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ON THE ROLE OF THIOL GROUPS IN THE INHIBITION OF LIVER MICROSOMAL Ca^{2+} SEQUESTRATION BY TOXIC AGENTS

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Abstract—ATP-dependent Ca^{2+} sequestration by rat liver microsomes was assayed using three different methods, and characterized with regard to the effect of various inhibitors. When glucose and hexokinase were added in combination to deplete ATP in the incubation, Ca^{2+} uptake was followed by rapid release of Ca^{2+} from the microsomes. Ca^{2+} sequestration was inhibited by reagents that cause alkylation (e.g. *p*-chloromercuribenzoate) or oxidation (e.g. diamide) of protein sulfhydryl groups. Moreover, pretreatment of the microsomes with cystamine, which causes formation of mixed disulfides with protein thiols, also resulted in the inhibition of Ca^{2+} sequestration. It is concluded that microsomal Ca^{2+} sequestration is critically dependent on protein sulfhydryl groups, and that modification of protein thiols may be an important mechanism for the inhibition of microsomal Ca^{2+} sequestration by a variety of toxic agents.

The active (ATP-dependent) sequestration of calcium ions by liver microsomes has been extensively studied in recent years because of its possible participation in the regulation of cytosolic free Ca^{2+} concentration [1–8]. The characteristics of this process include its relatively low capacity to accumulate Ca^{2+}, especially when compared to mitochondria [9], its optimal activity at slightly acidic pH [2], and its potentiation by oxalate [2]. It is still unclear whether liver microsomal Ca^{2+} transport is regulated by calmodulin, although two recent reports suggest calmodulin dependence of the phosphorylation-dephosphorylation cycle of the microsomal Ca^{2+}-ATPase [10, 11].

Recently, the endoplasmic reticular Ca^{2+} pool of rat hepatocytes has been shown to play a crucial role in the mediation of α-adrenergic hormone action [12]. Several reports [13–15] indicate that it may represent the target for inositol 1,4,5-trisphosphate formed at the plasma membrane level during the hormonal stimulation of the liver [12]. The subsequent interaction of the inositol trisphosphate with the Ca^{2+} sequestering system of the endoplasmic reticulum would result in a net efflux of sequestered Ca^{2+}, contributing to the transient increase in cytosolic Ca^{2+} level observed during α-adrenergic stimulation.

Because of its physiological role in controlling cytosolic Ca^{2+} concentration, perturbation of the endoplasmic reticular Ca^{2+} transport system could result in hepatocyte injury. In fact, evidence has been presented indicating that several well-known hepatotoxins, such as carbon tetrachloride, bromotrichloromethane and carbon disulfide, cause rapid destruction of the microsomal Ca^{2+} pump [16–21]. We have reported that toxicity induced by oxidative stress in isolated rat hepatocytes was associated with the depletion of intracellular Ca^{2+} pools, including the endoplasmic reticular Ca^{2+} pool [22]. Moreover, at least in the case of organic hydroperoxides, it has been demonstrated that depletion of the endoplasmic reticular Ca^{2+} pool was preceded by the oxidation of cellular glutathione (GSH) [23], indicating that an alteration of the thiol redox status could be involved in this process. Further support for this assumption has been provided by experiments with liver microsomes, in which the inhibition of Ca^{2+} sequestration by *t*-butylhydroperoxide was prevented by the thiol reducing agents GSH and dithiothreitol (DTT) [24].

In the present work we have studied the effects of agents that can react with protein thiols on ATP-dependent Ca^{2+} sequestration by rat liver microsomes. Three mechanisms have been investigated: alkylation, oxidation and mixed disulfide formation. Although a strict quantitative relationship between loss of protein thiols and inhibition of Ca^{2+} sequestration has not been found, evidence is presented indicating that modification of protein thiols may be an important mechanism for inhibition of microsomal Ca^{2+} sequestration by a variety of toxic agents.

