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Latency is the major determinant of UDP-glucuronosyltransferase activity in isolated hepatocytes

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The glucuronidation of *p*-nitrophenol was measured in intact, saponin- and alamethicin-treated isolated mouse hepatocytes. In saponin-permeabilized cells the elevation of extrareticular UDP-glucuronic acid concentration enhanced the rate of glucuronidation threefold. When intracellular membranes were also permeabilized by alamethicin, a further tenfold increase in the glucuronidation of *p*-nitrophenol was present. Parallel measurements of the ER mannose 6-phosphatase activity revealed that saponin selectively permeabilized the plasma membrane, whereas alamethicin permeabilized both plasma membrane and ER membranes The inhibition of *p*-nitrophenol glucuronidation by dbcAMP in intact hepatocytes was still present in saponin-treated cells and disappeared in alamethicin-permeabilized hepatocytes. It is suggested that the permeability of the endoplasmic reticulum membrane is a major determinant of glucuronidation not only in microsomes but in isolated hepatocytes as well.

UDP-glucuronosyltransferase; Compartmentation; Permeabilized cell; Alamethicin, Saponin; Mouse hepatocyte

1. INTRODUCTION

Glucuronidation is the major conjugation pathway in phase II of biotransformation. This reaction is catalyzed by the membrane-bound UDP-glucuronosyltransferases (UDPGT) [1]. The highest UDPGT activity is found in the endoplasmic reticulum (ER) membranes, but a significant activity is also present in the nuclear envelope.

The activity of ER enzymes is usually measured in microsomal preparations in which the ER is fragmented into right-side-out closed vesicles [2]. One of the most interesting features of several enzymes of ER (e.g. glucose-6-phosphatase, nucleoside diphosphatase, UDPGT) [3–6] is the latency; their activities are low in native microsomes compared to microsomes treated with membrane-disrupting agents. This phenomenon can be explained either by the compartmentation or the conformation hypothesis; according to the first the restricted accessibility of the water-soluble substrates, i.e. UDP-glucuronic acid (UDPGA), synthesized in the cytosol, to the intravesicular active site of the enzymes, i.e. UDPGTs, is responsible for the low activity in intact microsomes [6]. The compartmentation model postulates that enzymes latent in microsomal fractions face (at least with their catalytic site) the lumen of ER in situ. The conformation hypothesis, on the other hand, suggests that activation removes constraints that prevent full expression of enzyme activity [7].

The microsomal fraction is an artifact, and therefore the relevance of latency and the activity status of UDPGTs in vivo remains questionable. The aim of our study was to investigate whether the permeability of the ER membrane for UDPGA determines UDPGT activity in isolated hepatocytes. As the cytosolic UDPGA level is also known to influence the UDPGT activity, we compared the rate of glucuronidation of *p*-nitrophenol in the presence of exogenously added UDPGA in hepatocytes the plasma membranes of which were permeabilized selectively by saponin, and in hepatocytes in which both plasma membrane and endoplasmic reticulum membranes were permeabilized by the pore-forming antibiotic alamethicin.

2. EXPERIMENTAL

2.1 *Materials*

UDPGA (sodium salt), β-glucuronidase (type IX), saponin, alamethicin, dibutyryl cyclic AMP, UDP-*N*-acetylglucosamine (sodium salt) and collagenase (type IV) were from Sigma, St Louis, MO, USA.

2.2. *Preparation and incubation of isolated mouse hepatocytes*

Isolated hepatocytes were prepared from male CFLP mice (25–30 g body weight) with the collagenase perfusion method as detailed earlier [8]. Viability of the cells checked by the Trypan blue exclusion test was more than 90%. Isolated hepatocytes were resuspended (10^6 cells/ml) in a modified Hanks' medium [9] containing 2.6 mM $CaCl_2$ and incubated at 37°C with constant bubbling of CO_2/O_2 (5.95, v/v). In some experiments, isolated hepatocytes were treated with 1 mM dibutyryl cyclic AMP for 5 min.

