Intraluminal calcium of the liver endoplasmic reticulum stimulates the glucuronidation of *p*-nitrophenol

Gábor BÁNHEGYI,* Giorgio BELLOMO,† Rosella FULCERI,‡ József MANDL* and Angelo BENEDETTI‡§

*1st Institute of Biochemistry, Semmelweis Medical University, Budapest, Hungary, †Clinica Medica I, University of Pavia, 27100 Pavia, Italy, and ‡Istituto di Patologia Generale, University of Siena, 53100 Siena, Italy

The relationship between the intraluminal Ca^{2+} content of endoplasmic reticulum and the rate of the glucuronidation of *p*-nitrophenol was investigated in isolated rat hepatocytes. Different agents which decrease the Ca^{2+} level in the endoplasmic reticulum [calcium ionophores (A23187, ionomycin) or Ca^{2+}-ATPase inhibitors (thapsigargin, 2,5-di-(t-butyl)-1,4-benzohydroquinone)] inhibited the conjugation of *p*-nitrophenol. Depletion of intracellular Ca^{2+} stores by preincubation of hepatocytes in the absence of free Ca^{2+} (in the presence of excess EGTA) also decreased the rate of glucuronidation; Ca^{2+} re-admission to EGTA-treated hepatocytes restored glucuronidation. In intact liver microsomes the *p*-nitrophenol UDP-glucuronosyltransferase activity was not modified by varying the external free Ca^{2+} concentrations within a cytosol-like range. Emptying of the Ca^{2+} from the lumen of microsomal vesicles by A23187, after MgATP-stimulated Ca^{2+} sequestration, decreased the glucuronidation of *p*-nitrophenol. A similar effect was observed in filipin-permeabilized hepatocytes. In native and in detergent-treated microsomes, Ca^{2+} (1–10 mM) increased the *p*-nitrophenol UDP-glucuronosyltransferase activity. It is suggested that the physiological concentration of Ca^{2+} in the lumen of the endoplasmic reticulum is necessary for the optimal activity of *p*-nitrophenol UDP-glucuronosyltransferase; the depletion of Ca^{2+} decreases the activity of the enzyme.

INTRODUCTION

The endoplasmic reticulum (ER) is the major intracellular reservoir of Ca^{2+} [1]. The maintenance of physiological Ca^{2+} levels in the lumen of the ER is essential not only in signal transduction but also for the normal secretory pathway of proteins [2,3] and for the modulation of hepatocyte tight-junctional permeability [4]. Sequestered Ca^{2+} stores are essential for optimal rates of mRNA translation [5]. Depletion of ER Ca^{2+} pool induces secretion of luminal proteins [6], accelerates protein degradation in the reticular lumen [7], decreases the rate of protein synthesis by isolated hepatocytes [8], inhibits phosphatidylserine synthesis [9], and induces profound alterations in cell proliferation [10]. Intrareticular Ca^{2+} concentration may also influence the activity of lumenal enzymes. However, the activity of the intraluminal enzyme glucose-6-phosphatase has been reported to be influenced by neither cytosolic nor intrareticular 'physiological' Ca^{2+} concentrations [11]. In the present study, we have investigated from the same point of view another enzyme of the ER, the UDP-glucuronosyltransferase (UDPGT; EC 2.4.1.17).

UDPGT isoenzymes are integral membrane proteins of ER, and *in vitro*, i.e. in isolated ER-derived vesicles (microsomes), their activity is latent. The enzyme is in fact more active in disrupted microsomal vesicles than in intact microsomes [12–14]. The latency can be explained by a constraining effect of the intact ER membrane on the enzyme activity [13], or alternatively by the intraluminal compartmentation of the enzyme [14]. In the latter case, the 'intact' membrane would limit the enzyme activity by limiting the permeation of the intraluminal space by the cofactor UDP-glucuronic acid (UDGPA).

Glucuronidation is the highest capacity pathway in the second phase of biotransformation. In the liver, glucuronidation is important for the termination of drug action and for the disposition of endo- and xeno-biotics. In the liver cell the supply of UDGPA for glucuronidation depends mainly on the extent of glycogen pool and on the rate of glycogenolysis [15,16]. Therefore, activation of glycogen breakdown by different agents would be expected to stimulate glucuronidation of xenobiotics by increasing the UDGPA content in the cell. Contrary to this assumption, agents causing increased glycogenolysis, e.g. dibutyryl cyclic AMP [17,18], glucagon [19], adrenaline [20], hepatic nerve stimulation [21], endotoxin and prostaglandins (G. Bánhegyi and J. Mandl, unpublished work), inhibit glucuronidation despite the increased intracellular UDGPA levels. Some of these agents mobilize Ca^{2+} from the lumen of ER; therefore, as UDPGTs are embedded in the ER membrane, the putative role of intrareticular Ca^{2+} in the regulation of UDPGT activity was assumed.

EXPERIMENTAL

Materials

ATP, UDPGA (sodium salt), β-glucuronidase (type IX), phosphocreatine, creatine kinase, thapsigargin, filipin, A23187, ionomycin, carbonyl cyanide *p*-fluoromethoxyphenylhydrazone (FCCP) and Arsenazo III were from Sigma, St. Louis, MO, U.S.A. 2,5-Di-(t-butyl)-1,4-benzohydroquinone (tBuBHQ) was from EGA Chemie, Steinheim, Germany. Fura-2 AM (acetoxymethyl ester) was obtained from Molecular Probes, Eugene, OR, U.S.A., Percoll from Pharmacia, Uppsala, Sweden, and collagenase from Boehringer, Mannheim, Germany. Ca^{2+}-sensitive electrodes were purchased from Ionetics Inc., Palo Alto, CA, U.S.A. All other chemicals were of analytical grade.

