Biochemical Pharmacology, Vol. 52, pp. 1127–1131, 1996.

ISSN 0006-2952/96/$15.00 + 0.00
PII S0006-2952(96)00423-6

SHORT COMMUNICATION

Inhibition of Glucuronidation by an Acyl-CoA-Mediated Indirect Mechanism

Miklós Csala, Gábor Bánhegyi,* Tamás Kardon,* Rosella Fulceri,† Alessandra Gamberucci,† Roberta Giunti,† Angelo Benedetti† and József Mandl*‡*

*Department of Medical Chemistry, Semmelweis University of Medicine, Budapest, Hungary; †Istituto di Patologia Generale, University of Siena, Siena, Italy

ABSTRACT. The mechanism of the inhibition of glucuronidation by long-chain fatty acyl-CoAs was studied in rat liver microsomal membranes and in isolated hepatocytes. Palmitoyl- and oleoyl-CoA did not affect *p*-nitrophenol UDP-glucuronosyltransferase activity in native microsomes but were inhibitory in permeabilised vesicles. The extent of inhibition was dependent on the effectiveness of permeabilisation and was constant in time in fully permeabilised microsomes. Fatty acyl-CoAs mobilised calcium from calcium-loaded microsomes. Elevation of the intracellular acyl-CoA level by the addition of palmitate or oleate inhibited the glucuronidation of *p*-nitrophenol in isolated hepatocytes. This effect could be abolished by emptying the intracellular calcium stores. Therefore, it is concluded that fatty acyl-CoAs inhibit glucuronidation indirectly, presumably *via* calcium mobilisation. BIOCHEM PHARMACOL 52;7:1127–1131, 1996.

KEY WORDS. UDP-glucuronosyltransferase; fatty acids; fatty acyl-CoAs; calcium; endoplasmic reticulum; rat liver

Glucuronidation catalysed by different UDPGTs§ is an especially important pathway for detoxifying the reactive intermediates of biotransformation, which otherwise can be transformed into cytotoxic or carcinogenic compounds [1]. Therefore, any factor that modulates the rate of glucuronidation has an impact on long-term health. A strong positive correlation exists between dietary fat (especially polyunsaturated fatty acids) and death rates of certain types of cancer. It has been observed that long-chain fatty acids inhibit glucuronidation in perfused rat liver [2, 3]. This effect is probably mediated by long-chain acyl-CoAs, the obligatory intermediates of fatty-acid-metabolising pathways. Different pathophysiological, nutritional, and metabolic states that can be characterised by elevated acyl-CoA levels [4–6]—the addition of glucagon, starvation, oleate feeding, a high-fat diet, ethanol abuse, and diabetes mellitus—are accompanied by reduced glucuronidation. Previous data indicate that CoA derivatives of fatty acids inhibit UDPGTs noncompetitively [3] at physiological concentrations of acyl-CoAs [3, 7] in rat liver microsomal membranes. This effect is more pronounced in disrupted microsomes, indicating that intact microsomal membrane limits the accessibility of UDPGTs for fatty acyl-CoAs. It also has been suggested that, in addition to acyl-CoA binding, acyl-CoA-dependent fatty acylation may also decrease the activity of different UDPGTs [8].

The aim of the present study was to investigate the mechanism of acyl-CoA-dependent inhibition of glucuronidation, taking into account the possibility of indirect ways. Emptying of intracellular Ca^{2+} stores by various agents inhibits glucuronidation in isolated hepatocytes [9, 10]. It has also been shown that acyl-CoAs mobilise calcium from microsomal vesicles [11, 12]. Therefore, experiments were done to examine whether this mechanism was implicated in the inhibition of glucuronidation by fatty acids. Our data suggest that fatty acids inhibit glucuronidation by acyl-CoA-mediated calcium mobilisation.

MATERIALS AND METHODS

Hepatocytes were isolated from male Sprague-Dawley rats (180–220 g body weight) by collagenase perfusion [13] and incubated as reported previously [9]. Calcium-depleted cells were treated as described previously [9]. Fatty acids were added to the incubation medium [3]. Glucuronidation of *p*-nitrophenol was measured as described elsewhere [14]. Cell viability assessed by the Trypan Blue exclusion test was more than 90% in all experiments. None of the indicated treatments caused significant modification of cell viability.

