Biochimica et Biophysica Acta 862 (1986) 199–204
Elsevier

BBA 72956

A differential effect between the acute and chronic administration of ethanol on the endocytotic rate constant, k_e, for the internalisation of asialoglycoproteins by hepatocytes

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(Received 24 March 1986)
(Revised manuscript received 11 July 1986)

Key words: Endocytosis; Asialoglycoprotein; Ethanol

The endocytotic rate constant, k_e, originally described for the quantification of epidermal growth factor by fibroblasts (Wiley, H.S. and Cunningham, D.D. (1982) J. Biol. Chem. 257, 4222–4229) has been adapted to measure receptor-mediated endocytosis of asialoglycoproteins by hepatocytes. A k_e value of 0.21 min^{-1} was obtained for the internalisation of β-D-galactosyl bovine serum albumin by freshly isolated hepatocytes. The addition of ethanol to the incubation medium had a biphasic effect on k_e. The value of k_e was increased by up to 30% by low concentrations of ethanol, whereas higher concentrations progressively decreased k_e and in 500 mM ethanol the k_e value was 0.1 min^{-1}. The amount of ligand bound to the cell surface was independent of the extracellular concentration of ethanol and the changes in k_e were exclusively due to changes in the amount of internalised ligand. There was a progressive decrease in the value of k_e in hepatocytes prepared from rats that were maintained on an ethanol-impregnated liquid diet for up to 20 days. The decrease was already apparent by day 2 when blood alcohol levels were only 50 mg%, indicating that the effects of chronic alcoholism on endocytosis are manifested at an early stage.

Introduction

Apparently contradictory reports on the effects of ethanol on the biological activity of the same tissue or cell type may often be predetermined by the choice of an in vitro or in vivo experimental protocol and also by the mode of ethanol administration. For example, the liver, brain and small intestine appear to be more susceptible to the acute effects of ethanol, which can be administered either intragastrically to the intact animal or as a supplement in vitro to perfusion or culture medium, compared to those induced by the long-term chronic ingestion of ethanol [1–3]. Further variability is often introduced into chronic studies by the administration of ethanol in drinking water instead of as an isocaloric supplement to a liquid diet. This report compares the effect of extracellular ethanol on the endocytosis of asialoglycoproteins by isolated hepatocytes prepared from normal, chow-fed rats with the endocytotic activity of hepatocytes prepared from animals that were maintained for up to 20 days on an ethanol-impregnated liquid diet.

We have chosen to quantify endocytosis of the asialoglycoprotein β-D-galactosyl bovine serum albumin, D-Gal-BSA, by measuring k_e, the endocytotic rate constant, which was originally derived to quantify the endocytosis of epidermal growth factor by human fibroblasts [4]. k_e defines the prob-

Abbreviation: BSA, bovine serum albumin.

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ability of an occupied receptor being internalised in 1 min at 37°C. For example, a k_e value of 0.5 min^{-1} would indicate that there is approximately a 50% chance of an occupied receptor being internalised in 1 min. In preliminary experiments we established that the measurement of k_e could be successfully adapted from cells in culture to suspensions of freshly isolated hepatocytes prepared by collagenase perfusion of the liver [5]. This required that three conditions were satisfied:
(i) the successful discrimination of surface bound and internalised ligand;
(ii) that there was no degradation of ligand during the period of measurement; and
(iii) that the amount of ligand bound to the cell surface remained constant during the course of the experiment.