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Abbreviations. ER, endoplasmic reticulum, UDPGT(s), UDP-glucuronosyltransferase(s); UDPGA, UDP-glucuronic acid; UDPNAG, UDP-*N*-acetylglucosamine; dbcAMP, dibutyryl cyclic AMP.

2.3 *Permeabilization of the cells*

Cells were permeabilized in the incubation buffer with saponin (0 005%) or alamethicin (usually 0.02%) for 5 min. Both treatments rendered plasma membrane permeable to exogenous substrates since more than 95% of the cells became permeable to Trypan blue. Approx. 60% and 90% of the (cytosolic) lactate dehydrogenase activity was released by saponin and digitonin, respectively. In some experiments the incubation buffer was replaced by a medium reflecting the intracellular ionic environment (KCl. 100 mM, NaCl: 20 mM, $MgCl_2$: 5 mM, MOPS: 20 mM, pH 7.2).

2.4. *Measurement of enzyme activities*

Glucuronidation of *p*-nitrophenol in intact hepatocytes was measured as described previously [10] In permeabilized cells glucuronidation of *p*-nitrophenol was measured in the presence of *p*-nitrophenol (100 or 500 μM) and UDPGA (4 mM). The rate of glucuronide formation was calculated on the basis of aglycone disappearance according to Bock et al. [11]. Mannose 6-phosphatase activity was evaluated in the phosphate-free KCl-MOPS medium by measuring P_i according to Arion et al. [12]. Protein was measured according to Lowry et al. [13] using BSA as a standard

3. RESULTS

Glucuronidation of *p*-nitrophenol was measured in (i) saponin treated hepatocytes and (ii) in alamethicin permeabilized hepatocytes in the presence of exogenous UDPGA (4 mM). 0.1 mM aglycone concentration was chosen because the rate of *p*-nitrophenol glucuronidation of isolated hepatocytes is maximal at this concentration, and higher levels of *p*-nitrophenol inhibit its own conjugation [14–16].

Selective permeabilization of plasma membrane of hepatocytes by saponin enhanced the rate of *p*-nitrophenol glucuronidation threefold as compared to non-permeabilized, control cells (Table I). In saponin-permeabilized cells the addition of the UDPGT activator UDP-*N*-acetylglucosamine (UDPNAG; 1 mM) caused a further stimulation of glucuronidation. Treatment of the cells by the optimal concentration of alamethicin (0.02%) caused a tenfold activation compared to the saponin-treated hepatocytes (Table I). The magnitude of this activation was comparable with the unmasking effect of detergents on UDPGT activity in microsomal membrane [11]. In control, non-permeabilized hepatocytes exogenously added UDPGA resulted in a moderate increase of *p*-nitrophenol glucuronidation (1.33 ± 0.32 nmol/min/10^6 cells, mean ± S.D., $n = 4$) which was likely attributable to the permeation of UDPGA into the unviable cells, i.e. cells permeable for Trypan blue.

In alamethicin-permeabilized hepatocytes the high rate of *p*-nitrophenol glucuronidation indicated that alamethicin permeabilized not only the plasma membrane but the endoplasmic reticulum membranes as well. To further validate this possibility, *p*-nitrophenol UDPGT and mannose 6-phosphatase activities were detected in alamethicin-permeabilized cells. To this end isolated hepatocytes were permeabilized with various concentrations of alamethicin (0.01 to 0.5 mg/ml) and *p*-nitrophenol UDPGT and mannose 6-phosphatase activities were measured. Maximal activation was reached at 0.2 mg/ml alamethicin concentration in both cases (Fig. 1). UDPGT activity measured in the presence of 0.5 mM *p*-nitrophenol and 4 mM UDPGA in Hanks' medium or in the cytosol-like medium (see section 2) gave similar results (data not shown).