Abbreviations used: ER, endoplasmic reticulum; UDPGT(s), UDP-glucuronosyltransferase(s); UDPGA, UDP-glucuronic acid; tBuBHQ, 2,5-di-(t-butyl)-1,4-benzohydroquinone; FCCP, carbonyl cyanide *p*-fluoromethoxyphenylhydrazone.

§ To whom correspondence should be addressed.

Hepatocyte isolation and incubation

Collagenase-dispersed hepatocytes were obtained from fed male Sprague–Dawley rats (200–250 g), as previously described [22]. Isolated cells were resuspended (2×10^6 cells/ml) in a modified Hanks' medium [22] containing 1.3 mM $CaCl_2$ and incubated (37 °C) in a continuously rotated flask under CO_2/O_2 (1:19). After a 30 min equilibration period, the cell suspension was divided into two parts and 2.6 mM EGTA was added to one of them. Incubation was continued for a further 30 min, and then *p*-nitrophenol (100 μM) or the various Ca^{2+}-releasing agents followed by *p*-nitrophenol were added. Incubations were terminated 10 min after *p*-nitrophenol addition, and conjugation of *p*-nitrophenol was measured on the basis of aglycone disappearance as reported previously [19]. In some experiments *p*-nitrophenol glucuronide formation was measured enzymically [19].

Cell viability, as assessed by the Trypan Blue exclusion test, was over 85% in any experiment. None of the indicated treatments caused significant modification of cell viability.

Determination of cytosolic free Ca^{2+}

Cytosolic free Ca^{2+} concentration was measured with the fluorescent indicator Fura-2 as reported in detail elsewhere [22]. Briefly, cells were loaded for 30 min with Fura-2 AM in Hanks' medium including 1.3 nM $CaCl_2$ and 2% BSA. Cells were then washed, resuspended in Hanks' medium containing 1.3 mM $CaCl_2$ in the absence or presence of 2.6 mM EGTA and were incubated for a further 30 min. The various Ca^{2+}-releasing agents were then added and cytosolic free Ca^{2+} levels were measured 10 min later.

Measurement of mitochondrial and non-mitochondrial Ca^{2+} in isolated hepatocytes

Mitochondrial (protonophore-mobilizable) and non-mitochondrial (ionophore-mobilizable) Ca^{2+} pools were measured as previously described [23]. Briefly, hepatocytes were separated from the incubation medium by rapid centrifugation through a suspension of Percoll in Ca^{2+}/Mg^{2+}-free Hanks' medium and resuspended in the Ca^{2+}/Mg^{2+}-free Hanks' medium including Arsenazo III (40 μM final concen.). The amounts of Ca^{2+} released by the sequential addition of FCCP (10 μM) and A23187 (10 μM) were measured spectrophotometrically at the wavelength pair 654–685 nm.

Preparation of liver microsomes and filipin-permeabilized hepatocytes

Microsomes were prepared as reported in [24]. The microsomal fraction was resuspended (approx. 80 mg of protein/ml) in a medium of the following composition (mM): KCl, 100; NaCl, 20; $MgCl_2$, 5; Mops, 20, pH 7.2. The suspensions were frozen and maintained under liquid N_2 until work-up.

Intactness of the microsomal membrane was checked by measuring the latency of mannose-6-phosphatase activity [25], which was over 90% in all the preparations employed.

Hepatocytes were permeabilized with 100 μM filipin as reported [26]. Trypan Blue exclusion of the permeabilized cells was below 5%.

Incubation of microsomes and permeabilized cells

Microsomes (4 mg of protein/ml) and permeabilized cells (2×10^6/ml) were incubated in a thermostatically regulated (37 °C) Plexiglas vessel in which a Ca^{2+} electrode and a reference electrode (Radiometer K4040) were immersed. The incubation medium (1 ml) was as follows (mM): KCl, 100; NaCl, 20; $MgCl_2$, 5; Mops, 20 (pH 7.2); ATP, 3; phosphocreatine, 10; NaN_3 (as mitochondrial inhibitor), 5. Creatine kinase (10 units/ml) was also present. $CaCl_2$ (20 μM final concn.) was added to the medium. The amount of total Ca^{2+} present in the incubation medium before Ca^{2+} addition (i.e. Ca^{2+} already present as a routine contaminant of solutions) ranged from 12 to 20 nmol/ml, as measured by atomic-absorbance spectrophotometry.

MgATP-dependent Ca^{2+} accumulation by microsomes (and permeabilized cells) was evaluated by measuring free Ca^{2+} in incubations with a Ca^{2+} electrode, and was quantified by titrating the amount of Ca^{2+} released by using the Ca^{2+} ionophore A23187 (2 μM), by means of $CaCl_2$ additions to parallel incubations. Ca^{2+} electrodes were calibrated as described elsewhere [27].

p-Nitrophenol conjugation activity of microsomes and permeabilized cells was determined in the presence of 0.5 mM *p*-nitrophenol and 4 mM UDPGA by measuring *p*-nitrophenol disappearance as previously described [28].

