inhibitory effect of 250 nM $HgCl_2$ (Fig. 5). This concentration of mercury was used because it inhibits 40–60% of the myosin ATPase activity. Both compounds were able to reverse the inhibition produced by mercury.

4. Discussion

Very low concentrations of the metal inhibited the activity of myosin ATPase, suggesting a strong toxic effect. The kinetic analysis of the effects of mercury on the myosin ATPase activity indicated an uncompetitive inhibition. The inhibition was reversed by DTT and by reduced glutathione, suggesting the dependence of toxic actions of the metal on SH groups of the myosin ATPase.

These results indicated that mercury was acting on several sites including the contractile proteins. However, very few reports have analyzed the way by which mercury acts on the myosin ATPase, and we have extended a previous study on this subject. In a previous report (Vassallo et al., 1999) myosin ATPase was assumed to be the site of mercury action on the contractile proteins, mainly because myosins have several SH groups in their composition (Flink et al., 1978), which are sites that react with mercury (Clarkson, 1972; Halbach et al., 1981; Halbach, 1990). Several characteristics of the myosin ATPase are already described and our results reproduced those findings. In our assays the velocity of ATP hydrolysis by myosin ATPase showed the expected dependence on the concentration of the substrate. Values of V_{max} were very similar to previous findings obtained from ventricular myosin of rats (Bottinelli et al., 1995; Dowell, 1976) or from other species (Flink et al., 1978; Malhotra et al., 1992; Ianuzzo et al., 1991).

$HgCl_2$ reduced the myosin ATPase activity even at very low concentrations (50 nM) suggesting a strong toxic effect. This concentration of $HgCl_2$ (50 nM) is below the concentration that produces depressant effects on the contractile activity of papillary muscles (>1 μM $HgCl_2$) (Vassallo et al., 1999). This is a clear indication that the myocytes must have important buffer mechanism against mercury toxicity. Another interesting finding was related to the kinetic analysis of the inhibition of the myosin Ca^{2+}-ATPase. Results suggested that the metal acts as an uncompetitive inhibitor. This indicated that mercury acts at a site different from the active site of the enzyme. A similar uncompetitive inhibition of other Mg^{2+}-ATPases by mercury was reported for oligomycin-sensitive Mg^{2+}-ATPase and oligomycin-insensitive Mg^{2+}-ATPase by ATP in the rat brain (Chetty et al., 1990). The K_i value obtained for the inhibition of myosin ATPase by mercury was at the nanomolar level. These results suggested that the myosin Ca^{2+}-ATPase is more sensitive to mercury than other metabolizing enzymes such as ATP diphosphohydrolase (Oliveira et al., 1994b), acetylcholinesterase (Gill et al., 1990) and Na^+, K^+-ATPase (Anner et al., 1992).

SH groups are usually the preferential sites for mercury actions (Anner et al., 1992; Imesch et al., 1992). Flink et al. (1978) investigated the amino acid composition of ventricular cardiac myosins and determined the position of radiolabelled thiols in heavy chain peptides, and there were 27 labeled spots in ventricular myosin chains. Previous reports suggested that two important SH groups, SH1Cys-707 and SH2Cys-697, are the most reactive thiols of the myosin heavy chains (Geeves and Holmes, 1999; Bobkova et al., 1999). Both thiol groups are necessary for the generation of movements of myosin heads (for review see, Geeves and Holmes, 1999; Holmes and Geeves, 2000). SH2 was also suggested to be linked to the strong binding of the myosin head to actin (Bobkova et al., 1999). Moreover, SH1 and SH2 groups are contained in a helix, whose alterations are linked to the rotation of the converter domain during ATP hydrolysis coupled to force generation (Geeves and Holmes, 1999). Our results with DTT and glutathione support this idea. Both compounds protected SH groups against the effects of mercury. Moreover, SH groups exist in the myosin molecule, located in places other than the heavy chains of the myosin ATPase, such as the light chains L1 (Klotz et al., 1976). These facts reinforce our findings, which show that mercury inhibits the activity of the myosin Ca^{2+}-ATPase by an uncompetitive mechanism.

In summary, our results suggest that mercury reduces the activity of the myosin ATPase at a very low dose, by an uncompetitive mechanism acting at sites that are located away from the active site of the enzyme. DTT and glutathione are effective compounds for protection of SH groups against the observed effects of mercury. Hence, SH groups might be the sites of action of the metal on the myosin molecule, action that contributes for the reduction of twitch and tetanic force developed by the ventricular myocardium.

Acknowledgments

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