Rat liver microsomes were prepared as previously reported [15]. The microsomal fractions were resuspended (ca. 80 mg of protein/mL) in a medium of the following composition (mM): KCl, 100; NaCl, 20; $MgCl_2$, 3.5;

‡ Corresponding author: József Mandl, Department of Medical Chemistry, Semmelweis University of Medicine, H-1444, Budapest, P.O.B. 260, Hungary. Tel/Fax: (36) 1266 2615.

§ *Abbreviations:* ER, endoplasmic reticulum; UDPGT, UDP-glucuronosyltransferase.

Received 20 November 1995; accepted 15 May 1996.

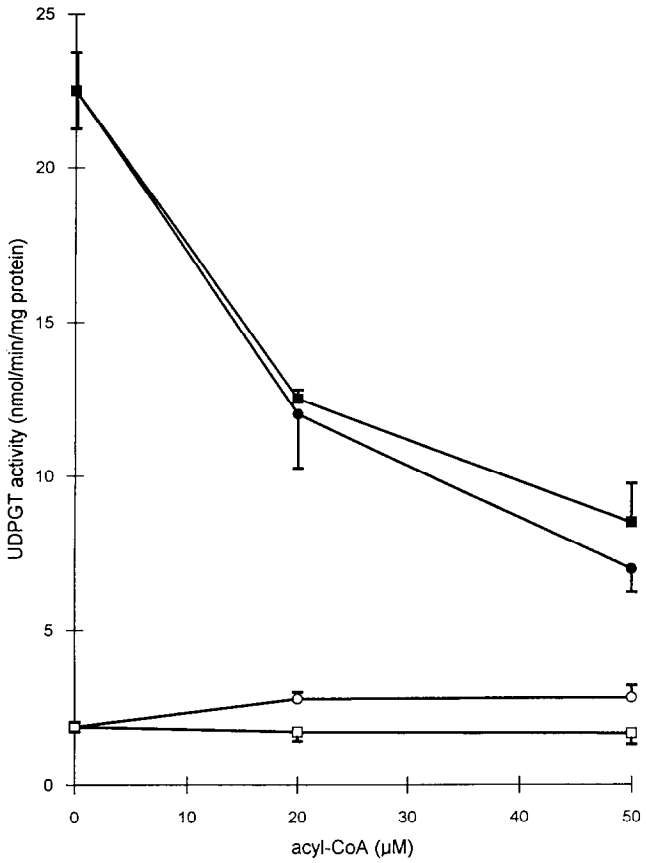

FIG. 1. Effect of fatty acyl-CoAs on *p*-nitrophenol UDPGT activity in rat liver microsomes. Rat liver microsomes (2 mg protein/mL) were incubated in the presence of 4 mM UDPGA, 0.5 mM *p*-nitrophenol, and different concentrations of palmitoyl- or oleoyl-CoA. Enzyme activities were determined after an incubation of 20 min (native vesicles: open circle, palmitoyl-CoA; open square: oleoyl-Coa) or 4 min (permeabilised vesicles: solid circle, palmitoyl-CoA; solid square, oleoyl-CoA) of incubation. Data are means ± SEM of four experiments.

MOPS, 20, pH 7.2. The suspensions were frozen and stored under liquid N_2 until use. Microsomal *p*-nitrophenol UDPGT activity was measured in the same medium [16]. Intactness of liver microsomes checked by measuring the mannose-6-phosphatase activity [17] was approximately 95% in all the preparations employed. Microsomes were permeabilised with alamethicin as reported previously [18].

For the measurement of Ca^{2+} fluxes with the Ca^{2+} indicator, Fluo 3 microsomes (1 mg of protein/mL) were incubated at 37°C in a temperature-controlled cuvette holder under continuous stirring. The incubation medium was supplemented with 3 mM ATP, 10 mM phosphocreatine, 10 mM NaN_3, 1 μM Fluo 3 (free acid), and 10 units/mL creatine kinase. The amount of Ca^{2+} present in the incubation medium (as a contaminant of solutions) ranged between 10 and 15 nmol/mL as measured by atomic absorption spectroscopy. Fluo 3 fluorescence was measured as described elsewhere [12].

Protein content was determined by the Lowry method [19] using bovine serum albumin as standard.

The chemicals ATP, phosphocreatine, creatine kinase (Type III), acyl-CoA esters, collagenase (Type IV), alamethicin, UDP-glucuronic acid, oleic acid, and palmitic acid were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Male Sprague-Dawley rats weighing 180–220 g were purchased from Nossan (Milan, Italy).