Incubations of detergent-treated microsomes

Microsomes were prepared as reported [24]. The microsomal fraction was resuspended (1 mg of protein/ml) in a medium of the following composition (mM): KCl, 100; NaCl, 20; Mops, 20, pH 7.2. The microsomal suspension was preincubated for 10 min at 0–4 °C in the presence of 0.01% (w/v) Triton X-100, and subsequently incubated at 37 °C in the presence of 0.5 mM *p*-nitrophenol and 4 mM UDPGA to measure *p*-nitrophenol-conjugation activity [28].

Other assays

Protein was determined as reported previously [29], with BSA as standard.

Table 1 Effect of various Ca^{2+}-mobilizing agents on *p*-nitrophenol conjugation in Ca^{2+}-supplemented and Ca^{2+}-depleted rat hepatocytes

Collagenase-dispersed hepatocytes (2×10^6 cells/ml) were preincubated in a modified Hanks' medium containing 1.3 mM $CaCl_2$. After a 30 min pre-equilibration period, the cell suspension was divided into two parts and 2.6 mM EGTA was added to one of them (Ca^{2+}-depleted hepatocytes). After a further 30 min of incubation, the various Ca^{2+}-releasing agents were added; 10 min later *p*-nitrophenol (pNP, 100 μM) was added and conjugation of *p*-nitrophenol was measured as described in the Experimental section. In some experiments *p*-nitrophenol glucuronide (pNPG) formation was measured enzymically [19]. Data are means ± S.D.; numbers of experiments are shown in parentheses. ND, not determined.

	Activity (pmol/min per 10^6 cells)			
	1.3 mM $CaCl_2$		1.3 mM $CaCl_2$ + 2.6 mM EGTA	
	pNP disapp.	pNPG formation	pNP disapp.	pNPG formation
None	739 ± 88 (8)	602 (2)	430 ± 126 (8)	310 (2)
1 μM A23187	522 ± 130 (4)	405 (2)	471 ± 38 (4)	321 (2)
1 μM ionomycin	570 (2)	ND	478 (2)	ND
1 μM thapsigargin	455 ± 113 (4)	352 (2)	306 ± 156 (4)	208 (2)
25 μM tBuBHQ	262 ± 51 (4)	188 (2)	281 ± 74 (4)	208 (2)

RESULTS

Depletion of reticular Ca^{2+} stores decreases *p*-nitrophenol conjugation in isolated intact hepatocytes

p-Nitrophenol disappearance and *p*-nitrophenol glucuronide formation were measured in intact hepatocytes, isolated from fed rats, after addition of 100 μM *p*-nitrophenol. In these experimental conditions, the rate of *p*-nitrophenol conjugation was maximal and the *p*-nitrophenol disappearance was mostly accounted for by formation of the glucuronide (see Table 1) as expected on the basis of previous data [16,17,20,30].

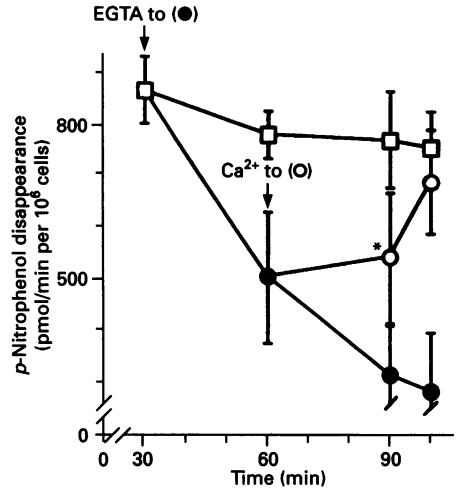

Figure 1 Reversibility of the inhibition of *p*-nitrophenol conjugation caused by EGTA in isolated rat hepatocytes

Isolated rat hepatocytes (2×10^6 cells/ml) were preincubated for 30 min in a modified Hanks' medium [20] containing 1.3 mM $CaCl_2$. When indicated by arrows, 2.6 mM EGTA and 2.6 mM $CaCl_2$ were added to the incubation system. The disappearance of *p*-nitrophenol was determined at the indicated times as detailed in the Experimental section. Data are means ± S.D. of four independent experiments. The value marked * is significantly different ($P < 0.05$) from the corresponding value (90 min) in the absence of Ca^{2+} addition (●) on the basis of an unpaired Student's *t* test.

Table 2 Effect of EGTA, thapsigargin and tBuBHQ on Ca^{2+} content of mitochondrial (FCCP-mobilizable) and non-mitochondrial (A23187-mobilizable) pools in isolated rat hepatocytes

Isolated rat hepatocytes were incubated and treated as described in Table 1, except that in 'EGTA followed by $CaCl_2$' samples 2.6 mM $CaCl_2$ was added after incubation with EGTA, and incubation was prolonged a further 30 min. FCCP- and A23187-mobilizable Ca^{2+} were measured as reported in the Experimental section. Data are means ± S.D.; numbers of experiments are given in parentheses.

	Ca^{2+} content (nmol/10^6 cells)	
	FCCP-mobilizable	A23187-mobilizable
None	1.54 ± 0.16 (5)	2.42 ± 0.20 (5)
EGTA (2.6 mM)	0.48 ± 0.06 (5)	0.53 ± 0.07 (5)
EGTA followed by $CaCl_2$	1.04 ± 0.09 (3)	2.15 ± 0.12 (3)
Thapsigargin (1 μM)	1.43 ± 0.34 (5)	0.62 ± 0.09 (5)
EGTA and thapsigargin	0.48 (2)	0.28 (2)
tBuBHQ (25 μM)	1.39 ± 0.45 (3)	0.77 ± 0.12 (3)

Table 3 Effect of various Ca^{2+}-mobilizing agents on cytosolic free Ca^{2+} concentration in Ca^{2+}-supplemented and Ca^{2+}-depleted rat hepatocytes

Isolated rat hepatocytes were incubated and treated as described in Table 1. Cytosolic free Ca^{2+} concentration was measured by using the Ca^{2+} indicator Fura 2 (see the Experimental section) 10 min after addition of the various agents. Data are means ± S.D.; numbers of experiments are given in parentheses.