Materials and Methods

Condition (i) was adequately met by exploiting the absolute requirement of the asialoglycoprotein receptor for Ca^{2+} for high-affinity ligand binding [6]. Cells were stripped of 90–95% of surface-bound ligand by the addition of 20 mM EDTA. Thus, the difference in radioactivity between cells that had either been stripped (equivalent to internalised ligand) or not stripped (equivalent to surface plus internalised ligand) before pelleting through a cushion of silicon oil represented the amount of surface-bound ligand (see legend to Fig. 1). Fluid phase pinocytosis of extracellular ligand was not significant, since less than 0.2% of extracellular non-endocytosed ^{125}I-labelled PVP (Amersham International, Amersham, U.K.) or ^{125}I-labelled bovine serum albumin was entrapped in the cell pellet after 20 min incubation with the isolated hepatocytes. The experimental period for the measurement of k_e was only 10 min. During this time, degradation of ^{125}I-labelled D-Gal-BSA, which would have been detected as acid-soluble product, was minimal. This was in agreement with other groups who have shown that less than 5% of endocytosed ligand is detected as acid-soluble material up to 20 min after internalisation [7–9]. Condition (ii) was therefore satisfied. The third requirement was demonstrated by a linear increase in the amount of ^{125}I-labelled D-Gal-BSA internalised over the 10 min period of measurement, whilst the amount of surface bound ligand remained constant (Fig. 1a, b). Thus there was no difference in the In/Sur ratio, from which k_e was derived, for the endocytosis of D-Gal-BSA as the extracellular concentration of ligand was increased from $0.25 \cdot 10^{-9}$ to $30 \cdot 10^{-9}$ M (Fig. 1c, d). Under these conditions, a k_e value of 0.21 ± 0.01 min^{-1} (mean ± S.E.) was estimated for receptor-mediated endocytosis of D-Gal-BSA by isolated hepatocytes. The non-dependence of k_e on the concentration of extracellular ligand confirmed, therefore, that the asialoglycoprotein receptor was continuously recycling to the cell surface [6].

The endocytotic activity of hepatocytes after the acute or chronic administration of ethanol was quantitated by measuring k_e with either normal cells that had been pre-incubated in medium supplemented with ethanol or by using cells that were prepared at 2 to 4 day intervals from rats which were pair-fed for up to 20 days with liquid diets containing 36% of total calories either as ethanol or sucrose [10]. When appropriate, blood ethanol concentrations from animals maintained on the ethanol-impregnated diet were measured using a commercially available diagnostic kit (TDx System, Abbot Diagnostics, Basingstoke, Hants., RH22 4EH, U.K.). At the completion of each study, 0.5 ml aliquots of cell suspension were mixed rapidly on ice with 0.15 ml 1.6 M $HClO_4$ for measurement of the total cellular content of ATP. ATP was quantified in neutralised extracts by HPLC with a Hypersil-APS weak anion-exchange column (HPLC Technology, Macclesfield, Cheshire, U.K.) using Waters pumps and system controller (Waters Associates, Harrow, U.K.). A linear gradient from 22 mM KH_2PO_4 (pH 2.8) to 700 mM KH_2PO_4 (pH 2.6) (Aristar Grade, BDH, Poole, Dorset, U.K.) was used to resolve the nucleotides.

Results and Discussion

Pre-incubation of normal hepatocytes for 20 min with increasing concentrations of ethanol prior to measurement of k_e elicited a biphasic response on the endocytotic activity of the cells (Fig. 2a). Extracellular concentrations of less than 100 mM ethanol increased k_e by up to 30% of that measured in normal cells. Thereafter, k_e progressively

