Inhibition of hepatocyte plasma membrane Ca^{2+}-ATPase activity by menadione metabolism and its restoration by thiols

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Incubation of isolated rat hepatocytes with cytotoxic concentrations of menadione resulted in inhibition of plasma membrane Ca^{2+}-ATPase activity. This could be restored by subsequent treatment with either dithiothreitol or reduced glutathione, suggesting that the inhibition by menadione was due to oxidation of sulfhydryl groups critical for Ca^{2+}-ATPase activity.

Hepatocyte *Plasma membrane* *Ca^{2+}-ATPase* *Menadione* *Glutathione* *Thiol*

1. INTRODUCTION

We have previously reported that the oxidative stress elicited by menadione metabolism in isolated hepatocytes is associated with a perturbation of intracellular Ca^{2+} homeostasis [1,2]. Thus, studies with intact cells [2], as well as isolated organelle fractions [3,4], have shown that during the metabolism of menadione Ca^{2+} is released from intracellular stores, and the ability of mitochondria and microsomes to sequester Ca^{2+} is impaired. Moreover, the menadione-induced release of Ca^{2+} from the intracellular stores has been found to cause a sustained increase in cytosolic free Ca^{2+} concentration in the hepatocytes [5].

The latter observation suggested that the perturbation of Ca^{2+} homeostasis caused by menadione metabolism also involves an impairment of Ca^{2+} extrusion by the hepatocytes. Since we have previously observed that the redox state of protein sulfhydryl groups is critical for both Ca^{2+} transport and Ca^{2+}-ATPase activity in liver plasma membrane vesicles [6], our recent finding that menadione metabolism by rat hepatocytes results in extensive oxidation of protein thiols [7], provided a possible mechanism by which menadione metabolism may inhibit Ca^{2+} efflux from hepatocytes.

Here, we demonstrate that the metabolism of menadione by isolated rat hepatocytes results in the inhibition of plasma membrane Ca^{2+}-ATPase activity. The critical involvement of protein sulfhydryl group oxidation in the menadione-mediated inhibition of the Ca^{2+}-ATPase is suggested by the finding that GSH and DTT were able to restore the impaired ATPase activity when added either to the cells (DTT) or directly to the isolated plasma membrane fraction (GSH).

2. MATERIALS AND METHODS

Collagenase (grade II) was obtained from Boehringer, Mannheim. Affi-Gel 731 polyacryl-

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Abbreviations: menadione, 2-methyl-1,4-naphthoquinone; GSH, glutathione (reduced form); DTT, dithiothreitol; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene bis (β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; NEM, *N*-ethylmaleimide; DTNB, 2,2'-dinitro-5,5'-dithiodibenzoic acid

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amide beads, coated with polyethyleneimine, were obtained from Bio-Rad Laboratories, Richmond, VA. *N*-Ethylmaleimide, ATP, GSH, glutathione reductase, and menadione were purchased from Sigma, St. Louis, MO. All other reagents were commercial products of highest available grade of purity.

Hepatocytes were isolated from male Sprague-Dawley rats (180–200 g, allowed food and water ad libitum) by collagenase perfusion as described previously [8]. Incubations were performed at 37°C in Krebs-Henseleit medium, supplemented with 12.6 mM Hepes (pH 7.4), at a concentration of 10^6 cells/ml. At the end of the incubation, the cells were sedimented by centrifugation, washed twice in sucrose-acetate buffer (pH 5.0) [9], and then used for isolation of plasma membrane fragments.

For isolation of the plasma membrane fraction, hepatocytes were attached to polyacrylamide beads (Affi-Gel 731) and treated as described in [10]. This procedure yields a highly-purified preparation of plasma membrane fragments attached to the beads and contamination with mitochondria and microsomes is negligible [10].

Ca^{2+}-ATPase activity was assayed in a medium containing 0.2 ml of a suspension of beads coated with plasma membrane fragments (50–60 μg protein), 1 mM ATP, 1 mM ouabain, 1 mM EGTA, 2 μg/ml oligomycin, 50 mM Tris-HCl (pH 8.0) and various concentrations of $CaCl_2$, in a final volume of 1 ml. Incubation was for 1 h at 37°C and the reaction was terminated by addition of ice-cold trichloroacetic acid. The inorganic phosphate released was measured as in [11]. The concentrations of free Ca^{2+} in the Ca^{2+}/EGTA buffer were calculated using the computer program developed by Fabiato and Fabiato [13] and the dissociation constants reported in [12].