MATERIALS AND METHODS

Materials. Arsenazo III, hexokinase, ruthenium red, rotenone, CCCP (carbonyl cyanide *m*-chlorophenylhydrazone), PCMB (*p*-chloromercuribenzoate), diamide, *t*-butylhydroperoxide, menadione, xanthine, xanthine oxidase, glutathione, glutathione

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disulfide, cystine, homocystine, lipoic acid (oxidized form), cystamine and cysteamine were purchased from Sigma. The cation ionophore A23187 was obtained from Calbiochem-Behring. $^{45}CaCl_2$ (specific activity: 10–40 Ci/g) was obtained from Amersham International. All other reagents were commercial products of the highest available grade of purity.

Animals. Male Sprague–Dawley rats (190–220 g, fed *ad libitum*), receiving sodium phenobarbital in their drinking water (1 mg/ml) for at least 7 days prior to use, were employed.

Microsomes. Liver microsomes were isolated essentially as described by Ernster *et al.* [25]. Briefly, the liver was homogenized in 0.25 M sucrose containing 1 mM EGTA and 10 mM Tris–HCl, pH 7.4. After centrifugation, the microsomal pellet was washed once and then resuspended in 0.15 M KCl. All incubations were performed at 25° in a medium containing 125 mM KCl, 2 mM K_2HPO_4, 4 mM $MgCl_2$, 25 mM Hepes, pH 7.2, and ~1 mg microsomal protein/ml.

Measurements of Ca^{2+} sequestration. Microsomal Ca^{2+} sequestration was assayed using three different methods: a spectrophotometric method employing Arsenazo III, a Ca^{2+} selective electrode, and millipore membrane filtration of microsome-associated $^{45}Ca^{2+}$.

The spectrophotometric method was modified from that originally developed by Jones *et al.* [24]. To the microsomal suspension, Arsenazo III (30 μM) was added and the absorbance changes were recorded using either a Sigma ZWSII or an Hitachi–Perkin Elmer DBOW 557 spectrophotometer, operating at the wavelength pair 654–685 nm. After baseline recording, ATP (1 mM, unless otherwise indicated) was added and the change in absorption was recorded. Calibration of the system was performed by adding different amounts of $CaCl_2$ to the same cuvette employed for the assay. Calculations were made using either a calibration curve obtained with $CaCl_2$, or an extinction coefficient of the Ca^{2+}–Arsenazo III complex of $2.14 \times 10^4\ M^{-1}cm^{-1}$ at $\Delta A_{654-685}$.

The electrode measurements were carried out using a Radiometer F2112 Ca ion selective electrode connected to the high impedance terminal of a Radiometer PHM 84 pH-meter. The reference part of a combined glass electrode (Radiometer GK 2401 B) connected to the reference terminal was used as reference electrode. The instrument was set in the pH mode in order to obtain maximal amplification of the electrode signal and it was connected to a recorder via an intermediate bucking device [26] for continuous monitoring of the electrode output. For studies of Ca^{2+} fluxes, 2 ml of freshly prepared microsomes (10 mg/ml) were added to 6 ml incubation medium in an open-loop thermostated vessel and the suspension was magnetically stirred at 25°. The electrodes and the measuring vessel were mounted into a thermally isolated Faraday cage to reduce electrical noise and baseline drift due to ambient temperature changes and electrical interference. The mV output was correlated to free Ca^{2+} concentration by addition of known amounts of $CaCl_2$ after each experiment.

$^{45}Ca^{2+}$ sequestration was measured as described by Moore *et al.* [2].

Measurement of protein thiols. Protein sulfhydryl groups were measured using the dye dithio-bis-dinitrobenzoic acid (DTNB). Briefly, DTNB (100 μM) was added to the microsomal suspension, containing 1 mg protein/ml, and the absorbance changes were measured spectrophotometrically at 412–520 nm. Quantitation of protein thiols was performed using a calibration curve obtained with GSH.

Protein concentration was measured according to Lowry *et al.* [27].