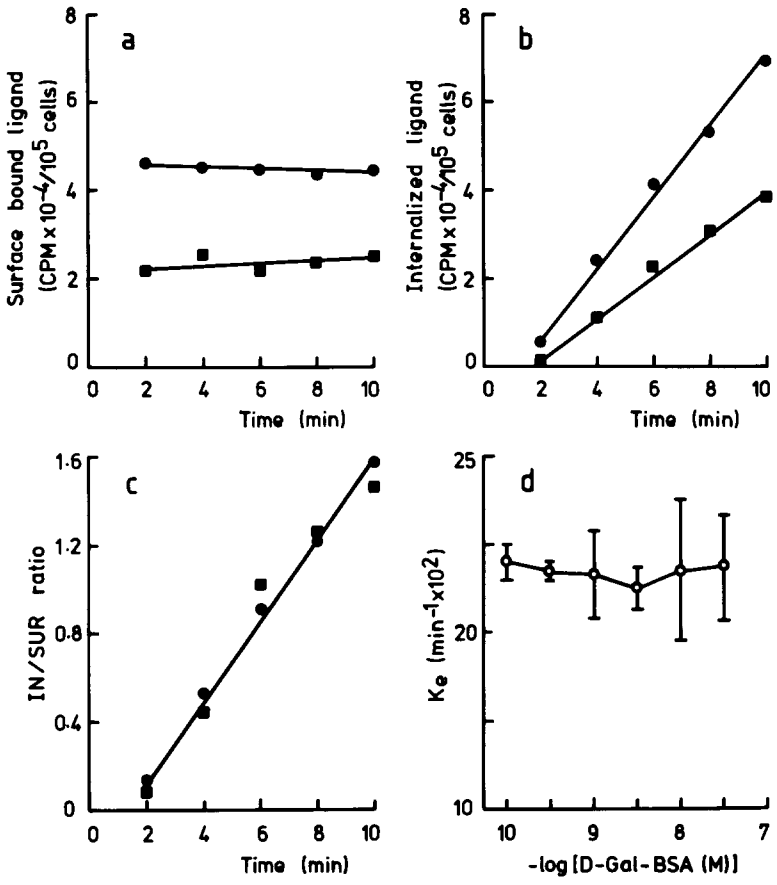

Fig. 1. Determination of the endocytotic rate constant, k_e, for the internalisation of D-Gal-BSA by isolated hepatocytes. Isolated hepatocytes were prepared from male Wistar rats as described [5] and suspended at a concentration of $3.9 \cdot 10^6$ cells/ml in Earle's basal salt solution, supplemented with 5% (v/v) heat-inactivated foetal calf serum, gassed with 95% O_2/5% CO_2, and pre-incubated at 37°C for 45 min to ensure complete re-expression of the cell surface asialoglycoprotein receptor and then placed on ice prior to the estimation of k_e. Viability was assessed both by exclusion of 0.2% Trypan blue and by release of lactate dehydrogenase into the culture medium. In all cases, cell viability was estimated to be more than 90% for the duration of the experiments. 2 ml aliquots of hepatocytes were transferred to wide-necked 20 ml plastic vials (Beckman, High Wycombe, Bucks., U.K.) containing 0.02 μCi of ^{125}I-labelled D-Gal-BSA [13] and the final extracellular concentration of D-Gal-BSA was in the range 0.25–30 nM. Two 0.1 ml aliquots were taken from each vial at 2 min intervals for 10 min to an equal volume of ice-cold medium in the presence or absence of 40 mM NaEDTA. The samples were mixed thoroughly and left on ice for no longer than 10 min before centrifuging 0.1 ml of the cell suspension through 0.5 ml silicon oil (specific gravity 1.04). The amount of ligand internalised and the total amount bound by the hepatocytes was calculated from the radioactivity in the cells that had been treated with or without NaEDTA, respectively; the difference in these values represented the surface-bound ligand. Non-specific binding was estimated from duplicate samples containing an approximate 400-fold molar excess of the appropriate ligand. The surface spillover value [4] was estimated by incubating 2 pmol of ^{125}I-labelled D-Gal-BSA (1 Ci/μmol) with 2 ml of hepatocytes on ice for 60 min to allow binding to reach equilibrium. Triplicate 0.1 ml samples were treated with or without NaEDTA as described above and the spillover value was estimated as the percentage of radioactivity that could not be removed by the chelating agent. Internal spillover was estimated by incubating 2 pmol of ^{125}I-labelled D-Gal-BSA (1 Ci/μmol) with 2 ml of hepatocytes at 37°C for 90 min. The cells were washed three times and resuspended in 2 ml of medium; 0.1 ml aliquots were treated in triplicate as described above and the spillover was estimated as the percentage of internalised radioactivity released by NaEDTA. k_e determinations were made by computational analysis using a programme kindly provided by Dr. Stephen Wiley [4]. The amount of (a) surface-bound and (b) internalised ligand was measured at extracellular concentrations of $0.25 \cdot 10^{-9}$ M (■) and $1.25 \cdot 10^{-9}$ M D-Gal-BSA (●) from which (c) the In/Sur ratio was derived to yield a k_e value of 0.21 min^{-1}. (d) This value remained unaltered as the ligand concentration was increased from $0.25 \cdot 10^{-9}$ M to $30 \cdot 10^{-9}$ M. Every point represents the mean of at least four separate determinations.