RESULTS

The effect of acyl-CoAs, the obligatory intracellular intermediates of fatty-acid-utilising pathways, was investigated in microsomal *p*-nitrophenol UDPGT activity. Palmitoyl- or oleoyl-CoA caused concentration-dependent inhibition only in disrupted microsomes permeabilised by alamethicin; in native microsomes up to 50 μM concentration, they did not affect *p*-nitrophenol UDPGT activity (Fig. 1). To investigate the relationship between permeabilisation and

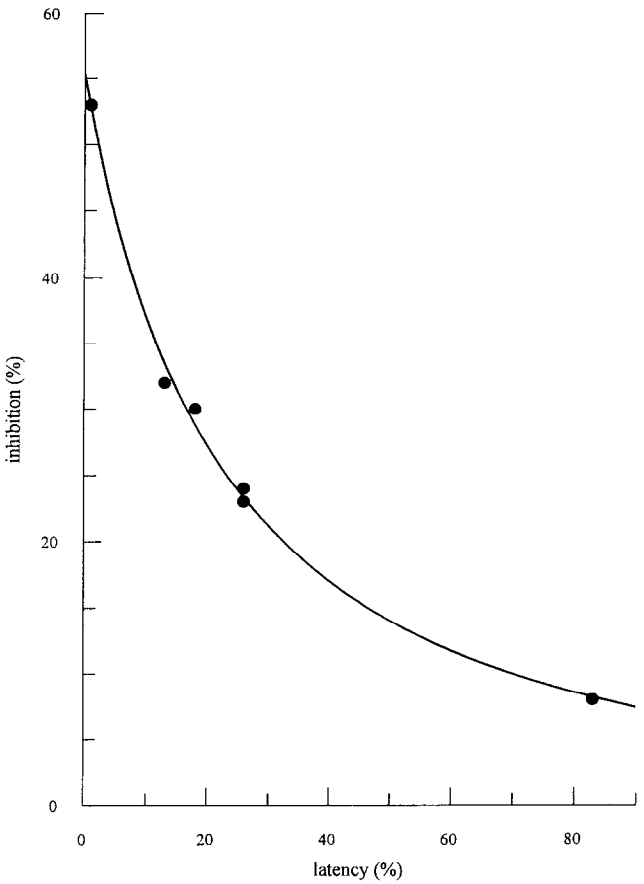

FIG. 2. Relation between the extent of permeabilisation and the inhibitory effect of palmitoyl-CoA on *p*-nitrophenol UDPGT activity in rat liver microsomes. Rat liver microsomes (2 mg protein/mL) were permeabilised by different concentrations of alamethicin (0.005–0.1 mg/mg protein) and incubated in the presence of 4 mM UDPGA and 0.5 mM *p*-nitrophenol for 4 min. The effect of 20 μM palmitoyl-CoA was investigated. UDPGT latency was expressed as maximal minus actual activity × 100 divided by maximal activity. Data are means of four experiments. SEMs were less than 10% of the means.

acyl-CoA-dependent inhibition of glucuronidation, microsomes were treated with suboptimal amounts of alamethicin. In these partially permeabilised vesicles, the inhibitory effect of 20 μM palmitoyl-CoA was dependent on the grade of permeabilisation detected on the basis of UDPGT latency (Fig. 2). In fully permeabilised vesicles, the extent of inhibition was constant in time. The rates of *p*-nitrophenol glucuronidation were 19.0 and 13.5 nmol/mg protein/min ($r = 0.9892$, $r = 0.9855$) in the absence and in the presence of 20 μM palmitoyl-CoA, respectively. Addition of 10 mM GSH or GSSG did not alter either UDPGT activity or the inhibition caused by 20 μM palmitoyl-CoA in permeabilised microsomes (data not shown).

The supposed Ca^{2+} releasing effect of acyl-CoAs was investigated in rat liver microsomes preloaded with Ca^{2+} in the presence of MgATP and an ATP-regenerating system. As was expected [12], addition of palmitoyl-CoA (50 μM) resulted in a prompt but partial release of intravesicular Ca^{2+} (Fig. 3).