	Cytosolic free Ca^{2+} (nM)	
	1.3 mM $CaCl_2$	1.3 mM $CaCl_2$ + 2.6 mM EGTA
None	156 ± 12 (4)	115 ± 17 (4)
1 μM thapsigargin	399 (2)	146 ± 12 (4)
25 μM tBuBHQ	385 ± 22 (3)	140 (2)
3 μM ionomycin	636 (2)	126 ± 15 (3)

Depletion of intracellular Ca^{2+} stores by preincubating the cells for 30 min in the presence of excess EGTA caused about 50% inhibition of *p*-nitrophenol glucuronidation (Table 1). The inhibition was reversible; addition of excess Ca^{2+} to EGTA-containing incubations resulted in the restoration of the conjugation activity (Figure 1), accompanied by the refilling of intracellular Ca^{2+} stores (see Table 2).

In the presence of extracellular Ca^{2+}, the reticular Ca^{2+} stores were depleted by using low concentrations of Ca^{2+} ionophores [31] and inhibitors of ER Ca^{2+}-ATPase. All the agents employed (the ionophores A23187 and ionomycin, and the Ca^{2+}-ATPase inhibitors thapsigargin [32] and tBuBHQ [33]) inhibited the conjugation of *p*-nitrophenol in intact hepatocytes incubated in the Ca^{2+}-supplemented medium (Table 1). In the hepatocytes whose conjugation activity has already been decreased by preincubation with excess EGTA, a further inhibition was observed after the treatment with the Ca^{2+}-ATPase inhibitors (Table 1).

The possibility for a direct inhibitory effect of the above-mentioned agents on the *p*-nitrophenol UDPGT activity was evaluated in isolated liver microsomes. At the concentrations used in intact hepatocytes, A23187, ionomycin and thapsigargin did not modify the microsomal conjugation activity. A moderate inhibitory effect (approx. 10%) was present with tBuBHQ. This effect may somehow contribute to the marked inhibition of *p*-nitrophenol conjugation by tBuBHQ in intact hepatocytes (see Table 1).

UDPGTs are embedded in the microsomal membrane and both the extra- (cytosolic) and intra-reticular compartment are involved in the pathway of glucuronidation. In parallel experiments, we checked the effect of Ca^{2+} depletion by EGTA and also the effect of the Ca^{2+}-mobilizing agents on cytosolic free Ca^{2+} levels and on the Ca^{2+} content of sequestered intracellular pools.

Preincubation of cells with EGTA caused a marked depletion of both mitochondrial and non-mitochondrial Ca^{2+} pools (Table 2), whereas the level of cytosolic free Ca^{2+} was only slightly decreased (Table 3). Emptying of the intracellular Ca^{2+} stores by EGTA could be reversed by re-addition of excess Ca^{2+} during a further 30 min incubation (Table 2).

As expected, thapsigargin and tBuBHQ specifically depleted the non-mitochondrial (reticular) Ca^{2+} pool (Table 2) and increased the cytosolic free Ca^{2+} concentration more than 2-fold in Ca^{2+}-supplemented hepatocytes (Table 3). In cells depleted of Ca^{2+} by EGTA preincubation, thapsigargin caused a further decrease in the Ca^{2+} content of the non-mitochondrial pool (Table 2). Ionomycin elevated the cytosolic free Ca^{2+} concentration in Ca^{2+}-supplemented cells, whereas in Ca^{2+}-depleted

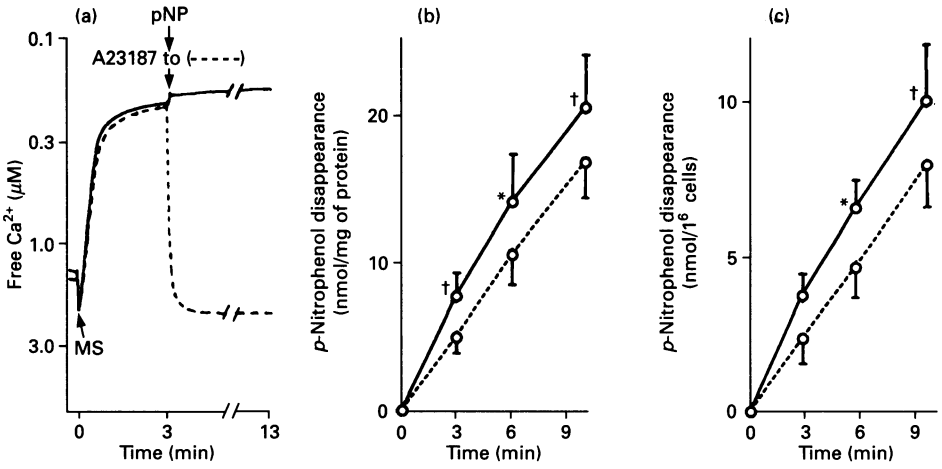

Figure 2 Effect of Ca^{2+} accumulation (a) on p-nitrophenol UDPGT activity in rat liver microsomes (b) or filipin-permeabilized rat hepatocytes (c)