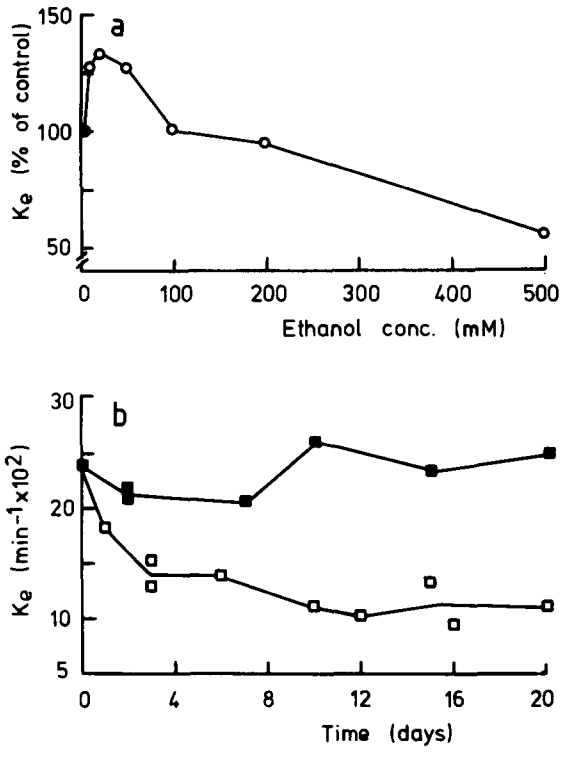

Fig. 2. The effect of acute and chronic ethanol administration on k_e for the endocytosis of D-Gal-BSA by isolated hepatocytes. (a) k_e was estimated in normal hepatocytes after 20 min pre-incubation with 5 to 500 mM extracellular ethanol. Each point represents the mean of four determinations. (b) k_e values over a 20-day period for rats pair-fed liquid diets containing either 5% (w/v) ethanol (□), or iso-caloric sucrose (■). Each point represents an individual animal.

declined as the ethanol concentration was increased and pre-incubation in 500 mM ethanol reduced k_e to 0.1 min^{-1} which was half the normal value. The increase in k_e in the presence of relatively low extracellular concentrations of ethanol was due entirely to an increase in the amount of internalised ligand, since the amount of surface bound ligand remained unaltered from that measured in ethanol-free medium. Similarly, the decrease in k_e in the presence of extracellular concentrations of ethanol in excess of 100 mM was due to a reduction in the amount of internalised ligand rather than the amount of surface bound ligand. A non-specific change to k_e as a result of altered fluid-phase pinocytotic activity of the cells was discounted, since the amount of entrapped inert, non-endocytosed ligand in the cell pellets remained at no more than 0.2% in the presence of all extracellular ethanol concentrations tested. The biphasic effect of ethanol on the endocytotic activity of hepatocytes appeared to be a property of the intact cell since similar concentrations of ethanol had no effect on the binding at 37°C of ^{125}I-labelled-D-Gal-BSA to the asialoglycoprotein receptor associated with either freshly prepared sinusoidal plasma membrane or smooth microsomes [11,12] (Table I). The apparent equilibrium binding constant, K_D, and the apparent maximum number of binding sites, B_{max}, for these two membranes were identical to those reported previously for ligand binding in the absence of ethanol [13].

The internalisation of asialoglycoproteins by hepatocytes is an energy-dependent process and the transit of ligands through specialised vesicles requires metabolic energy, not least for the formation of coated pits and the acidification of intravesicular compartments [14]. Consequently, the apparently opposing effects of low and high concentrations of extracellular ethanol on the endocytotic activity of normal hepatocytes could have been explained by concentration-dependent changes to energy metabolism within the cell [15]. However, under the experimental conditions described here where hepatocytes were incubated with ethanol-containing medium for a period of only 30 min (20 min preincubation plus 10 min experimental time), the cellular content of ATP at the end of the study was not significantly different from normal (1.83 ± 0.25 μmol/g wet wt., mean $\pm$ S.E.) at any of the ethanol concentrations tested. This was in agreement with earlier studies using the intact perfused liver [16,17]. Nonetheless, a differential effect on the endogenous concentration of ATP in the mitochondria could not be excluded. Alternatively, the higher concentrations of ethanol may have had a direct effect on the structural integrity of the hepatocyte plasma membrane which could have affected k_e [18,19]. However, this would not explain the absence of any direct effect of ethanol at any concentration tested on the binding of D-Gal-BSA to the asialoglycoprotein receptor associated with purified membrane preparations.