RESULTS

Validation of the method used to measure Ca^{2+} sequestration by rat liver microsomes

The metallochromic dye Arsenazo III has been used previously to study Ca^{2+} fluxes in isolated liver mitochondria [28] and plasma membrane vesicles [29] and in sarcoplasmic reticulum vesicles from skeletal muscle [30]. In this study we have used Arsenazo III to monitor Ca^{2+} transport in rat liver microsomes. As illustrated in Fig. 1, the addition of ATP resulted in a decrease in the Ca^{2+} concentration of the medium. When a low ATP concentration

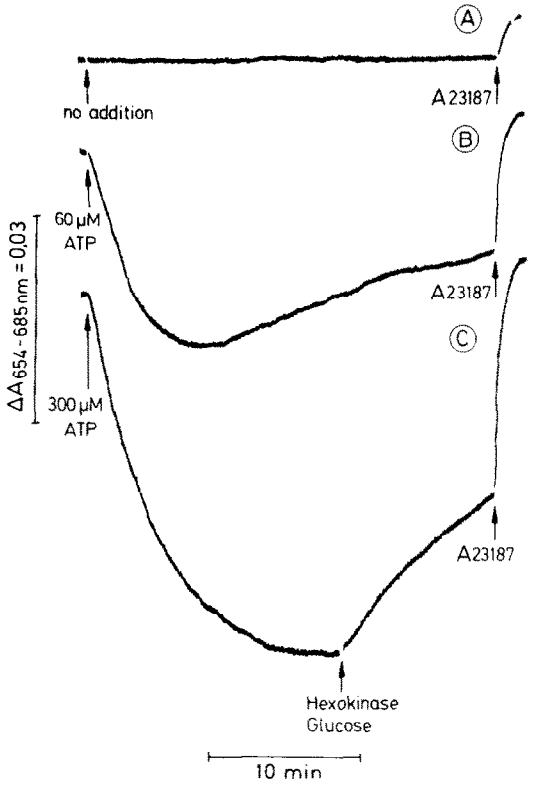

Fig. 1. ATP-induced Ca^{2+} uptake by isolated rat liver microsomes. Liver microsomes were incubated at 1 mg protein/ml and then processed for spectrophotometric assay of Ca^{2+} uptake as described in Methods: (A) control, no ATP was added. (B) 60 μM ATP was added. (C) 300 μM ATP was added. When indicated, hexokinase (2 μg/ml) and glucose (13 mM) were added in combination to deplete ATP. At the end of each experiment, ionophore A23187 (15 μM) was added to release the sequestered Ca^{2+}.

Table 1. ATP-dependent Ca^{2+} sequestration in rat liver microsomes measured by three different methods (see Methods)

Methods	Ca^{2+} sequestration* (nmol Ca^{2+}/mg protein)
Millipore membrane filtration ($^{45}Ca^{2+}$)	5.8 ± 0.17
Ca^{2+}-selective electrode	6.6 ± 0.53
Arsenazo III	7.6 ± 0.39

The results are expressed as mean ± S.E. of three to four separate experiments.

* Ca^{2+} sequestration was measured at 25° using 1 mg/ml of microsomal protein, 20 μM $CaCl_2$ and 1 mM ATP. The values reported in the table refer to the maximal amount of Ca^{2+} sequestered in microsomal vesicles under the conditions employed.

was used, Ca^{2+} uptake was not complete and was followed by a slow, spontaneous release of the sequestered Ca^{2+}. Addition of hexokinase and glucose to the medium, containing microsomes which had accumulated Ca^{2+}, resulted in release of the sequestered Ca^{2+} due to the consumption of available ATP. When ionophore A23187 was added to permeabilize the vesicles to cations, an immediate release of the sequestered Ca^{2+} occurred, indicating that all the Ca^{2+} which had disappeared from the medium in response to ATP addition was sequestered in a compartment not accessible to Arsenazo III.