As the permeation of ER membrane seemed to be a major limit for the UDPGT activity in isolated hepatocytes, it can be supposed that glucuronidation is regulated through the modification of the permeability of ER membrane towards UDPGA. Since dibutyryl cyclic AMP was reported to inhibit the glucuronidation of *p*-nitrophenol [16,17], we have also examined whether this effect was related to a decreased UDPGA penetration through the ER membrane.

As it was observed previously [17], preincubation of intact hepatocytes with dbcAMP (1 mM) inhibited the glucuronidation of *p*-nitrophenol. The inhibitory effect

Table I

Glucuronidation of *p*-nitrophenol in intact, saponin- and alamethicin-treated isolated mouse hepatocytes

Treatment	*p*-Nitrophenol glucuronidation (nmol/min/10^6 cells)			Mannose 6-phosphatase activity (nmol/min/10^6 cells)
	Control	dbcAMP pretreated	+ UDPNAG	
None	0.94 ± 0.32	0.32 ± 0 17	N.M.	N.M.
Saponin	2.77 ± 0.68*	2.12 ± 0 55*	8.37 ± 0.11	17 ± 5
Alamethicin	26.5 ± 4.8	27.6 ± 5.2	N M	413 ± 22

Hepatocytes (10^6 cells/ml) were permeabilized in the presence of 0.005% saponin or 0.02% alamethicin for 5 min. Dibutyryl cyclic AMP (dbcAMP, 1 mM) was added to the cells 5 min prior to the addition of the permeabilizing agents. Glucuronidation was measured in the presence of 0 1 mM *p*-nitrophenol and, in the case of permeabilized cells only, 4 mM UDPGA plus or minus 1 mM UDPNAG. In intact cells *p*-nitrophenol glucuronide formation was measured at 10 min of incubation. In permeabilized cells *p*-nitrophenol disappearance was measured at 5 (saponin) or at 1 min (alamethicin) of incubation. To evaluate mannose 6-phosphatase activity cells were washed and resuspended in the phosphate-free, KCl-MOPS medium and were permeabilized as above After permeabilization, P_i formation 1 min after the addition of 1 mM mannose 6-phosphate was measured. Data are means ± S.D. of 5 (*p*-nitrophenol glucuronidation) or 3 (mannose 6-phosphatase) experiments N.M = not measured. *Statistically significant difference: $P < 0.01$

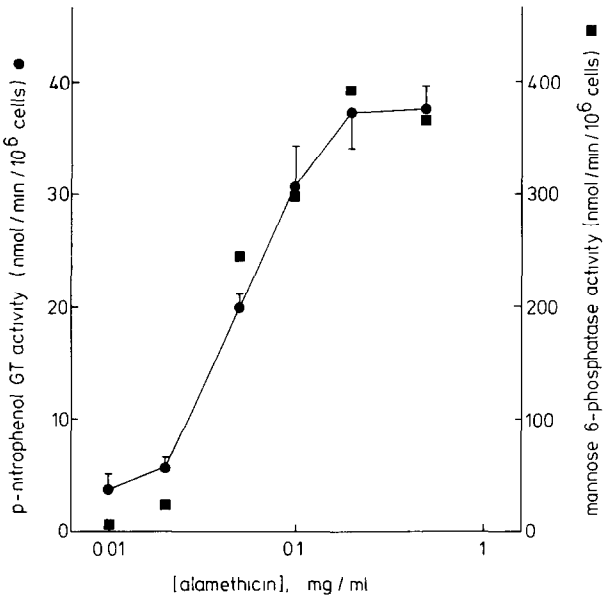

Fig. 1. Effect of alamethicin on *p*-nitrophenol UDPGT and mannose 6-phosphatase activity in isolated mouse hepatocytes. Hepatocytes (10^6 cells/ml) were incubated in the presence of the indicated concentrations of alamethicin. UDPGT and mannose 6-phosphatase activity were measured as detailed in the legend to Table I, except that UDPGT activity was evaluated at 3 min of incubation with 0.5 mM *p*-nitrophenol. Data are means ± S.D. of 4 (UDPGT) or means of 2 (mannose 6-phosphatase) experiments.

due to dbcAMP-preincubation was still present in cells subsequently permeabilized by saponin, but was no more evident in cells subsequently permeabilized by alamethicin (Table I).