The intracellular acyl-CoA level of isolated hepatocytes was elevated by the addition of palmitate or oleate. The cells were incubated in the presence of 100 μM *p*-nitrophenol. At this aglycone concentration, the rate of conjugation is maximal, and mainly glucuronides are formed [9, 14]. Addition of palmitate or oleate to the cells at 300 μM concentration, reported to inhibit glucuronidation in perfused rat liver [2], decreased the rate of *p*-nitrophenol disappearance (Table 1). The inhibition of conjugation was due to the diminished glucuronidation in both fatty acids. Depletion of intracellular Ca^{2+} stores by preincubating the cells for 30 min in the presence of excess EGTA [7] caused an approximate 80% inhibition of *p*-nitrophenol conjugation; addition of palmitate did not result in a further decrease in these hepatocytes (Table 1).

DISCUSSION

Our results indicate that permeabilisation of microsomal vesicles is a precondition for the inhibition of glucuronidation caused by acyl-CoAs (Fig. 1) and that the extent of permeabilisation determines its effectiveness (Fig. 2). Previous conflicting observations can be explained by the different intactness of microsomal membranes. Because fatty acids have been reported to be ineffective in microsomal experiments [20], we suppose that the inhibition of glucuronidation caused by fatty acids has an indirect mechanism. Fatty acids and their CoA derivatives have been reported to inhibit Ca^{2+} accumulation and stimulate Ca^{2+} efflux from endoplasmic reticulum in both isolated hepatocytes and liver microsomal vesicles [11, 12]. Considering that UDPGT activity is dependent on the presence of divalent cations [1] and that mobilisation of intrareticular calcium by ionophores, Ca^{2+} ATPase inhibitors [9], or hormones acting by Ca^{2+} mobilisation [10] inhibited the con-

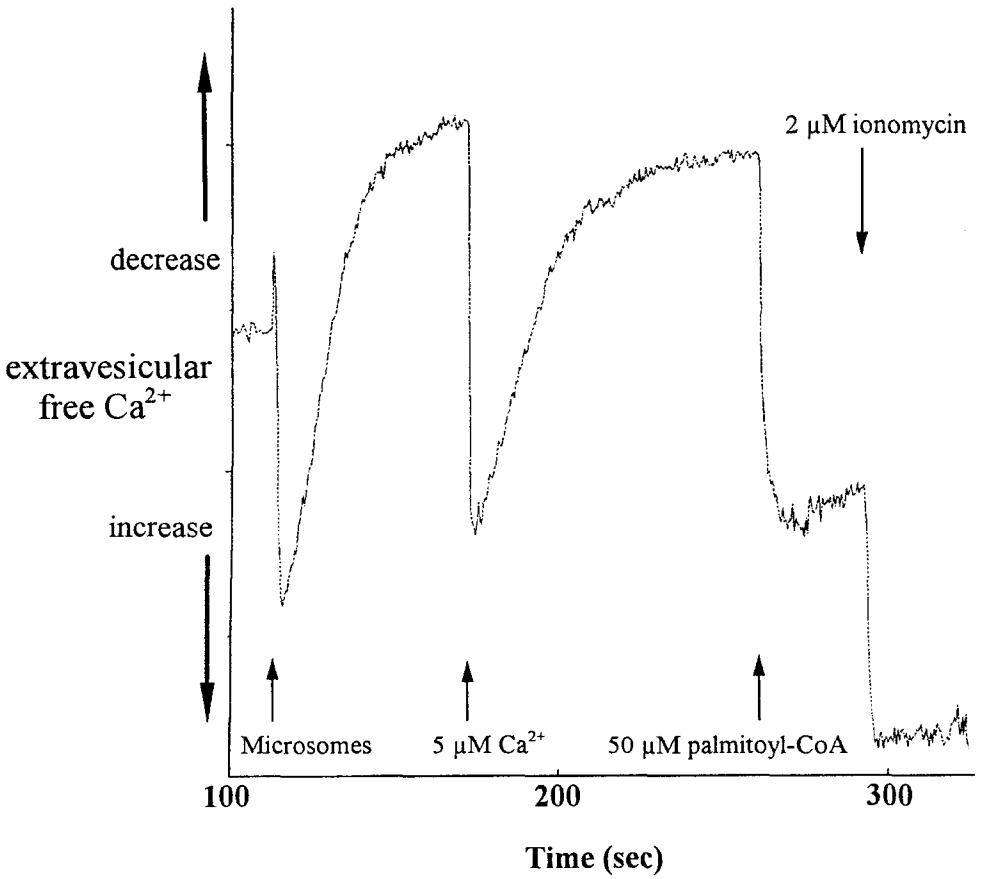

FIG. 3. Ca^{2+}-releasing effect of palmitoyl-CoA in Ca^{2+}-loaded liver microsomes. The volume of the reaction mixture was 2 mL. Ca^{2+} fluxes were evaluated by monitoring Fluo 3 fluorescence emission at 526 nm (excitation at 506 nm) as detailed in Materials and Methods. Incubation was started by the addition of 2 mg of microsomal protein. Microsomes were loaded by the addition of 5 μM Ca^{2+}. At steady-state microsomal Ca^{2+} accumulation, palmitoyl-CoA was added. One typical experiment of four.