To study the reversibility of the menadione-mediated inhibition of plasma membrane Ca^{2+}-ATPase activity, cells incubated with menadione for 1 h as described above were recovered by centrifugation, resuspended in fresh Krebs-Henseleit medium, with or without 1 mM DTT, and incubated for 15 min at 37°C before being processed for isolation of plasma membrane fraction. In another set of experiments, the plasma membrane fraction isolated from hepatocytes incubated (1 h) with or without menadione was preincubated for 15 min at 37°C in the presence or absence of 2 mM GSH, 1 mM NADPH, and 2 U/ml of glutathione reductase prior to addition of 1 mM ATP and measurement of Ca^{2+}-ATPase activity. Glutathione reductase and NADPH were added to prevent accumulation of glutathione disulfide during incubation.

Protein was measured according to Lowry et al. [14].

3. RESULTS

Incubation of the hepatocyte plasma membrane fraction with ATP and various Ca^{2+} concentrations resulted in ATP hydrolysis exhibiting two distinct kinetic components with low and high affinity for Ca^{2+}, respectively (fig.1) [12,15]. Some characteristics of the high affinity component of the Ca^{2+}-ATPase activity are presented in table 1. The kinetic parameters are similar to those reported by others for plasma membrane Ca^{2+}-ATPase activity [12], Ca^{2+} transport [16] and phosphorylated intermediate formation [16],

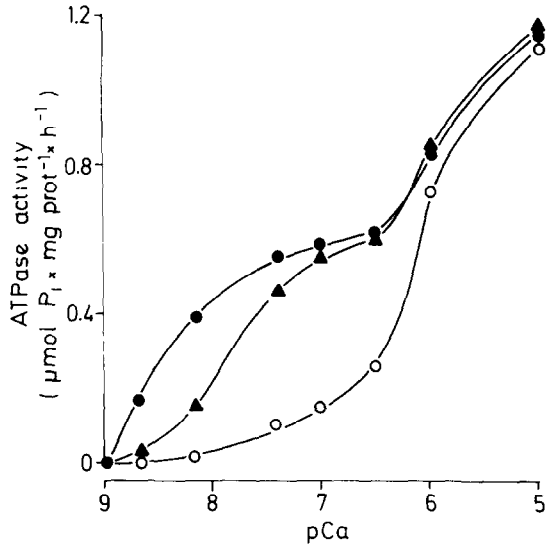

Fig.1. Effects of menadione metabolism on hepatocyte plasma membrane Ca^{2+}-ATPase activity. Isolated hepatocytes were incubated in the absence (●), or presence (○, ▲) of 200 μM menadione for 30 (▲) or 60 (○) min. At the end of the incubation, the plasma membrane fraction was isolated and Ca^{2+}-ATPase activity was assayed as described in section 2.

Table 1

Characteristics of the high affinity Ca^{2+}-ATPase activity present in the plasma membrane fraction of rat hepatocytes

V_{max} (μmol P_i/mg protein per h)	0.85
K_m for Ca^{2+}	9 μM
K_m for ATP	40 μM
pH optimum	8.0
Mg^{2+} requirement[a]	micromolar
Calmodulin stimulation[b]	absent

[a] Mg^{2+} requirement was investigated by including $MgCl_2$ concentrations ranging from 0 to 5 mM in the assay medium. Mg^{2+} concentrations up to 7.5 μM were found to stimulate Ca^{2+}-ATPase activity whereas higher concentrations had an inhibitory effect

[b] Calmodulin sensitivity was tested by including calmodulin (0.1–10 μg/ml) in the incubation medium. The experiments were performed with either control membranes or membranes washed with 1 mM EGTA to displace endogenous calmodulin

suggesting that the activity detected in our plasma membrane preparation is associated with Ca^{2+} transport in the plasma membrane. Several other properties of this preparation, i.e., optimal activity at basic pH, inhibition by high Mg^{2+} concentrations, and insensitivity to added calmodulin, are also characteristics of the hepatic plasma membrane Ca^{2+}-ATPase, suggesting that there was no apparent contribution to the recorded Ca^{2+}-ATPase activity by contaminating microsomes (cf. [17,18]).