Rat liver microsomes or filipin-permeabilized rat hepatocytes were incubated in the presence of 4 mM UDPGA, MgATP and an ATP-regenerating system (see the Experimental section). Microsomal or cellular Ca^{2+} uptake was measured by monitoring the free Ca^{2+} concentration of the medium with Ca^{2+} electrodes: a typical experiment with isolated microsomes (ms) is shown in (**a**). At steady-state levels of Ca^{2+} accumulation, 0.5 mM *p*-nitrophenol (pNP; continuous line) or A23187 (5 μM) and *p*-nitrophenol (broken line) were added, as depicted in (**a**). (**b**) *p*-Nitrophenol UDPGT activity of isolated microsomes measured in control incubation (continuous line) or after A23187 addition (broken line). (**c**) *p*-Nitrophenol UDPGT activity of filipin-permeabilized hepatocytes measured in control incubation (continuous line) or after A23187 addition (broken line). Data are means $\pm$ S.D. of four (**b**) or five (**a**) independent experiments. Values marked * and † are significantly different from the corresponding values obtained in microsomes or cells treated with A23187 ($P < 0.05$ and $P < 0.01$ respectively) on the basis of a paired Student's *t* test.

cells it was ineffective (Table 3). Data reported in Tables 1–3 were at 10 min after the addition of the various Ca^{2+}-discharging agents. At later times after the treatment with tBuBHQ (45 min), and in the presence of extracellular Ca^{2+}, the inhibition of *p*-nitrophenol conjugation was still present (61 %; $n = 2$), although the cytosolic free Ca^{2+} returned to near the basal level (167 nM; $n = 2$).

Effect of Ca^{2+} on UDPGT activity in isolated rat liver microsomes

The maximal rate of UDPGT activity in the presence of 0.5 mM *p*-nitrophenol was unaffected by changing the extravesicular free Ca^{2+} concentration within a physiological cytosol-like range. In fact, in the presence of 1 mM EGTA and various amounts of added Ca^{2+} the free [Ca^{2+}] of the incubation system was varied from 0.1 to 3.0 μM (as measured with Ca^{2+} electrodes) and the rate of *p*-nitrophenol conjugation by intact microsomes was not modified (results not shown).

In a second set of experiments, liver microsomes were incubated in the presence of UDPGA and MgATP (plus an ATP-regenerating system). Under these conditions, microsomal vesicles sequestered external Ca^{2+} until a steady-state level corresponding to approx. 10 nmol/mg of protein, as revealed by measuring extravesicular [Ca^{2+}] with a Ca^{2+} electrode (see Figure 2a for a typical experiment). At steady-state level, *p*-nitrophenol was added to the system and its glucuronidation was detected. The *p*-nitrophenol conjugation was linear over a period of 10 min (Figure 2b, continuous line). After the release of sequestered Ca^{2+} by addition of A23187, even in the presence of higher extravesicular free [Ca^{2+}] (Figure 2a, broken trace), the rate of *p*-nitrophenol conjugation was diminished (Figure 2b, broken line). Similar results were obtained by using filipin-permeabilized hepatocytes (Figure 2c).

As intravesicularly accumulated Ca^{2+} appeared to increase the UDPGT activity in intact microsomes, we studied the effect of millimolar Ca^{2+} concentrations (similar to the intravesicular ones) on *p*-nitrophenol UDPGT activity of detergent-disrupted

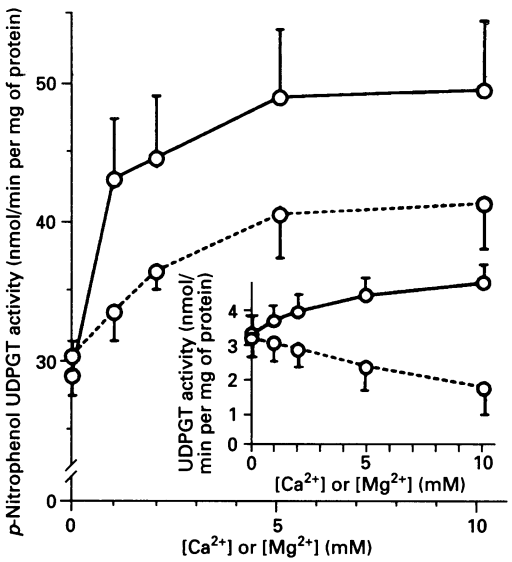

Figure 3 Effect of Ca^{2+} and Mg^{2+} on *p*-nitrophenol UDPGT activity of detergent-disrupted rat liver microsomes

p-Nitrophenol UDPGT activity was measured in detergent-disrupted and in native (inset) microsomal vesicles in the presence of Ca^{2+} (as $CaCl_2$, continuous line) or Mg^{2+} (as $MgCl_2$, broken line) as reported in the Experimental section. Data are means $\pm$ S.D. of 4–5 experiments.

microsomes. As shown in Figure 3 (continuous line), Ca^{2+} increased the *p*-nitrophenol UDPGT activity in disrupted microsomes. The stimulatory effect was evident at 1 mM and maximal at 5 mM. In intact microsomes, Ca^{2+} had a similar, although less expressed, effect (Figure 3, inset). We also examined the effect of the other physiological bivalent cation, Mg^{2+}, on microsomal *p*-nitrophenol UDPGT activity. Unlike Ca^{2+}, Mg^{2+} increased *p*-nitrophenol conjugation only in disrupted vesicles, whereas

in intact microsomes it was slightly inhibitory (Figure 3, broken lines).