The validity of the spectrophotometric method used routinely throughout this study to measure microsomal Ca^{2+} sequestration is supported by the data reported in Table 1, which compares the Arsenazo III method with two other commonly used methods to measure Ca^{2+} transport, namely use of a Ca^{2+} selective electrode and millipore membrane filtration of microsome-associated $^{45}Ca^{2+}$. As indicated by the data in Table 1, no appreciable differences were found in the capability of liver microsomes to actively sequester Ca^{2+} as measured by the three methods.

Table 2. Effects of some mitochondrial Ca^{2+} transport inhibitors on Ca^{2+} sequestration by liver microsomes

Additions	Ca^{2+} sequestration* (nmol Ca^{2+}/mg protein)
None	7.6 ± 0.39
Ruthenium Red, 1.2 μM	7.4 ± 0.24
CCCP, 3.3 μM	7.4 ± 0.27
Rotenone, 7 μM	8.2 ± 0.36

The inhibitors were added to the incubation just before the addition of ATP. The results are expressed as mean ± S.E. of three separate experiments.

* Ca^{2+} sequestration was measured by the Arsenazo II method as described in Table 1.

To exclude a possible contribution to ATP-dependent Ca^{2+} sequestration by mitochondria contaminating the microsomal suspension, the effect of three well-known inhibitors of mitochondrial Ca^{2+} transport was investigated. As shown in Table 2, these inhibitors were without effect on Ca^{2+} sequestration by the fraction used. Moreover, investigation of the pH-dependency of Ca^{2+} sequestration indicated that this process was maximal at pH 7–7.2, with considerably less activity at pH 8, suggesting that inverted plasma membrane vesicles did not contribute significantly to the recorded activity (data not shown).

Alterations of microsomal Ca^{2+} sequestration by agents affecting protein sulfhydryl groups

It has been reported previously [2] that the thiol reagent PCMB is extremely effective in preventing the active Ca^{2+} accumulation by liver microsomes. As illustrated in Fig. 2, 50 μM PCMB was able to completely prevent ATP-dependent Ca^{2+} sequestration by the microsomes, and also increased the rate of Ca^{2+} release triggered by ATP consumption. Another thiol reagent, *N*-ethylmaleimide, had similar, but less pronounced, effects on both Ca^{2+} uptake and release (data not shown).

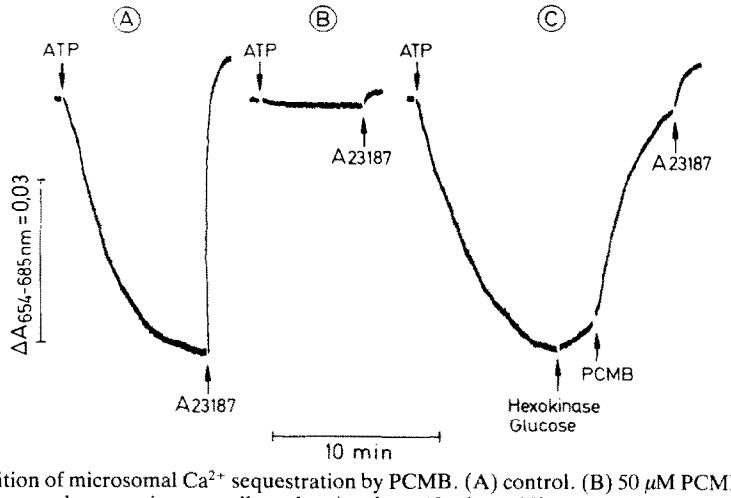

Fig. 2. Inhibition of microsomal Ca^{2+} sequestration by PCMB. (A) control. (B) 50 μM PCMB was added and the microsomal suspension was allowed to incubate 10 min at 25° before measuring Ca^{2+} uptake. (C) microsomes were allowed to actively sequester Ca^{2+}, then hexokinase (2 μg/ml) and glucose (13 mM) were added to deplete ATP; after 2 min, PCMB (50 μM) was also added. At the end of each experiment, ionophore A23187 (15 μM) was added to release the sequestered Ca^{2+}.