TABLE 1. Effect of fatty acids on the conjugation of *p*-nitrophenol in isolated rat hepatocytes

	p-Nitrophenol disappearance	*p*-Nitrophenol glucuronide formation	(pmol/min/10^6 cells) *p*-Nitrophenol disappearance in Ca^{2+}-depleted cells
Control	1061 ± 34	783 ± 56	189 ± 9
300 μM Palmitate	718 ± 95*	486 ± 82*	208 ± 6
300 μM Oleate	868 ± 35*	547 ± 67*	N.M.

Cells were incubated in the presence and in the absence of fatty acids with 100 μM *p*-nitrophenol for 15 min. The disappearance of the aglycone and the formation of *p*-nitrophenol glucuronide were measured. Data are means ± SEM, n = 4, N.M., not measured.

* Different from control at $P < 0.01$.

jugation of *p*-nitrophenol in isolated hepatocytes, it seems likely that fatty acids inhibit glucuronidation *via* acyl-CoA-mediated Ca^{2+} mobilisation.

According to this assumption, palmitoyl-CoA at a physiological cytosolic concentration [12] mobilised calcium from preloaded microsomal vesicles (Fig. 3). In hepatocytes, the calcium-filled state of intracellular stores was the precondition for fatty-acid-dependent inhibition (Table 1). These findings raise the possibility of an indirect mechanism of inhibition: the intracellular metabolites of fatty acids, i.e. acyl-CoAs (and presumably fatty acids) mobilise calcium from the endoplasmic reticulum, and the decreased lumenal calcium level in turn diminishes UDPGT activity.

Our findings show that long-chain fatty acyl-CoAs do not inhibit glucuronidation in an intact microsomal system. This observation indicates that the binding of these compounds to UDPGTs and the consequent noncompetitive inhibition of the enzyme and the acyl-CoA-dependent fatty acylation [8] are unlikely to play a role in intact cells. ER membrane represents a strict barrier for hydrophilic compounds of higher molecular mass (e.g. sugar nucleotides, ATP, glucuronides). The poor penetration of palmitoyl-CoA was also observed in phospholipid monolayers [21]. The intraluminal positioning of UDPGTs [18], therefore, excludes a direct interaction between acyl-CoAs and the enzyme. Beyond the limited accessibility of acyl-CoAs to UDPGTs, the oxidative environment of the lumen of ER and the high GSSG/GSH ratio [22] make the acylation of a thiol group of UDPGTs less probable. Fatty acylation was observed in disrupted microsomes and on purified enzymes in reductive (dithiothreitol-containing) medium [8]. Moreover, the extent of inhibition was independent of the reductive or oxidative environment achieved by the addition of GSH or GSSG, respectively, and proved to be constant in time. These observations do not support an inhibition caused by a progressive fatty acylation.

In conclusion, our data suggest that long-chain fatty acids inhibit glucuronidation in the liver *via* a fatty acyl-CoA-mediated indirect way. Their Ca^{2+} mobilising effect and the calcium dependency of the intrareticular UDPGTs suggest that this mechanism may be responsible for the inhibition.

We are grateful to Gizella Ferencz for skillful technical assistance. G. Bánhegyi was a recipient of an ESF-PFT fellowship. This work was also supported by OTKA, Hungary.