Incubation of isolated hepatocytes with 200 μM menadione resulted in a time-dependent inhibition of the plasma membrane Ca^{2+}-ATPase (fig.1). This inhibition was restricted to the high affinity component of the ATPase, the low affinity component being almost completely unaffected. The menadione-induced inhibition was dose-dependent; lower concentrations of menadione (50–100 μM) did not cause inhibition of activity, whereas higher concentrations (200–400 μM) had the same effect as 200 μM but required shorter incubation time (not shown).

As mentioned above, we have previously found that incubation of hepatocytes with cytotoxic concentrations of menadione is associated with depletion of protein sulfhydryl groups [5,7]. To investigate whether the observed inhibition of the plasma membrane Ca^{2+}-ATPase by menadione could be related to the disappearance of such thiol groups, the hepatocyte suspension was incubated for 20 min with the SH-complexing agent, NEM. Under these conditions, more than 80% of the protein sulfhydryl groups were complexed by NEM, as demonstrated by their inability to interact with DTNB (not shown). As illustrated in fig.2, the Ca^{2+}-ATPase of the plasma membrane fraction isolated from NEM-treated hepatocytes exhibited the same pattern of inhibition as seen with menadione, suggesting that the inhibition was closely associated with the disappearance of critical sulfhydryl groups.

Since it seemed possible that the inhibition of the Ca^{2+}-ATPase by menadione metabolism could be due to oxidation of protein thiols critical for ATPase activity (cf. [5]), two sets of experiments were performed to investigate this hypothesis: (i) Menadione-treated cells were suspended in fresh medium and treated with DTT before isolation of the plasma membrane fraction and measurement of Ca^{2+}-ATPase activity, and (ii) the plasma membrane fraction isolated from menadione-treated

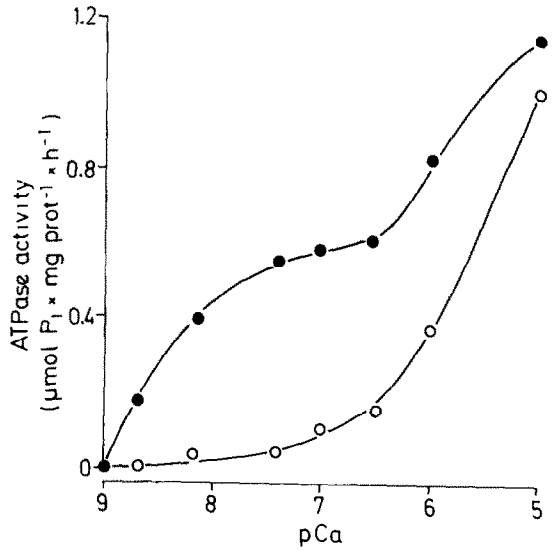

Fig.2. Effects of *N*-ethylmaleimide on hepatocyte plasma membrane Ca^{2+}-ATPase activity. Isolated hepatocytes were incubated in the absence (●) or presence (○) of 250 μM NEM for 30 min. At the end of the incubation, the plasma membrane fraction was isolated and the Ca^{2+}-ATPase activity assayed as described in section 2.