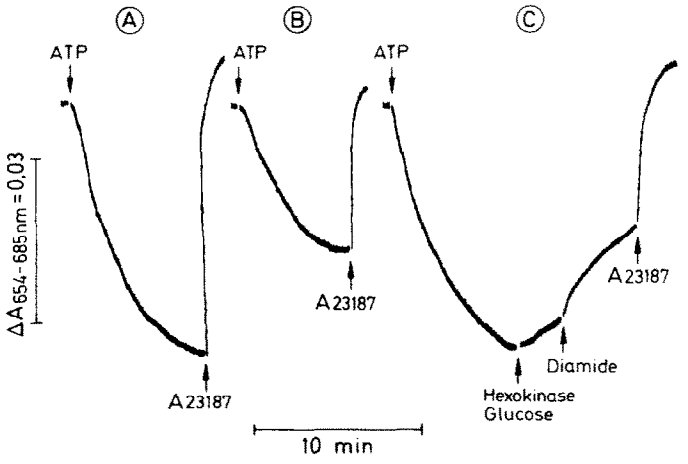

Fig. 3. Inhibition of microsomal Ca^{2+} sequestration by diamide: (A) control. (B) 1 mM diamide was added to the microsomal suspension which was then incubated at 25° for 10 min before measuring Ca^{2+} uptake. (C) Microsomes were allowed to actively sequester Ca^{2+}, then hexokinase (2 μg/ml) and glucose (13 mM) were added to deplete ATP; after 2 min, diamide (1 mM) was also added. At the end of each experiment, ionophore A23187 (15 μM) was added to release the sequestered Ca^{2+}.

To compare the effects of protein thiol alkylation with those of oxidation, the modification of microsomal Ca^{2+} sequestration induced by a variety of oxidizing agents was investigated. In Fig. 3 are reported the alterations of microsomal Ca^{2+} fluxes induced by the oxidizing agent diamide. Both inhibition of Ca^{2+} sequestration and potentiation of Ca^{2+} release were observed in diamide-treated microsomes although quantitatively the effects were much less pronounced than those seen with the alkylating agents.

We have previously found that the intracellular production of active oxygen species can cause a depletion of the endoplasmic reticular Ca^{2+} pool in intact hepatocytes [22, 31, 32]. In the present study, we have extended this investigation to isolated microsomes. As illustrated in Table 3, both peroxides and superoxide radicals, generated either by the xanthine–xanthine oxidase system or by the metabolism of menadione via microsomal NADPH-cytochrome P-450 reductase, are powerful inhibitors of microsomal Ca^{2+} sequestration. Moreover, the inclusion of GSH in the incubation medium, at millimolar concentrations, prevented this inhibition of Ca^{2+} sequestration as well as that seen with diamide.

In addition to alkylation and oxidation, protein sulfhydryl groups can be modified by formation of mixed disulfides [33]. We have therefore tested the possibility that naturally occurring disulfides may inhibit microsomal Ca^{2+} sequestration by forming mixed disulfides with protein sulfhydryl groups critical for Ca^{2+}-ATPase activity. Table 4 shows that of the disulfides employed cystamine was most effective in decreasing the activity of microsomal Ca^{2+} sequestration. That the inhibition of microsomal Ca^{2+} sequestration by cystamine was in fact related to its effect on protein thiols is supported by the results of the following experiments. Firstly, the addition of the thiol reducing agent DTT to microsomes, whose Ca^{2+} sequestering activity was inhibited by cystamine, resulted in a rapid and almost complete restoration of Ca^{2+} sequestration (Fig. 4). Secondly,

Table 3. Inhibition of microsomal Ca^{2+} sequestration by oxidizing agents, and its prevention by reduced glutathione

