Histone 2A stimulates glucose-6-phosphatase activity by permeabilization of liver microsomes

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Histone 2A increases glucose-6-phosphatase activity in liver microsomes. The effect has been attributed either to the conformational change of the enzyme, or to the permeabilization of microsomal membrane that allows the free access of substrate to the intraluminal glucose-6-phosphatase catalytic site. The aim of the present study was the critical reinvestigation of the mechanism of action of histone 2A. It has been found that the dose-effect curve of histone 2A is different from that of detergents and resembles that of the pore-forming alamethicin. Inhibitory effects of EGTA on glucose-6-phosphatase activity previously reported in histone 2A-treated microsomes have been also found in alamethicin-permeabilized vesicles. The effect of EGTA cannot therefore simply be an antagonization of the effect of histone 2A. Histone 2A stimulates the activity of another latent microsomal enzyme, UDP-glucuronosyltransferase, which has an intraluminal catalytic site. Finally, histone 2A renders microsomal vesicles permeable to non-permeant compounds. Taken together, the results demonstrate that histone 2A stimulates glucose-6-phosphatase activity by permeabilizing the microsomal membrane.

Key words: alamethicin, detergent, enzyme latency, glucose 6-phosphate transporter, UDP-glucuronosyltransferase.

INTRODUCTION

Liver glucose-6-phosphatase (G-6-Pase; EC 3.1.3.9) catalyses the terminal reaction of gluconeogenesis and glycogenolysis and plays a major role in the control of blood glucose levels [1,2]. It has been recognized since the 1950s [3] that the enzyme is intimately associated with the endoplasmic reticulum (ER) [4]. A main feature of liver G-6-Pase activity is its latency. The enzyme is more active *in vitro* in disrupted microsomal vesicles than in intact microsomes (for reviews see [1,2,5]). To explain this latency of the enzyme activity two different major hypotheses have been proposed. The earliest to be proposed was the conformational model [6–12], which postulated that the G-6-Pase enzyme changes its molecular conformation and increases its activity when the microsomal membrane structure is altered by various treatments, e.g. by detergents. An alternative hypothesis called the substrate-transport model of G-6-Pase was originally proposed more than 20 years ago [13,14]. In this model the enzyme is compartmentalized within the ER lumen, and therefore requires a substrate-transport protein to allow cytosolic glucose 6-phosphate (G-6-P) to reach the active site of the enzyme. This hypothesis predicted the existence of at least two different genes, one encoding the G-6-Pase enzyme and the other encoding an ER transporter for G-6-P. This is consistent with the existence of two different forms of type 1 glycogen storage disease (GSD) [2,15]. The first, called type 1a GSD, is characterized by the loss of enzyme activity in both intact and disrupted liver microsomes [2,3,15,16]. The second form, or type 1b GSD, is characterized by the loss of activity in intact microsomes while the activity can be revealed in the test tube upon disruption of microsomal vesicles [2,16,17]. Two different genes are mutated in type 1a and 1b GSD: a gene encoding the liver G-6-Pase enzyme protein [18] and a gene encoding a putative ER transporter for G-6-P (G-6-PT1) [19–22]. Previous work from our laboratories [23] and recent work from other laboratories [24] also demonstrate that the G-6-Pase enzyme acts on G-6-P that has entered the intraluminal space of liver microsomes, giving strong biochemical evidence for the substrate-transport model.

Nonetheless there is not yet a consensus view on the transport hypothesis. A revisited and updated conformational model has been proposed. This model, called the combined conformational flexibility substrate-transport model, views the G-6-Pase system as a multifunctional enzyme embedded deeply within the ER membrane that possesses both catalytic and substrate/product-transport activities [25]. Obviously this model does not require a G-6-P transport protein and the gene product of the G-6-PT1 gene should have a still undefined but crucial function.

An argument recently used in favour of the revisited conformational model is the assumption that activation of G-6-Pase by histone 2A (H2A) is not the result of permeabilization of microsomal membrane by H2A [25] but the result of a sort of perturbation of the enzyme protein conformation by H2A [26,27]. H2A has been used as a permeabilizing agent in microsomes in a variety of studies in our laboratory [28–30] and in other laboratories [31,32].

