Critical role of sulfhydryl group(s) in ATP-dependent Ca^{2+} sequestration by the plasma membrane fraction from rat liver

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The ATP-dependent sequestration of Ca^{2+} by the plasma membrane fraction from rat liver is stimulated by reduced glutathione and dithiothreitol and inhibited by diamide and *t*-butyl hydroperoxide. The inhibitory effect on Ca^{2+} sequestration by the oxidizing agents is prevented in the presence of the thiols. Our results therefore suggest that free sulfhydryl group(s) may be critical for the activity of hepatic plasma membrane Ca^{2+} translocase, and that inhibition of this activity by the oxidation of such group(s) may contribute to the perturbation of Ca^{2+} homeostasis during oxidative stress.

Liver *Plasma membrane* *Calcium translocase* *ATPase* *Sulfhydryl group* *Glutathione*

1. INTRODUCTION

Intracellular Ca^{2+} compartmentation in hepatocytes is regulated by Ca^{2+} translocases present in both the mitochondria and endoplasmic reticulum [1,2]. However, because of the concentration gradient across the plasma membrane, long-term regulation of cellular Ca^{2+} homeostasis further requires that Ca^{2+} influx is balanced by the active extrusion of calcium ions from the cell.

ATP-dependent Ca^{2+} translocases have been identified in the plasma membrane fraction of different mammalian cells and tissues [3] and, in some cases, they have been solubilized, purified and characterized in detail [4,5]. Recently, a Ca^{2+}-dependent, calmodulin-insensitive ATPase activity has been demonstrated in the plasma membrane fraction of rat liver [6–8]. In addition, inverted plasma membrane vesicles from the same tissue have been shown to actively sequester Ca^{2+} by a process which is dependent on ATP, but not on calmodulin, and inhibited by vanadate [9,10].

Studies in our laboratories have revealed that perturbation of intracellular Ca^{2+} homeostasis is an early phenomenon during oxidative damage to isolated hepatocytes [11,12]. Moreover, agents causing oxidative stress, notably *t*-butyl hydroperoxide (*t*-BH) and menadione, have been shown to impair Ca^{2+} sequestration in both isolated liver mitochondria [13,14] and microsomes [15]. We here report that ATP-dependent Ca^{2+} sequestration by the plasma membrane fraction from rat liver is also inhibited by *t*-BH, and that this effect appears to be related to oxidation of free sulfhydryl group(s) critical for translocase activity.

2. MATERIALS AND METHODS

Arsenazo III, *t*-butyl hydroperoxide, diamide (diazinedicarboxylic acid bis dimethylamide), dithiothreitol and Ruthenium red were obtained from Sigma, the calcium ionophore A23187 was from Calbiochem-Behring, and ATP was from Boehringer-Mannheim; all other chemicals were at least of reagent grade and purchased from local commercial sources.

Liver plasma membrane vesicles were isolated from adult, male Sprague–Dawley rats (180–230 g), fed ad libitum, by the discontinuous sucrose gradient centrifugation method in [16].

Ca^{2+} fluxes were monitored spectrophotometric-

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ally using the metallochromic dye Arsenazo III in a Hitachi-Perkin Elmer 557 spectrophotometer operating with the wavelength pair 654–685 nm. Plasma membrane vesicles (0.5 mg protein/ml) were incubated in a medium containing 100 mM KCl, 5 mM $MgCl_2$, 20 μM ouabain, 2 μM Ruthenium red, 20 μM $CaCl_2$, 60 μM Arsenazo III and 20 mM HEPES (pH 7.8) at 37°C for 10 min. ATP (1 mM final conc.) was then added, and Ca^{2+} uptake was monitored; after its completion, ionophore A23187 (0.3 μg) was added to release the sequestered Ca^{2+}. The system was calibrated before each experiment by adding known amounts of $CaCl_2$.

ATPase activity was assayed by determination of inorganic phosphate formed from ATP as in [17]. The incubation medium was the same as that employed for the study of Ca^{2+} fluxes; the presence of ouabain in the medium prevents any contribution by (Na^+ + K^+)-stimulated ATPase to the recorded ATPase activity.

Free sulfhydryl groups were measured by the absorbance at 520–412 nm after treatment of the samples with dithio-bis-dinitrobenzoic acid (100 μM) as in [18]. Protein concentration was assayed as in [19].

3. RESULTS

As shown in fig.1, addition of ATP to the plasma membrane fraction preincubated for 10 min in presence of Ca^{2+}, resulted in a decrease in the Ca^{2+} concentration of the medium. This could be restored to the original level by the subsequent addition of the cation ionophore A23187. Similarly, ATP addition caused no change in Ca^{2+} concentration when the ionophore was already present in the medium. It therefore appears that the ATP-dependent decrease in the concentration of Ca^{2+} in the medium was in fact due to the active sequestration of Ca^{2+} by the plasma membrane fraction. Further, in agreement with [9], the pH optimum of the reaction was at 7.8 (not documented), where the contribution to Ca^{2+} sequestration by contaminating microsomes is negligible.