DISCUSSION

The ER has a prominent role in the Ca^{2+} homoeostasis of the cell related to the hormonal mechanisms. Conventionally, cytosolic free Ca^{2+}, rather than sequestered Ca^{2+}, is considered as a regulator of metabolic processes, but certain lines of evidence support the possibility of a regulatory role of sequestered Ca^{2+}. For example, in the matrix of mitochondria Ca^{2+} play a role in the regulation of dehydrogenases of the citrate cycle [34,35]. A similar mechanism for the regulation of intralumenal enzymes of the ER can be also postulated on the basis of the marked effects of Ca^{2+} depletion of the ER on different biological events of the cell [2–10].

UDPGTs are microsomal enzymes which have an active site of lumenal orientation. Previous observations have shown that bivalent cations may affect their activity [36–45]. It was tempting to suppose that fluctuations of lumenal Ca^{2+}, an important bivalent cation in the lumen of the ER, may regulate the rate of glucuronidation.

In a first series of experiments, Ca^{2+} was depleted from isolated hepatocytes by preincubation with EGTA. EGTA decreased both mitochondrial and non-mitochondrial (reticular) Ca^{2+} contents (Table 2), whereas the cytosolic free Ca^{2+} was slightly diminished (Table 3). *p*-Nitrophenol conjugation and glucuronide formation were also decreased by EGTA (Table 1). All these effects were reversible; the re-admission of Ca^{2+} to the system restored both the reticular Ca^{2+} content (Table 2) and the rate of *p*-nitrophenol conjugation (Figure 1). UDPGA supply for glucuronidation is connected to glycogenolysis [15,16]. Therefore, it can be supposed that a decreased cytosolic free Ca^{2+} concentration (in the presence of EGTA) is accompanied with a decreased glycogen phosphorylase activity and a concomitant decreased cytosolic UDPGA level. Nevertheless, it has been reported that excess EGTA does not significantly change either glucose production or glycogen breakdown in isolated rat hepatocytes [46].

Similarly to EGTA, low concentrations of Ca^{2+} ionophores (1 μM A23187 or 1 μM ionomycin) inhibited *p*-nitrophenol conjugation in hepatocytes, but in Ca^{2+}-depleted cells they failed to decrease the already diminished rate of conjugation (Table 1). Ca^{2+} ionophores at relatively low concentrations have been widely used to discharge intracellularly stored Ca^{2+} (e.g. [3,5–7,31]) and to assess the dependency on the reticular stored Ca^{2+} of several cellular functions [3,5–7]. An inhibitory effect of A23187 on glucuronidation has also been observed in perfused rat livers [20].

Preincubation with EGTA or addition of Ca^{2+} ionophores acts on different cellular Ca^{2+} pools unspecifically. To obtain more direct evidence on the role of reticular Ca^{2+} in the regulation of glucuronidation, selective inhibitors of ER Ca^{2+}-ATPases, namely thapsigargin [32] and tBuBHQ [33], were used. These inhibitors have been extensively used to discharge the agonist-sensitive reticular Ca^{2+} pool (e.g. [3,6,7,9,10,31,33]). Thapsigargin and tBuBHQ decreased the Ca^{2+} content in the non-mitochondrial pool (Table 2), indicating a selective depletion of the ER compartment. Both agents inhibited *p*-nitrophenol conjugation strongly in Ca^{2+}-supplemented cells and slightly in Ca^{2+}-depleted hepatocytes (Table 1). In the hepatocytes already depleted of Ca^{2+} by EGTA, Ca^{2+}-ATPase inhibition by thapsigargin caused a further decrease in the non-mitochondrial Ca^{2+} pool (Table 2). Possibly, EGTA by itself did not fully deplete the ER compartment of Ca^{2+}, although it markedly decreases the Ca^{2+} content in non-mitochondrial pool(s). Non-mitochondrial Ca^{2+} in fact appears to be mostly, but not exclusively, accounted for by the intrareticular Ca^{2+} [31]. In line with this assumption, thapsigargin (and tBuBHQ) still mobilized stored Ca^{2+} in EGTA-treated hepatocytes (Table 3).

Since ionophores and thapsigargin did not modify *p*-nitrophenol glucuronidation in isolated liver microsomes, their effect is probably not on UDPGA or *p*-nitrophenol transport into the ER. A competition between *p*-nitrophenol and the phenolic tBuBHQ, however, may partially contribute to the inhibitory effect of tBuBHQ. After a prolonged incubation in the presence of tBuBHQ, when cytosolic free Ca^{2+} returned to the resting level, the conjugation of *p*-nitrophenol remained inhibited.

Ca^{2+} ionophores and Ca^{2+}-ATPase inhibitors, unlike preincubation with EGTA, elevated the cytosolic free Ca^{2+} (Table 2). Elevated cytosolic Ca^{2+} ought to increase glucuronidation, because of the increased glycogenolysis and the consecutively higher UDPGA level. However, a change of glucuronidation in the opposite direction was observed. It is concluded that the decrease in reticular Ca^{2+} content and not the increase in cytosolic free Ca^{2+} is inhibitory toward glucuronidation.