In the present study we put forward direct evidence that H2A treatment does permeabilize the membrane of liver microsomes, which further supports the substrate transport model of G-6-Pase.

EXPERIMENTAL

Materials

1-[1-^{14}C]Naphthol, [U-^{14}C]glucose and [U-^{14}C]G-6-P were obtained from Amersham Biosciences (Little Chalfont, Bucks.,

Abbreviations used: G-6-Pase, glucose-6-phosphatase; ER, endoplasmic reticulum; G-6-P, glucose 6-phosphate; GSD, glycogen storage disease; G-6-PT1, glucose 6-phosphate transporter; H2A, histone 2A; M-6-P, mannose 6-phosphate; UDPGT, UDP-glucuronosyltransferase.

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U.K.). Nitrocellulose blotting membrane was obtained from Schleicher and Schuell (Dassell, Germany); Hecameg was from Vegatec (Villejuif, France); and G-6-P (monosodium salt), mannose 6-phosphate (M-6-P; disodium salt), uridine 5′-diphosphoglucuronic acid triammonium salt, 1-naphthol, Lubrol PX and H2A were all obtained from Sigma Chemicals Co. (Poole, Dorset, U.K.). All other chemicals were Analar grade.

Preparation and enzyme assays of microsomal fractions

Rat liver microsomes were prepared as described previously [26]. G-6-Pase activity with G-6-P and M-6-P as substrates were measured as described previously [16] and expressed as nmol/min per mg of microsomal protein. Non-specific hydrolysis of G-6-P was assayed and corrected for as described previously [16]. Microsomal intactness was measured using mannose-6-phosphatase activity [16]. UDP-glucuronosyltransferase (UDPGT) activity towards 1-naphthol was measured at pH 7.4 with 2 mM UDP-glucuronic acid and 500 μM 1-naphthol as substrates, essentially as described in [33], and expressed as nmol/min per mg of microsomal protein. Microsomal protein content was determined by the method of Peterson [34].

Rapid filtration of microsomes

Rat liver microsomes prepared from fed rats (20 μg of protein) were incubated with increasing concentrations of H2A (0.25–10 mg) in 0.25 M sucrose/5 mM Hepes, pH 7.4, at 20 °C. This mixture was then applied to Protran nitrocellulose membrane (Schleicher and Schuell; 0.45 μm) on a Bio-Rad dot-blot apparatus under a gentle vacuum. When all the solution was pulled through, 50 μl of 0.2 mM [^{14}C]G-6-P (5 mCi/ml, 0.185 MBq/ml) or 50 μl of 0.2 mM [^{14}C]glucose (5 mCi/ml, 0.185 MBq/ml) was added. When this was pulled through the membrane was dried and the radioactivity retained on the filter acquired on a GS250 Molecular Imager (Bio-Rad).

Transport measurements by the light-scattering technique

Osmotically induced changes in microsomal vesicle size and shape [35] were monitored at 400 nm at right angles to the incoming light beam, using a fluorimeter (Hitachi F-4500) equipped with a temperature-controlled cuvette holder (37 °C) and magnetic stirrer. Liver microsomal vesicles (100 μg of protein/ml) were equilibrated for 1 h in a hypotonic medium (5 mM K-Pipes, pH 7.0). The osmotically induced changes in light scattering were measured after the addition of a small volume ($< 5\%$) of the total incubation volume of concentrated and neutralized solutions of the compounds to be tested, as described in detail elsewhere [36,37].

RESULTS AND DISCUSSION

G-6-Pase activity in microsomal vesicles is latent. It can be activated by preincubation prior to assay with a wide variety of detergents that for many years have been thought to disrupt the structure of microsomal vesicles. Recently such increases in enzyme activity have been explained according to the 'revisited conformational model' by a specific change in the conformation of the G-6-Pase enzyme, which then allows direct access of G-6-P substrate from outside the vesicles [11,12,25,26]. One of the major arguments recently used in favour of the revisited conformational model is that H2A increases G-6-Pase activity (and hence removes latency) without disrupting microsomal vesicles [25]. We have therefore compared the effects of H2A, a variety of detergents and the pore-forming antibiotic alamethicin on G-6-Pase and other activities requiring vesicular intactness in liver microsomal vesicles.