Treatment	Ca^{2+} sequestration (nmol/mg protein)
None	7.6
Diamide, 1 mM (60 min)*	4.5
Diamide, 1 mM + GSH, 2 mM (60 min)	7.2
t-Butylhydroperoxide, 1 mM (10 min)	1.9
t-Butylhydroperoxide, 1 mM + GSH, 1 mM (10 min)	6.8
H_2O_2, 1 mM (10 min)	4.0
H_2O_2, 1 mM + GSH, 1 mM (10 min)	7.4
Menadione, 400 μM + NADPH, 1 mM (60 min)	4.6
Menadione, 400 μM + NADPH, 1 mM + GSH, 1 mM (60 min)	7.4
Xanthine, 1 mM + xanthine oxidase, 10 μl (15 min)	3.1

Ca^{2+} sequestration was measured by the Arsenazo method as described in Table 1. When indicated, GSH was included in the incubation medium together with the oxidizing agent. One experiment typical of four.

* Time of preincubation of microsomes before ATP-dependent Ca^{2+} sequestration was assayed is given within parentheses.

Table 4. Effects of various disulfides on microsomal Ca^{2+} sequestration

Addition	Ca^{2+} sequestration (nmol/mg protein)
None	7.6 ± 0.39
GSSG, 5 mM	6.3 ± 0.89
Cystine, 5 mM	6.3 ± 1.15
Homocystine, 5 mM	7.3 ± 0.88
Lipoic acid (oxidized), 5 mM	7.4 ± 1.31
Cystamine, 100 μM	4.5 ± 0.51
1 mM	2.2 ± 0.39
5 mM	2.1 ± 0.45

The disulfides were included in the incubation at the concentrations indicated. The pH of the medium was carefully controlled, and microsomes (1 mg protein/ml) were added. After 1 hr incubation, Ca^{2+} sequestration was measured using the Arsenazo method as described in Table 1. The results are expressed as mean ± S.E. of five different experiments.

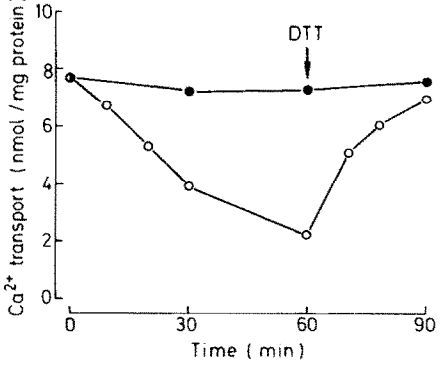

Fig. 4. Inhibition of microsomal Ca^{2+} transport by cystamine, and its restoration by dithiothreitol. Cystamine (1 mM) was included in the incubation medium together with microsomes, and the suspension was incubated at 25°; Ca^{2+} uptake was measured at different times. After 60 min, dithiothreitol (DTT, 2 mM) was added and Ca^{2+} uptake was measured after 10, 20 and 30 min: (●) microsomes pre-incubated without cystamine; (○) microsomes pre-incubated with 1 mM cystamine.

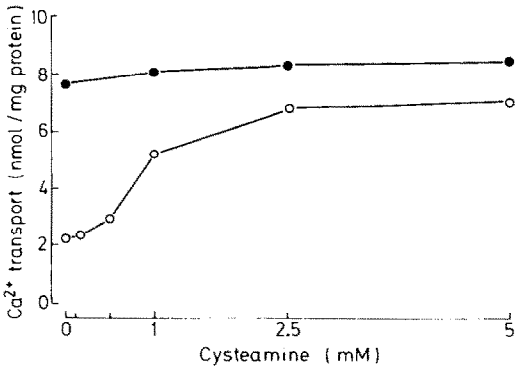

Fig. 5. Modulation of microsomal Ca^{2+} transport by changing the cystamine/cysteamine ratio. Microsomes (1 mg protein/ml) were incubated for 60 min at 25° with 1 mM cystamine and various concentrations of cysteamine to achieve cysteamine/cystamine ratios ranging from 0 to 5. At the end of the incubation period, Ca^{2+} transport was assayed as described in Methods: (●) microsomes incubated with cysteamine alone; (○) microsomes incubated with 1 mM cystamine + cysteamine.

cysteamine, the reduced equivalent of cystamine, counteracted the inhibitory effect of cystamine (Fig. 5), suggesting that the balance of these two agents may represent a factor modulating Ca^{2+} transport in microsomes.