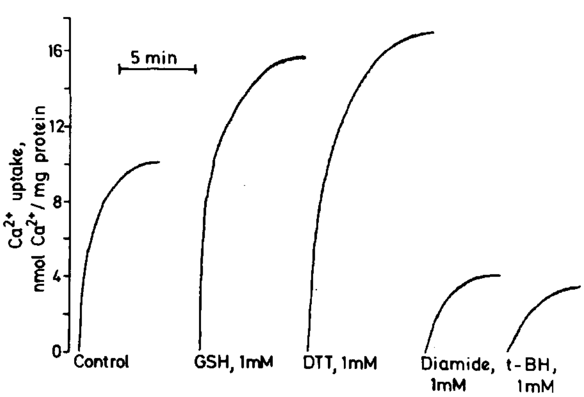

Fig.1. Effects of *t*-butyl hydroperoxide and thiol reagents on the ATP-dependent Ca^{2+} sequestration by liver plasma membrane fraction. The plasma membrane fraction was incubated for 10 min at 37°C alone (control), or in combination with 1 mM GSH, DTT, diamide or *t*-BH. ATP (1 mM) was then added and Ca^{2+} uptake was monitored continuously.

As also shown in fig.1, the presence of either reduced glutathione (GSH) or dithiothreitol (DTT) during the preincubation period, markedly stimulated ATP-dependent Ca^{2+} sequestration by the plasma membrane fraction; in the presence of 1 mM GSH or DTT the amount of Ca^{2+} sequestered was 14.3 ± 2.3 nmol/mg protein and 17.4 ± 3.2 nmol/mg protein, respectively, as compared to 9.9 ± 2.2 nmol/mg protein when preincubation was performed in the absence of added thiol. Interestingly, a similar stimulatory effect was observed in the absence of added thiol in the incubation medium, when 1 mM DTT had been included in the various media used during isolation of the plasma membrane fraction (17.3 ± 3.2 nmol Ca^{2+}/mg protein). Conversely, the presence of the thiol-oxidizing agent diamide during incubation, or during isolation of the plasma membrane fraction, caused an inhibition of Ca^{2+} sequestration by this fraction (4.0 ± 1.1 nmol/mg protein and 5.1 ± 2.4 nmol/mg protein, respectively; cf. fig.1).

The presence of *t*-BH during preincubation also inhibited Ca^{2+} sequestration by the plasma membrane fraction (fig.1). This effect was dependent on peroxide concentration and preincubation time; preincubation with 1 mM *t*-BH for 10 min caused an approximate 40% decrease in Ca^{2+} sequestration (3.8 ± 1.2 nmol/mg protein as compared to 9.9 ± 2.2 nmol/mg protein when preincubation was performed in the absence of peroxide).

To investigate the mechanism of the *t*-BH-induced impairment of Ca^{2+} sequestration by the plasma membrane fraction, the effects of the hydroperoxide on Ca^{2+} sequestration, Ca^{2+}-Mg^{2+}

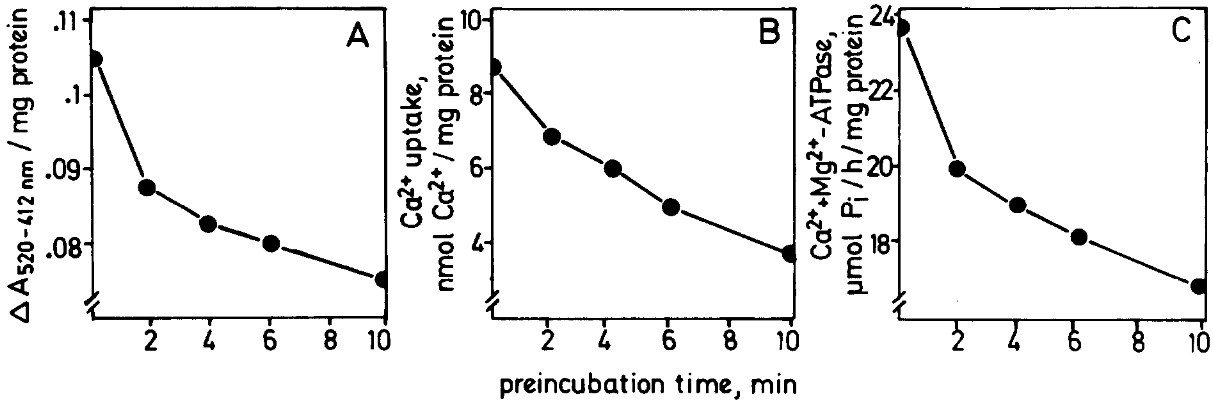

Fig.2. *t*-Butyl hydroperoxide-induced thiol oxidation and inhibition of Ca^{2+} sequestration and Ca^{2+}–Mg^{2+}-ATPase activity in liver plasma membrane fraction. The plasma membrane fraction was incubated at 37°C with 1 mM *t*-BH. At the times indicated samples were taken for measurement of total thiol groups (A), Ca^{2+} sequestration (B) and Ca^{2+}–Mg^{2+}-ATPase activity (C).