Effects of H2A on G-6-Pase activity

All the compounds tested were able to activate G-6-Pase activity to similar extents (Figure 1). Figures 1(A)–1(C) show the effects of non-ionic detergents (Lubrol and Hecameg) and an ionic detergent (deoxycholate). Low concentrations of all three types of detergent disrupt microsomal vesicles and activate G-6-Pase activity. Unfortunately, most detergents are also potent non-competitive inhibitors of G-6-Pase activity [38] and they also make the activity of the enzyme unstable at temperatures commonly used in assays [6,39–41] and hence at higher detergent concentrations the activity of the enzyme falls (Figures 1A–1C). It can therefore be difficult to fully permeabilize the membrane with detergents without any inhibition of G-6-Pase activity. H2A and the pore-forming antibiotic alamethicin differ from detergents in that increasing concentrations of the compounds do not result in a loss of G-6-Pase activity (Figures 1D and 1E). This indicates that alamethicin and H2A may interact with membranes in a different way to detergents. Pore-forming antibiotics form pores in membranes. In contrast, detergents perturb both membrane structure and membrane protein conformation; indeed, at high concentrations detergents can completely solubilize membrane proteins. It is logical that H2A may be pore forming in microsomal vesicles because it has previously been shown that basic proteins are pore forming in other membranes (e.g. [42,43]).

Effect of H2A on UDPGT activity

UDPGTs are ER membrane proteins that have their active site inside the ER lumen [44,45]. Their activity is latent and increases on membrane disruption. It is very difficult to envisage how a direct effect of H2A on the conformation of the G-6-Pase enzyme could alter UDPGT activity. In contrast, if H2A makes the membrane permeable to small molecules then it would be expected to increase UDPGT activity. We therefore compared the effects of the non-ionic detergent Lubrol and H2A on UDPGT activity. Figure 2 shows that Lubrol and H2A both activate UDPGT to a similar extent (although again detergent is inhibitory at high concentrations). H2A, similarly to alamethicin [46], does not inhibit the enzyme, even at high concentrations. This demonstrates that H2A has a general effect on microsomal membrane permeability rather than interacting directly with the enzyme. UDPGTs are integral membrane proteins but they have only one transmembrane section and the vast majority of the protein is inside the lumen of the ER [44,45]. It is therefore not possible for the addition of H2A to the outside of the vesicles to cause a conformational shift in UDPGT that moves the protein through the membrane to the outside of the vesicle, as in the revisited conformational model for G-6-Pase.

Effect of H2A on microsomal permeability

Traditionally, uptake of small molecules into microsomal vesicles has been measured by using radioactive compounds in combination with a variety of rapid filtration methods [26,36,47]. We therefore attempted to apply rapid filtration methods using ^{14}C-labelled G-6-P as a substrate in the presence and absence of H2A. The binding of high levels of protein to the filters in the presence of H2A slowed the rapid filtration. In addition, routine controls demonstrated that the presence of H2A caused a high