Physiological cytosol-like concentrations of Ca^{2+} in the incubation medium did not influence the *p*-nitrophenol UDPGT activity in microsomes. To model the normal Ca^{2+} gradient between the cytosol and the lumen of ER, microsomes were allowed to accumulate Ca^{2+} until a steady-state level in the presence of ATP was reached (Figure 2a). Microsomes with accumulated Ca^{2+} showed higher UDPGT activity than did microsomes emptied by A23187 (Figure 2b). The UDGPT activity of Ca^{2+}-filled microsomes was independent of the extravesicular steady-state level of free Ca^{2+}. In filipin-permeabilized hepatocytes the Ca^{2+}-filled state of the ER affected the rate of glucuronidation similarly (Figure 2c). The difference observed was not so marked as in intact hepatocytes. This may be due to the inhibitory effect of ATP on UDPGT (see [18]). Actually, under the present experimental conditions ATP inhibited (by approx 40%; not shown) *p*-nitrophenol conjugation in microsomes and permeabilized cells.

Metals have been known to influence the activity of UDPGTs for several years. Most of the bivalent cations (including Ca^{2+} and Mg^{2+}, but not Zn^{2+}) have been reported to stimulate *p*-nitrophenol UDPGT activity in microsomes from guinea-pig liver [36]. Several authors suggested the application of Mg^{2+} routinely in the UDPGT assay mixture [28,37,41]. In rabbit hepatic microsomes [39] and in rat liver postnuclear supernatant [40], $MgCl_2$ (10 and 5–20 mM respectively) stimulates UDPGT activity towards *p*-nitrophenol. Other authors found no effect of 10 mM Mg^{2+} on rat liver microsomal *p*-nitrophenol UDPGT activity. On the contrary, it has been found that *p*-nitrophenol UDPGT activity of mouse [41] and rabbit [42] liver homogenates could be inhibited by 1–10 mM Mg^{2+}. Moreover, 0.5–10 mM Mg^{2+} decreased the activity of solubilized UDPGT from rabbit liver microsomes with *p*-nitrophenol as aglycone [43]. The contradictions may originate from the species differences and the different incubation conditions applied.

In our experimental system, both Ca^{2+} and Mg^{2+} stimulated the *p*-nitrophenol UDPGT activity in disrupted microsomes. The stimulatory effect of Ca^{2+} was more marked and appeared at 1 mM concentration (Figure 3). In native microsomes only Ca^{2+} increased, whereas Mg^{2+} decreased, the UDPGT activity (Figure 3, inset). These results suggest that bivalent cations may act directly on the UDPGT catalytic site; the different behaviour of the two cations in native microsomes may be related to their different capacity to enter into the lumen of microsomal vesicles. The data obtained in isolated microsomes are in accordance with

the results obtained in intact hepatocytes. In fact, the concentration of Ca^{2+} in the lumen of the ER in isolated hepatocytes is approx. 2 mM, and after the addition of thapsigargin or tBuBHQ it decreases to 0.5–0.3 mM (calculated from the data of Table 2 and ref. [22]).

The measurement of *p*-nitrophenol glucuronide content reflects the balance between its synthesis and hydrolysis by β-glucuronidase in isolated liver cells and in microsomes as well. Thus activities of both UDPGT and β-glucuronidase can theoretically be modified by Ca^{2+}. It was reported [20] that α-adrenergic agonists and A23187 inhibit *p*-nitrophenol glucuronidation in perfused rat liver, with the conclusion that this effect is mediated by the increased β-glucuronidase activity in the presence of elevated cytosolic free [Ca^{2+}]. However, the β-glucuronidase activity is low compared with that of UDPGT, even in the presence of very high concentration of *p*-nitrophenol glucuronide. According to the observation that the glucuronidation of harmol is slower in isolated hepatocytes in the absence of extracellular Ca^{2+} [47] or to our results with EGTA (i.e. conditions of low cytosolic free Ca^{2+}), these effects might be the consequence of the diminished Ca^{2+} content in the ER.

UDPGA is synthesized in the liver at the expense of glycogen. Ca^{2+}-mobilizing agents, via increased glycogenolysis, elevate the UDPGA level in the cytosol. At the same time UDPGT activity is decreased by the decrease in intrareticular Ca^{2+}. Therefore, the Ca^{2+} release from the ER has a dual function which may ensure the priority of glucose production over the glucose-consuming glucuronidation. In this respect, the fact that another intralumenal enzyme, glucose-6-phosphatase, lacks Ca^{2+}-dependency [11] has a particular importance.

This work was supported by grants from the Italian National Research Council (C.N.R. Rome) and the Italian Ministry of University and Scientific Research (Progetto 'Patologia da Radicali Liberi e dell'Equilibrio Redox').