References

1. Dutton GJ, *Glucuronidation of Drugs and Other Compounds*. CRC Press, Boca Raton, 1980.
2. Zhong Z, Gao W, Kauffman FC and Thurman RG, Effect of fatty acids on formation, distribution, storage, and release of benzo(a)pyrene phenols and glucuronides in the isolated perfused rat liver. *Cancer Res.* **49:** 2012–2016, 1989.
3. Zhong Z, Kauffman FC and Thurman RG, Inhibition of glucuronidation of benzo(a)pyrene phenols by long-chain fatty acids. *Cancer Res.* **51:** 4511–4515, 1991.
4. Siess EA, Brocks DG, Lattke HK and Wieland OH, Effect of glucagon on metabolite compartmentation in isolated rat liver cells during gluconeogenesis from lactate. *Biochem J* **166:** 225–235, 1977.
5. Corkey BE, Analysis of acyl-coenzyme A esters in biological samples. *Methods Enzymol* **166:** 55–70, 1988.
6. Williamson JR, Scholtz R and Browning ET, Control mechanisms of gluconeogenesis and ketogenesis. II. Interactions between fatty acid oxidation and the citric acid cycle in perfused rat liver. *J Biol Chem* **244:** 4617–4627, 1969.
7. Krcmery M and Zakim D, Effects of oleoyl-CoA on the activity and functional state of UDP-glucuronosyltransferase. *Biochem Pharmacol* **46:** 897–904, 1993.
8. Yamashita A, Watanabe M, Tonegawa T, Sugiura T and Waku K, Acyl-CoA binding and acylation of UDP-glucuronosyltransferase isoforms of rat liver: their effect on enzyme activity. *Biochem J* **312:** 301–308, 1995.
9. Bánhegyi G, Bellomo G, Fulceri R, Mandl J and Benedetti A, Intraluminal calcium of the liver endoplasmic reticulum stimulates the glucuronidation of *p*-nitrophenol. *Biochem J* **292:** 99–104, 1993.
10. Braun L, Fulceri R, Benedetti A, Mandl J and Bánhegyi G, Inhibition of *p*-nitrophenol glucuronidation by calcium mobilizing hormones. *Xenobiotica* **25:** 1073–1079, 1995.
11. Comerford JG and Dawson AP, Effects of CoA and acyl-CoAs on GTP-dependent Ca^{2+} release and vesicle fusion in rat liver microsomal vesicles. *Biochem J* **289:** 561–567, 1993.
12. Fulceri R, Gamberucci A, Bellomo G, Giunti R and Benedetti A, CoA and fatty acyl-CoA derivatives mobilize calcium from a liver reticular pool. *Biochem J* **295:** 663–669, 1993.
13. Moldeus P, Hogberg J and Orrenius S, Isolation and use of liver cells. *Methods Enzymol* **52:** 60–71, 1978.
14. Bánhegyi G, Garzó T, Antoni F and Mandl J, Glycogenolysis—and not gluconeogenesis—is the source of UDP-

glucuronic acid for glucuronidation. *Biochim Biophys Acta* **967:** 429–435, 1988.
15. Benedetti A, Fulceri R, Romani A and Comporti M, MgATP-dependent glucose 6-phosphate-stimulated Ca^{2+} accumulation in liver microsomal fractions. Effects of inositol 1,4,5-triphosphate and GTP. *J Biol Chem* **263:** 3466–3473, 1988.
16. Bock KW, Burchell B, Dutton GJ, Hanninen O, Mulder GJ, Owens IS, Siest G and Tephly TR, UDP-glucuronosyltransferase activities. Guidelines for consistent interim terminology and assay conditions. *Biochem Pharmacol* **32:** 953–956, 1983.
17. Waddell ID, Gibb L and Burchell A, Calcium activates glucose-6-phosphatase in intact rat hepatic microsomes. *Biochem J* **267:** 549–551, 1990.
18. Fulceri R, Bánhegyi G, Gamberucci A, Giunti R, Mandl J and Benedetti A, Evidence for the intraluminal positioning of *p*-nitrophenol UDP-glucuronosyltransferase activity in rat liver microsomal vesicles. *Arch Biochem Biophys* **309:** 43–46, 1994.
19. Lowry OH, Rosebrough NJ, Farr AL and Randall RJ, Protein measurement with the Folin phenol reagent. *J Biol Chem* **193:** 265–275, 1951.
20. Graham AB and Wood GC, On the activation of microsomal UDPglucuronyltransferase by phospholipase A. *Biochim Biophys Acta* **370:** 431–440, 1974.
21. Requero AM, González M, Goñi FM, Alonso A and Fidelio G, Differential penetration of fatty acyl-coenzyme A and fatty acylcarnitines into phospholipid monolayers. *FEBS Lett* **357:** 75–78, 1995.
22. Hwang C, Sinskey AJ and Lodish HF, Oxidized redox state of glutathione in the endoplasmic reticulum. *Science* **257:** 1496–1502, 1992.