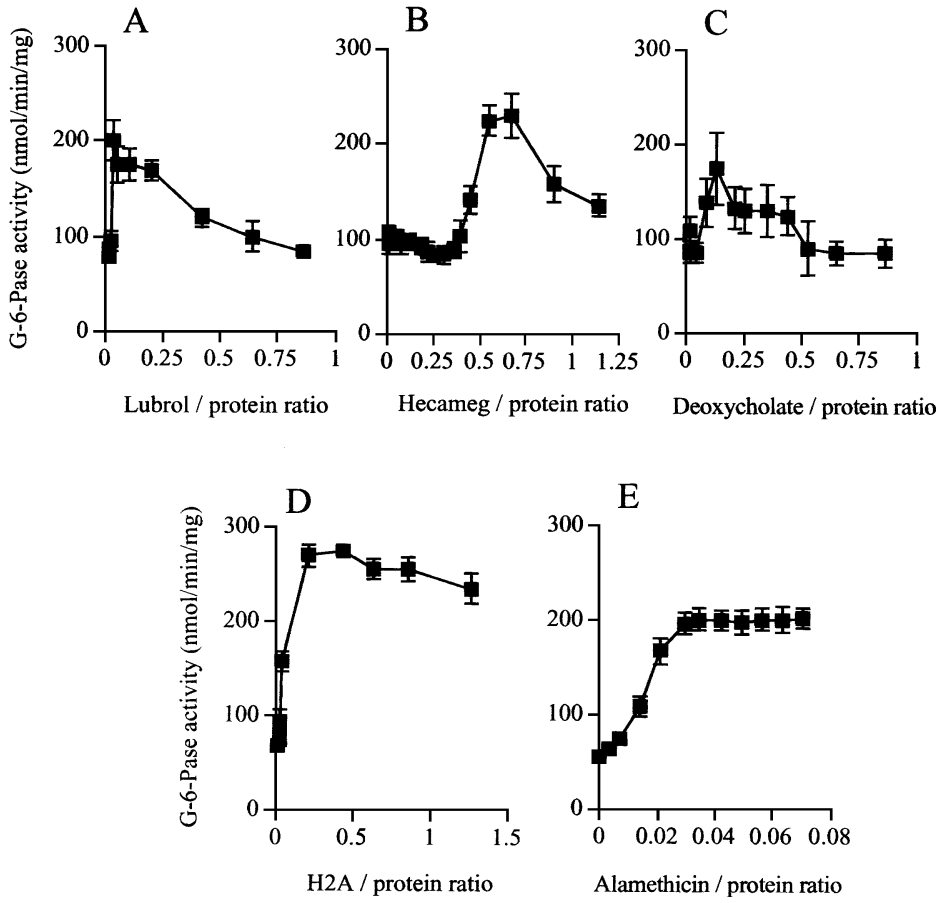

Figure 1 Effect of treatment with Lubrol (A), Hecameg (B), deoxycholate (C), H2A (D) and alamethicin (E) on G-6-Pase activity in rat liver microsomes

Microsomes were preincubated for 20 min on ice with the indicated compounds at different ratios (w/w) with microsomal protein prior to assay of G-6-Pase with G-6-P as the substrate, as described in the Experimental section. Data are means ± S.E.M. from three separate experiments.

background level of radioactivity on the filters; this occurred when the ^{14}C-labelled substrate was either glucose or G-6-P. The controls were done using both ^{14}C-labelled glucose and G-6-P because most of the radioactivity measured in filtration-based microsomal transport assays where [^{14}C]G-6-P has been used as substrate is not [^{14}C]G-6-P but [^{14}C]glucose, as the G-6-P is rapidly hydrolysed by G-6-Pase during the assay (e.g. see [23]). The increased background combined with the slowing of the rate of filtration in the presence of H2A make it very difficult to interpret the results of rapid filtration assays carried out in the presence of H2A. These problems may also have contributed to the previously reported greater accumulation in rapid filtration assays of ^{14}C from [^{14}C]G-6-P in microsomes treated with H2A [26].

We therefore decided to use an alternative method that does not involve filtration to study the effect of H2A on microsomal permeability. The effect of H2A on permeability of liver microsomal vesicles was assessed by a light-scattering technique [35–37]. Compounds not entering the microsomal lumen, e.g. sucrose (Figure 3A) or M-6-P (Figure 3G), are known to cause a sustained shrinking of microsomal vesicles as revealed by the increase in light scattering [36,37]. Permeant compounds like KCl (Figure 3C) or G-6-P (Figure 3E) cause a transient shrinking followed by a swelling phase (decrease in light scattering), whose rate reflects the rate of entry of these compounds into the vesicles [36,37]. The pre-treatment of microsomes with H2A [at an H2A/microsomal protein ratio of 1 (w/w; see Figure 1)] resulted in the loss of the rapid shrinking phase upon the addition of each of the investigated osmolites (Figures 3B, 3D, 3F and 3H), which indicated a very high permeability to all compounds of the microsomal vesicles. In the case of H2A-treated microsomes, M-6-P addition resulted in a slow increase in light-scattering intensity. However, this effect is likely to be due to an interaction between M-6-P and H2A, since very similar increases in light-scattering intensity were also observed in control samples where microsomes were omitted from the medium (Figure 3H, lower trace). The nature of this interaction was not investigated as it was out of the scope of this paper. The net changes in light scattering caused by M-6-P addition to H2A-treated microsomes (Figure 3H, trace 3; calculated by graphically subtracting trace 1 from trace 2) had a profile very similar to that observed with sucrose, KCl and G-6-P (Figures 3B, 3D and 3F, respectively). The H2A treatment does not substantially affect the basal values of light-scattering intensity of the microsomal suspension (compare Figures 3A, 3C, 3E and 3G with Figures 3B, 3D, 3F and 3H, respectively), indicating the maintenance of the vesicular structure in H2A-treated microsomes. Permeabilization by alamethicin of microsomes pre-treated with H2A resulted in small decreases of the signal as compared with the control microsomes. Solubilization of both H2A-treated and control microsomes