Relationship between loss of protein sulfhydryl groups and inhibition of microsomal Ca^{2+} *sequestration*

To investigate the relationship between inhibition of microsomal Ca^{2+} sequestration and loss of protein thiols, we measured free sulfhydryl groups remaining after treatment of isolated microsomes with some of the agents employed in this study (Table 5). Although there was a rather good correlation between disappearance of protein thiols and inhibition of Ca^{2+} sequestration, conflicting results were obtained with diamide. In spite of a 90% decrease in protein thiols, Ca^{2+} sequestration was inhibited by less than 50%. To investigate the possibility that this discrepancy could reflect a relative insensitivity

Table 5. Relationship between the inhibition of microsomal Ca^{2+} sequestration and the loss of protein thiols induced by sulfhydryl reagents, oxidizing agents, and disulfides

Addition	Ca^{2+} sequestration	Protein SH-groups
	% of control	
None	100	100
PCMB, 50 μM (15 min)*	0	0
Diamide, 1 mM (60 min)	55	10
t-Butylhydroperoxide, 1 mM (10 min)	25	30
Menadione, 400 μM + NADPH, 1 mM (60 min)	57	17
Cystamine, 5 mM (60 min)	29	35

Ca^{2+} sequestration and protein sulfhydryl groups were measured as described in Table 1 and Methods, respectively and expressed as % of control. One experiment typical of four.

* Time of preincubation of microsomes before assay of ATP-dependent Ca^{2+} sequestration and protein thiols is given within parentheses.

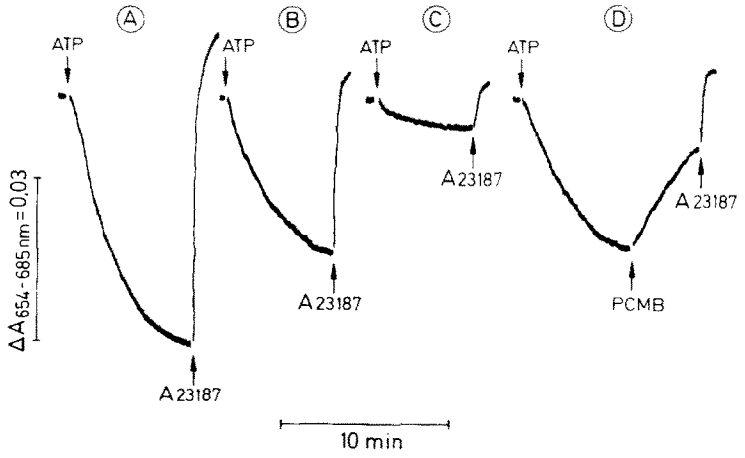

Fig. 6. Potentiation of diamide-induced inhibition of Ca^{2+} sequestration in microsomes by PCMB: (A) control. (B) microsomes treated with 1 mM diamide for 10 min at 25°. (C) as in (B) except that 25 μM PCMB was added to the incubation medium just before measuring Ca^{2+} uptake. (D) as in (B) except that 25 μM PCMB was added at the end of Ca^{2+} uptake. At the end of each experiment, ionophore A23187 (15 μM) was added to release the sequestered Ca^{2+}.

of the sulfhydryl groups critical for Ca^{2+}-ATPase activity to diamide treatment, we investigated the efficiency of PCMB to further inhibit Ca^{2+} sequestration in diamide-treated microsomes. As illustrated in Fig. 6, PCMB greatly potentiated the inhibitory effect of diamide on Ca^{2+} sequestration, and was also effective in promoting a spontaneous release of the sequestered Ca^{2+}.