4. DISCUSSION

In vivo and in intact isolated hepatocytes glucuronidation is regulated by the level of UDPGTs and the availability of UDPGA. UDPGA availability can be influenced by the synthesis of the cofactor or by its access to the active site of the enzyme. The existence of the latter possibility has been proved true in microsomal preparations but it was uncertain in intact hepatocytes.

In the present paper we have shown that selective permeabilization of the plasma membrane of hepatocytes by saponin, accompanied with the addition of UDPGA, caused about a threefold increase of glucuronidation. This stimulatory effect is probably due to the relatively higher (extrareticular) UDPGA concentration attained after plasma membrane permeabilization as 4 mM UDPGA was added to the medium; in intact hepatocytes the cytosolic UDPGA concentration was reported to be around 0.5 mM [18–20]. A similar stimulatory effect was reported in hepatocytes treated with another plasma membrane permeabilizing agent, i.e. filipin [21]. Saponin and filipin are widely used as selective plasma membrane permeabilizing agents; their effect is based on complex formation with cholesterol in the membranes of animal cells. The treatment is highly selective for the plasma membrane, because, relative to intracellular membranes, it is enriched in cholesterol. Although little permeabilization of the ER membrane cannot be excluded, we measured high latency of mannose 6-phosphatase activity (4% of the total) in saponin-permeabilized cells. An additional sensitive marker of the intactness of the ER membrane, the stimulatory effect of UDPNAG on UDPGT activity [22], was also present in saponin-treated cells. Indeed, this effect appears to be caused by the facilitation of UDPGA transport through the intact ER membrane [23].

Permeabilization of hepatocytes with alamethicin caused a tenfold enhancement of UDPGT activity compared to the saponin-treated cells. Alamethicin is a pore-forming antibiotic [24,25] and its effect is independent on the cholesterol content of the membrane. Alamethicin appeared to permeabilize both the plasma and the ER membranes, as revealed by the full activation of the intrareticular mannose 6-phosphatase. The optimal alamethicin concentration was the same, 0.2 mg/ml, in the unmasking of both mannose 6-phosphatase and *p*-nitrophenol UDPGT activity.

The inhibitory effect of dbcAMP on *p*-nitrophenol glucuronidation in intact hepatocytes [16,17] was retained following plasma membrane permeabilization by saponin, but was lost following the permeabilization of ER (and plasma membrane) by alamethicin. This indicates that the target of the inhibitor is the permeability of the ER membrane for UDPGA, perhaps an UDPGA transporter protein [26].

The use of alamethicin-permeabilized cells has some advantage over the conventional microsomal systems. First, it renders the measurement of the total cellular UDPGT activity possible while during the preparation of microsomes at least the nuclear activity is lost. The maximal activity measured in alamethicin-treated cells (approx. 36/min/10^6 cells) would correspond to an activity of the ER in situ of 125 nmol/min/mg protein microsomal activity when calculated with 1.7 mg protein/10^6 cells and 20% ER protein. This activity is much higher than that measured in detergent activated liver microsomes (approx. 40 nmol/min/mg protein) or even in alamethicin permeabilized microsomes (approx. 60 nmol/min/mg protein; R. Fulceri, G. Bánhegyi, J. Mandl and A. Benedetti, unpublished work). Secondly, the method may be suitable for the detection of glucuronidation in cells of low UDPGT activity.

The results in the present study indicate that the phenomenon of latency does exist in isolated intact hepatocytes; the permeability of the ER membrane for UDPGA is rate limiting for UDPGT activity and it is a possible regulation site in the control of glucuronidation. Based on the mechanism of permeabilization of saponin or alamethicin it is less probable that these agents would modify the sideness of ER membranes.

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