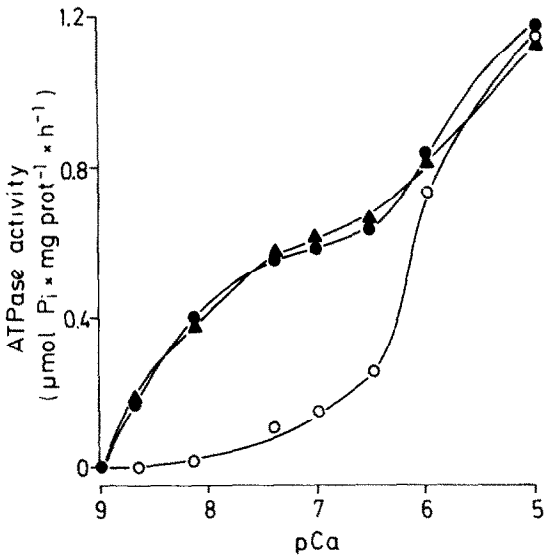

Fig.3. Restoration of menadione-inhibited plasma membrane Ca^{2+}-ATPase. Isolated hepatocytes were incubated with 200 μM menadione for 60 min and then exposed to thiols by one of the following methods: (i) Cells were subsequently resuspended in fresh buffer containing DTT (1 mM) and incubated at 37°C for an additional 15 min prior to isolation of the plasma membrane fraction (●), or (ii) plasma membranes were isolated from the menadione-treated cells and incubated with GSH (2 mM)/glutathione reductase (2 U/ml)/ NADPH (1 mM) for 15 min at 37°C before starting the assay for Ca^{2+}-ATPase activity (▲). The Ca^{2+}-ATPase activity of membranes receiving no thiol treatment is shown as (○). Enzyme activity was measured as described in section 2.

cells was preincubated with GSH prior to assay of Ca^{2+}-ATPase activity. As shown in fig.3, both treatments resulted in restoration of the menadione-inhibited Ca^{2+}-ATPase activity.

4. DISCUSSION

Intracellular Ca^{2+} homeostasis is regulated by the concerted action of several Ca^{2+} translocases [19], by Ca^{2+} sequestration into intracellular stores [20], and by the buffering capacity of Ca^{2+}-binding proteins such as calmodulin [21]. The metabolism of menadione by isolated hepatocytes causes a perturbation of normal Ca^{2+} homeostasis, which represents an early step in menadione-induced cytotoxicity [2,22]. The mechanisms by which menadione disrupts intracellular Ca^{2+} homeostasis have been shown to involve release of Ca^{2+} from mitochondria [3] as well as inhibition of the ability of both mitochondria and endoplasmic reticulum to sequester Ca^{2+} [3,4]. However, the exclusive impairment of intracellular sequestration processes does not fully explain the sustained increase in the cytosolic free Ca^{2+} level observed during menadione metabolism [5] because, due to the very high affinity for Ca^{2+}, the plasma membrane Ca^{2+}-translocase should be able to maintain cytosolic Ca^{2+} concentration at the physiological, submicromolar level.

Here, we have shown that the metabolism of menadione by isolated hepatocytes can also cause an inhibition of plasma membrane Ca^{2+}-ATPase activity. The pronounced effect of menadione metabolism on the high affinity component of the Ca^{2+}-ATPase may have important consequences since the cytosolic Ca^{2+} level is normally in the range of 0.1–0.2 μM [23], a concentration at which menadione inhibits Ca^{2+}-ATPase activity by more than 60%.

The metabolism of menadione by isolated hepatocytes has also been demonstrated to cause a pronounced decrease in protein thiols by both oxidation and arylation, oxidation being the quantitatively more important process [5]. Since the hepatic plasma membrane Ca^{2+}-ATPase was previously found to depend on reduced sulfhydryl groups for activity [7], it appeared that, during the metabolism of menadione, the oxidation of such sulfhydryl groups could impair Ca^{2+}-ATPase activity. This hypothesis is supported by the present finding that NEM, used at a concentration which specifically complexes with SH-groups, induced the same pattern of Ca^{2+}-ATPase inhibition as observed with menadione. Moreover, these experiments demonstrated that either GSH or DTT were able to reactivate the menadione-inhibited Ca^{2+}-ATPase while the same agents could not restore enzyme activity in plasma membrane isolated from NEM-treated cells (not shown). In addition, preliminary evidence from our laboratory using other thiol-specific agents such as *p*-chloromercuribenzoate and methane sulfonic acid have also shown an inhibition of the high affinity Ca^{2+}-ATPase. These data provide further evidence for an involvement of protein thiol oxidation in the inhibition of plasma membrane

Ca^{2+}-ATPase. Thus, our results indicate that liver plasma membrane Ca^{2+}-ATPase activity can be modulated by a process involving thiol/disulfide exchange as has been demonstrated for other transport systems [24,25].

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