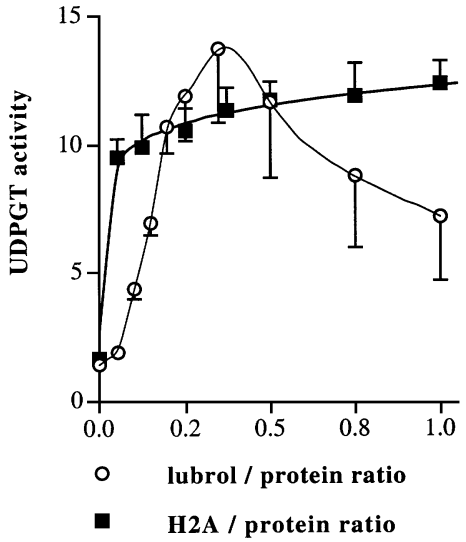

Figure 2 Effect of treatment with Lubrol and H2A on UDPGT activity in rat liver microsomes

Microsomes were preincubated for 20 min on ice with either Lubrol (○) or H2A (■) at various ratios (w/w) with microsomal protein. UDPGT activity was then assayed using 1-naphthol as the substrate as described in the Experimental section. Data are means ± S.E.M. from three separate experiments.

(upon addition of Triton X-100) resulted in similar large decreases in light scattering, indicating the destruction of the vesicular structure in all cases.

Effect of EGTA on G-6-P activity in alamethicin- or H2A-treated microsomes

One of the major arguments used in favour of the revisited conformational model is that H2A increases G-6-Pase activity (and hence removes latency) without disrupting microsomal vesicles. This idea was apparently supported by the observation that EGTA had little effect on G-6-Pase activity in intact microsomes but it inhibited or decreased G-6-Pase activity in H2A-treated microsomes (see Figure 3 in [26]). The level of G-6-Pase activity in the presence of H2A and EGTA was found to be very similar to the activity in untreated microsomes (see Figure 3 in [26]). This was interpreted as a demonstration that the H2A effect could not be due to permeabilization of the vesicles as it was reversible. We therefore compared the effect of EGTA on G-6-Pase activity in the presence and absence of either the pore-forming antibiotic alamethicin or H2A. We obtained very similar results with H2A (Figure 4A) to those reported previously by others [26]. We also obtained very similar results with alamethicin to those with H2A (Figure 4A). EGTA had little effect on G-6-Pase activity of intact vesicles but inhibited (or decreased) G-6-Pase activity of alamethicin- or H2A-treated microsomes (Figure 4A). In the presence of higher concentrations of EGTA, the level of G-6-Pase activity of alamethicin- or H2A-treated microsomes was very similar to that of control microsomes. Clearly alamethicin creates pores in membranes and H2A appears to act in a very similar manner. A simple explanation for the results in Figure 4(A) of this study and in Figure 3 of [26] is that EGTA inhibits G-6-Pase activity in disrupted but not intact microsomes. If that is correct then untreated microsomes should not be very permeable to EGTA whereas H2A- or alamethicin-treated microsomes should be very permeable to EGTA.