DISCUSSION

The intramicrosomal Ca^{2+} content depends on the balance between uptake and release processes. Ca^{2+} uptake has been demonstrated to be strictly associated with the operation of a Ca^{2+}-stimulated, Mg^{2+}-dependent ATPase [4, 6]. The route by which Ca^{2+} is released has not been characterized in detail, although its existence has been proven by the demonstration that physiological stimuli (inositol 1,4,5-trisphosphate) specifically induce Ca^{2+} efflux from liver endoplasmic reticulum [14, 15].

The results reported in this study clearly show that agents which are able to modify the thiol status of microsomal proteins can also affect Ca^{2+} sequestration by the microsomes. The observed effects could be related either to the inhibition of the Ca^{2+}-ATPase or to the potentiation of Ca^{2+} efflux from the vesicles. The unavailability of a specific inhibitor of microsomal Ca^{2+} uptake makes it difficult to discriminate between these effects. Binoldi and Fleischer [34] recently reported that mercurials were able to induce the release of Ca^{2+} from sarcoplasmic reticulum vesicles at a rate which was more rapid than that of passive Ca^{2+} leakage when the transport ATPase was inhibited by quercetin. We also found that several of the agents employed in our study could stimulate the rate of Ca^{2+} efflux from preloaded microsomes in which the uptake process was minimized by the removal of ATP. However, it is clear that Ca^{2+} uptake could also be affected, particularly by alkylation of protein thiols (cf. Fig. 2). When assayed under similar experimental conditions, the activity of Ca^{2+}-ATPase was markedly depressed by the sulfhydryl reagents (Thor, H., unpublished results).

It is a common feature of a large number of enzymes to be irreversibly inactivated by thiol alkylating reagents such as PCMB and *N*-ethylmaleimide. More recently, it has become clear that the activity of certain enzymes can also be modulated by a thiol–disulfide exchange induced by several naturally occurring disulfides [35–37]. Together with the demonstration of a cyclic diurnal fluctuation of the hepatic thiol/disulfide ratio [38, 39], these observations have led to the proposal of a hypothetical 'third messenger' function of biological disulfides. Our finding that microsomal Ca^{2+} sequestration can be modulated by the cysteamine/cystamine ratio may be interpreted as further support of this hypothesis. Moreover, the demonstration that protein mixed disulfides are formed during oxidative stress [32, 33] suggests that these findings may have toxicological relevance.

Since this study was performed with intact microsomes, it is not possible to conclude how many sulfhydryl groups may be involved in the active sequestration of Ca^{2+}. Such considerations are further complicated by the quantitatively different effects induced by oxidative vs alkylating processes. For example, it has been reported that oxidation and alkylation of free sulfhydryl groups of the 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase complex have markedly different effects [40]. The oxidative process involves disulfide bond formation between two sulfhydryl groups located close to each other, whereas alkylation and formation of mixed disulfides do not require this prerequisite. It is therefore possible that protein thiol oxidation may not involve all of the critical sulfhydryl groups required for the full activity of the Ca^{2+}-ATPase. This may explain the lack of correlation between the loss of protein thiols and the inhibition

of microsomal Ca^{2+} sequestration observed in the case of diamide. The potentiation of the diamide effect by PCMB seems to substantiate this view.

In summary, the data presented in this study demonstrate that a variety of toxic agents can inhibit Ca^{2+} sequestration in liver microsomes by affecting protein sulfhydryl groups. In the intact cell, this inhibition may not only result in a decreased sensitivity to physiological stimuli such as α-adrenergic hormones, but also to a loss of the contribution by the endoplasmic reticulum in buffering cytosolic free Ca^{2+} concentration. In addition recent evidence indicates that the plasma membrane Ca^{2+}-ATPase is also inhibited under similar conditions [29]. The simultaneous inhibition of both transport systems would be expected to result in an uncontrollable increase in cytosolic Ca^{2+} concentration and a rapid progression of the toxic effects.

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