-ATPase activity and the amount of free sulfhydryl groups in the plasma membrane preparation, were investigated in parallel experiments. As shown in fig.2, incubation with *t*-BH caused a progressive decrease in all 3 parameters, and a very good correlation was observed when disappearance of free sulfhydryl groups was compared to either the impairment of Ca^{2+} sequestration (0.977) or the inhibition of Ca^{2+}, Mg^{2+}-ATPase activity (0.997).

It thus appears that ATP-dependent Ca^{2+} sequestration by the plasma fraction is dependent on free sulfhydryl group(s) for activity, and that the inhibition by diamide and *t*-BH may be due to oxidation of such group(s). Further support for this hypothesis is provided by the data in table 1, which show that inclusion of either GSH or DTT in the medium during preincubation of the plasma membrane fraction with *t*-BH, caused complete protection against the inactivating effect on Ca^{2+} sequestration by the peroxide.

Table 1

Protection by GSH and dithiothreitol against *t*-butyl hydroperoxide-induced inhibition of Ca^{2+} sequestration and Ca^{2+}–Mg^{2+}-ATPase activity in liver plasma membrane fraction

	Ca^{2+} sequestration (nmol Ca^{2+}/ mg protein)	Ca^{2+}–Mg^{2+}-ATPase activity (μmol $P_i.h^{-1}.mg\ protein^{-1}$)
Control	9.9 ± 2.2	25 ± 4.6
Diamide (1 mM)	4.0 ± 1.1	16 ± 3.4
t-BH (1 mM)	3.8 ± 1.2	17 ± 4.0
t-BH (1 mM) + GSH (1 mM)	8.5 ± 1.8	24 ± 6.0
t-BH (1 mM) + DTT (1 mM)	10.0 ± 3.0	27 ± 4.7

Plasma membrane vesicles were incubated for 10 min at 37°C in the absence or presence of 1 mM diamide, or with 1 mM *t*-BH in the absence or presence of 1 mM GSH or 1 mM DTT. ATP (1 mM) was then added, and Ca^{2+} uptake and Ca^{2+}–Mg^{2+}-ATPase activity were measured

4. DISCUSSION

Although the plasma membrane fraction employed in this study consists of a mixture of right-side-out and inverted vesicles [16], the observations that Ca^{2+} sequestration occurred only in the presence of ATP and was strongly inhibited by vanadate (unpublished), suggest that it was due mainly to the presence of inverted vesicles in the preparation. Based on a detailed kinetic analysis of Ca^{2+} uptake under similar experimental conditions, authors in [9] have concluded that Ca^{2+} sequestration by this fraction is attributable to the presence of inverted plasma membrane vesicles. However, under our experimental conditions we cannot exclude a minor contribution to Ca^{2+} sequestration by contaminating microsomes, even though this could be minimized by taking advantage of the known difference in pH optimum of the microsomal and plasma membrane Ca^{2+} translocases [9].

Several transport ATPases, including the Mg^{2+}-dependent, $(Na^+ + K^+)$-stimulated ATPase [20] and the liver microsomal and sarcoplasmic reticular Ca^{2+}, Mg^{2+}-ATPases [21,22], have previously been found to depend on free sulfhydryl groups for activity. It therefore appears likely that the observed effects of GSH and DTT, and of the thiol-oxidizing agent diamide on Ca^{2+} sequestration by the plasma membrane fraction are related to a similar dependence of the hepatic plasma membrane Ca^{2+} translocase on free sulfhydryl group(s) for activity. Oxidation of critical sulfhydryl group(s) may also explain the inhibitory effect of *t*-BH on Ca^{2+} sequestration by the plasma membrane fraction, although the detailed mechanism by which the hydroperoxide exerts this effect remains to be elucidated. It is of interest to note that both diamide and *t*-BH have also been found to inhibit Ca^{2+} sequestration in liver microsomes, although this system appears to be more resistant to inhibition by the oxidizing agents than the plasma membrane fraction used here (cf. [15]).

Depletion of intracellular GSH has been found to affect Ca^{2+} compartmentation in hepatocytes [11,12], and oxidation of thiol groups are known to affect the ability of both liver mitochondria and microsomes to sequester Ca^{2+} [13,15]. In view of these results, it appears likely that any marked alteration in the cytosolic glutathione redox level will also affect the activity of the plasma membrane Ca^{2+} translocase. An inhibition of this activity may therefore contribute to the increase in cytosolic-free Ca^{2+} concentration that appears to be a result of GSH depletion in hepatocytes, and to the possible toxicological consequences thereof [11]. Whether the cytosolic glutathione redox system also plays a role in the physiological regulation of Ca^{2+} transport across the plasma membrane is the subject of a current investigation in our laboratories.

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