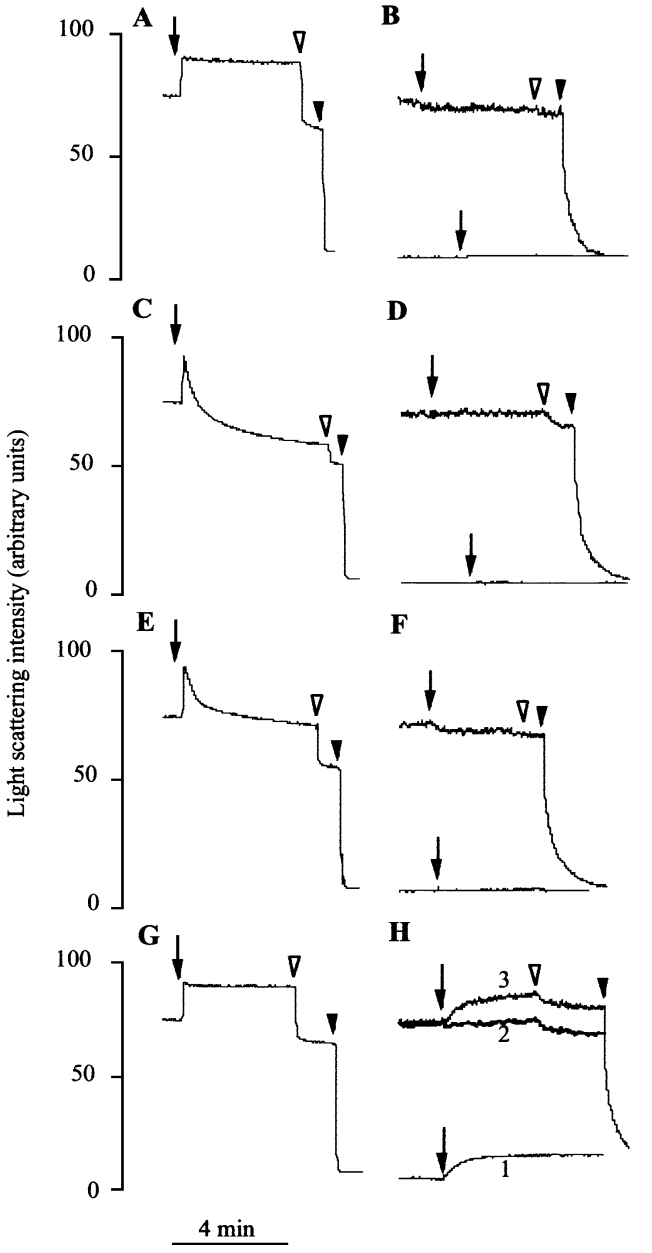

Figure 3 Influx of sucrose, KCl, G-6-P and M-6-P in rat liver microsomal vesicles treated or not with H2A monitored by light scattering

Rat liver microsomes (70 μg/ml of protein) were pre-treated (10 min on ice) with (**B**, **D**, **F** and **H**) or without (**A**, **C**, **E** and **G**) H2A (70 μg/ml) and equilibrated in a low-osmolarity buffer (5 mM K-Pipes, pH 7) until a stable light-scattering baseline was obtained. Concentrated solutions (1 M in K-Pipes buffer, pH 7) of sucrose (**A**, **B**), KCl (**C**, **D**), G-6-P (**E**, **F**) and M-6-P (**G**, **H**) were added (black arrows) to 2.0 ml of microsomal suspension, giving a 50 mM final concentration for each compound. Alamethicin (10 μg per ml; white arrowheads) was then added to permeabilize microsomal vesicles, and subsequently microsomes were solubilized by adding (black arrowheads) 0.1 ml of 1% Triton X-100 to the reaction mixture. The lower traces in the right-hand panels represent controls in which no microsomes were present. Representative traces are shown from 5–8 similar experiments.