REFERENCES

1 Carafoli, E. (1987) Annu. Rev. Biochem. **56**, 395–433
2 Sambrook, J. F. (1990) Cell **61**, 197–199
3 Lodish, H. F., Kong, N. and Wikström, L. (1992) J. Biol. Chem. **267**, 12753–12760
4 Llopis, J., Kass, G. E. N., Duddy, S. K., Farell, G. C., Gahm, A. and Orrenius, S. (1991) FEBS Lett. **280**, 84–86
5 Brostrom, C. O. and Brostrom, M. A. (1990) Annu. Rev. Physiol. **52**, 577–590
6 Booth, C. and Koch, G. E. L. (1989) Cell **59**, 729–737
7 Wilemann, T., Kane, L. P., Carson, G. R. and Terhorst, C. (1991) J. Biol. Chem. **266**, 4500–4507
8 Kalapos, M. P., Garzó, T., Bánhegyi, G., Mucha, I., Antoni, F. and Mandl, J. (1987) Acta Biochim. Biophys. Hung. **22**, 59–66
9 Czarny, M., Sabala, P., Ucieklak, A., Kaczmarek, L. and Baranska, J. (1992) Biochem. Biophys. Res. Commun. **186**, 1582–1587
10 Ghosh, T. K., Bian, J., Short, A. D., Rybak, S. L. and Gill, D. L. (1991) J. Biol. Chem. **266**, 24690–24697
11 Fulceri, R., Bellomo, G., Gamberucci, A. and Benedetti, A. (1991) Biochem. J. **275**, 805–807
12 Mulder, G. J. (1992) Annu. Rev. Pharmacol. Toxicol. **32**, 25–49
13 Zakim, D. and Dannenberg, A. J. (1992) Biochem. Pharmacol. **43**, 1385–1393
14 Jansen, P. L. M., Mulder, G. J., Burchell, B. and Bock, K. W. (1992) Hepatology **15**, 532–544
15 Reinke, L. A., Kauffman, F. C., Evans, R. K., Belinsky, S. A. and Thurman, R. G. (1979) Res. Commun. Chem. Pathol. Pharmacol. **23**, 185–193
16 Bánhegyi, G., Garzó, T., Antoni, F. and Mandl, J. (1988) Biochim. Biophys. Acta **967**, 429–435
17 Shipley, L. A., Eacho, P. I., Sweeny, D. J. and Weiner, M. (1986) Drug Metab. Dispos. **14**, 526–531
18 Bánhegyi, G., Garzó, T., Mészáros, Gy., Faragó, A., Antoni, F. and Mandl, J. (1988) Biochem. Pharmacol. **37**, 849–854
19 Bánhegyi, G., Puskás, R., Garzó, T., Antoni, F. and Mandl, J. (1992) Biochem. Pharmacol. **42**, 1299–1302
20 Belinsky, S. A., Kauffman, F. C., Sokolove, P. M., Tsukuda, T. and Tharman, R. G. (1984) J. Biol. Chem. **259**, 7705–7711
21 Beuers, U., Pogonka, T., Esterline, R., Ji, S. and Jungermann, K. (1986) Toxicol. Lett. **34**, 247–252
22 Bánhegyi, G., Fulceri, R., Bellomo, G., Romani, A., Pompella, A. and Benedetti, A. (1991) Arch. Biochem. Biophys. **287**, 320–328
23 Fulceri, R., Bellomo, G., Mirabelli, F., Gamberucci, A. and Benedetti, A. (1991) Cell Calcium **12**, 431–439
24 Henne, V. and Söling, H. D. (1986) FEBS Lett. **202**, 267–273
25 Waddell, I. D., Gibb, L. and Burchell, A. (1990) Biochem. J. **267**, 549–551
26 Jorgenson, R. A. and Nordlie, R. C. (1980) J. Biol. Chem. **255**, 5907–5915
27 Tsien, R. Y. and Rink, T. J. (1981) J. Neurosci. Methods **4**, 73–86
28 Bock, K. W., Burchell, B., Dutton, G. J., Hanninen, O., Mulder, G. J., Owens, I. S. and Tephly, T. R. (1983) Biochem. Pharmacol. **32**, 953–956
29 Lowry, O. H., Rosebrough, N. H., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. **193**, 265–275
30 Orrenius, S., Andersson, B., Jernstrom, B. and Moldeus, P. (1978) in Conjugation Reactions in Drug Biotransformation (Aitio, A., ed.), pp. 273–282, Elsevier/North Holland, Amsterdam
31 Fasolato, C., Zottini, M., Clementi, E., Zacchetti, D., Meldolesi, J. and Pozzan, T. (1991) J. Biol. Chem. **266**, 20159–20167
32 Thastrup, O. (1990) Agents Actions **29**, 8–15
33 Kass, G. E. N., Duddy, S. K., Moore, G. A. and Orrenius, S. (1989) J. Biol. Chem. **264**, 15192–15198
34 Denton, R. M. and McCormack, J. G. (1980) FEBS Lett. **119**, 1–8
35 Lukács G. L., Kapus, A. and Fonyó, A. (1988) FEBS Lett. **229**, 219–223
36 Zakim, D., Goldenberg, J. and Vessey, D. A. (1973) Biochemistry **12**, 4068–4074
37 Dutton, G. J. (1980) in Glucuronidation of Drugs and Other Compounds, pp. 47–48, CRC Press, Boca Raton, FL
38 Burchell, B. and Weatherill, P. (1981) Methods Enzymol. **77**, 169–177
39 Yost, G. S. and Finley, B. L. (1983) Biochem. Biophys. Res. Commun. **111**, 219–223
40 Mulder, G. J. (1971) Biochem. J. **125**, 9–15
41 Storey, I. D. E. (1965) Biochem. J. **95**, 209–214
42 Tomlinson, G. A. and Yaffe, S. J. (1966) Biochem. J. **99**, 507–512
43 Isselbacher, K. J., Chrabas, M. F. and Quinn, R. C. (1992) J. Biol. Chem. **237**, 3033–3036
44 Hauser, S. C., Ziurys, J. C. and Gollan, J. L. (1988) Biochim. Biophys. Acta **967**, 149–157
45 Schöllhammer, I., Poll, D. S. and Bickel, M. H. (1975) Enzyme **20**, 269–275
46 Hems, D. A., Rodrigues, L. M. and Whitton, P. D. (1978) Biochem. J. **172**, 311–317
47 Andersson, B., Jones, D. P. and Orrenius, S. (1979) Biochem. J. **184**, 709–711

Received 19 August 1992/10 December 1992; accepted 6 January 1993