The permeability of microsomes to EGTA

The impermeability of microsomes to EDTA has been studied previously [24,27,48,49]. Here we used light scattering to monitor the influx of EGTA into liver microsomal vesicles (Figure 4B). EGTA caused a sustained shrinking of untreated microsomal

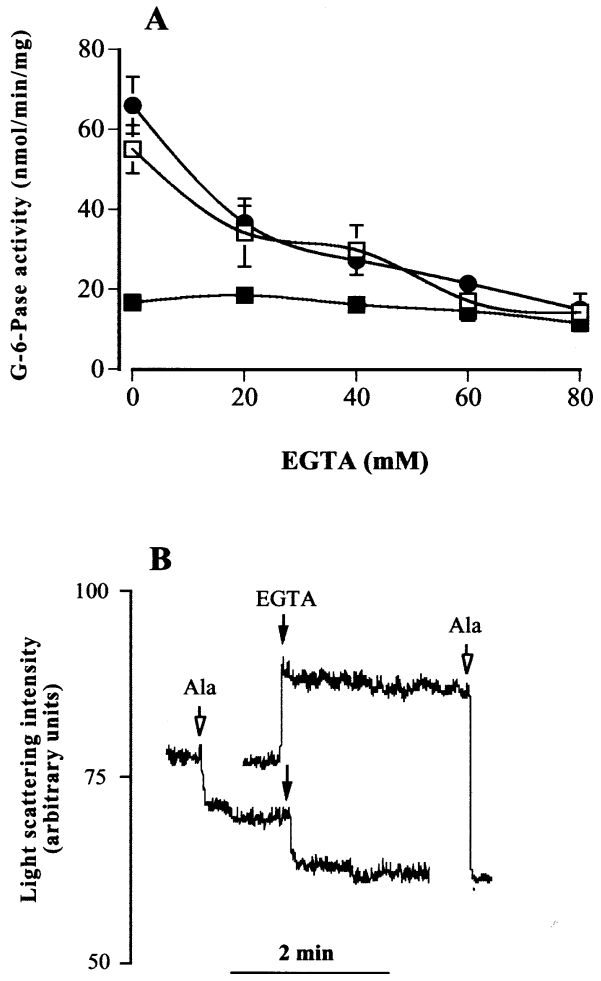

Figure 4 Effect of EGTA on G-6-Pase activity in alamethicin- or H2A-treated microsomes (A) and permeability towards EGTA of liver microsomal vesicles (B)

(**A**) G-6-Pase activity (nmol/min per mg) was measured in the presence of increasing concentrations of EGTA with 0.2 mM G-6-P as the substrate in untreated (■), H2A-treated (●; 1 mg/mg of microsomal protein) and alamethicin-treated (□; 0.1 mg/mg of microsomal protein) microsomes. (**B**) The influx of EGTA into liver microsomal vesicles was monitored by light scattering as described in the legend to Figure 3; alamethicin (Ala, 10 μg/ml; white arrows) was added to permeabilize microsomal vesicles prior to or after the addition of a concentrated solution (0.5 M in K-Pipes buffer, pH 7) of EGTA to 2.0 ml of the microsomal suspensions (black arrows) to a final concentration of 50 mM.

vesicles (Figure 4B). In addition, alamethicin-pre-treated microsomes did not have a rapid shrinking phase upon the addition of EGTA, which indicated a very high permeability to EGTA of the treated microsomal vesicles (Figure 4B). These results confirm that untreated microsomes are not very permeable to EGTA whereas alamethicin-treated microsomes are very permeable to EGTA. The simplest explanation for the results shown in Figure 4(A) of this study and in Figure 3 of [26] is that EGTA inhibits G-6-Pase enzyme activity and that it does so by interaction/inhibition at the enzyme's active site, which is on the luminal side of the microsomal membrane, when it is able to cross the microsomal membrane (i.e. in the presence of H2A or alamethicin). In contrast, in intact vesicles no inhibition is seen as EGTA cannot cross the intact membrane and hence cannot interact with the luminal active site.

Concluding remarks

The results presented here clearly demonstrate that H2A (i) has an effect on the microsomal membrane that is comparable with that of the pore-forming alamethicin, (ii) activates not only G-6-Pase but also UDPGT, another microsomal enzyme with an intraluminal active site, and (iii) promotes the permeation of various small molecules through the microsomal membrane. Consequently, the action of H2A on G-6-Pase activity is due to the permeabilization of microsomes. The action of H2A is therefore not compatible with the revisited conformational model of the G-6-Pase system.

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