Over the 20 day period that rats were main-

TABLE I

LIGAND BINDING TO SMOOTH MICROSOMES AND SINUSOIDAL PLASMA MEMBRANE AFTER ACUTE OR CHRONIC ETHANOL ADMINISTRATION

Membranes were prepared from normal rat liver and livers of animals that had been maintained on either the ethanol-impregnated or control diets for 10 to 20 days. Ligand binding studies with these membrane preparations were carried out with ^{125}I-labelled D-Gal-BSA at 37°C, essentially as described [13] except for the following modifications: 10 mM *p*-aminobenzamidine and 500 units/ml Trasylol were added to minimise proteolysis, the assay time was reduced to 45 min, and the receptor-ligand complex was harvested with 5% (w/v) PEG 6000 at the same temperature. Results are mean ± S.E. for 4–6 estimations.

Diet	Smooth microsomes		Plasma membrane	
	K_D (10^{-9} M)	B_{max} (pmol/mg)	K_D (10^{-9} M)	B_{max} (pmol/mg)
Chow-fed				
+ 5 mM ethanol	1.45 ± 0.20	20 ± 2	1.25 ± 0.11	40 ± 5
+ 500 mM ethanol	1.15 ± 0.15	20 ± 1	1.5 ± 0.17	42 ± 6
Pair-fed liquid diet				
Day 10: + ethanol	2.10 ± 0.22	22 ± 2	1.05 ± 0.12	38 ± 4
+ sucrose	1.85 ± 0.12	20 ± 2	0.95 ± 0.10	40 ± 6
Day 20: + ethanol	1.60 ± 0.08	20 ± 3	1.25 ± 0.18	40 ± 3
+ sucrose	1.25 ± 0.14	21 ± 4	1.1 ± 0.08	42 ± 2

tained on an ethanol-impregnated liquid diet, there was a progressive decrease in the value of k_e for the internalisation of ^{125}I-labelled D-Gal-BSA by hepatocytes (Fig. 2b). Over this period the k_e value declined by 60% from a mean value of 0.24 min^{-1} to 0.1 min^{-1}, whilst blood alcohol concentration increased steadily to reach about 400 mg% (or, for man, 5-times the U.K. legal limit for driving). This would be equivalent in vitro to an 'extracellular' concentration of 90 mM. In pair-fed control animals, which had received isocaloric sucrose instead of ethanol, there was a 10–20% decrease in k_e over the first 5 days of the study, but this was not maintained and after 20 days there was no difference in k_e between the pair-fed control animals and rats maintained on normal chow. In all cases, there was no significant change, when compared with the normal hepatocytes, in the asialoglycoprotein receptor surface density (B_{max}) and K_D for the D-Gal-BSA ligand. In addition, there was no significant difference from normal chow fed animals in K_D and B_{max} values for the binding of D-Gal-BSA to the asialoglycoprotein receptor associated with smooth microsomes or sinusoidal plasma membrane prepared from rats chronically fed ethanol for 10 or 20 days (Table I). There was no correlation between the period of ethanol ingestion and the cellular content of ATP which was maintained at a level close to that measured for normal chow-fed rats. However, a 57% decrease in mitochondrial ATP content has been demonstrated in rats maintained on a similar ethanol-impregnated liquid diet for 31 days [20]. The decrease was not due to any adaptation in the composition of bulk-phase phospholipids but rather due to perturbation in mitochondrial energy-linked properties. Therefore, the effect of chronic ethanol ingestion in reducing the endocytotic activity of the hepatocyte was most likely manifested, like the acute study, by an imbalance in the metabolic activity of the cell [20,21]. It should also be recognised, however, that chronic alcoholism (both clinically and experimentally) induces adaptive changes in the lipid composition of the plasma membrane [22,23] which may also affect receptor-mediated endocytosis. The significance of any ethanol-induced changes in membrane lipid composition on the function of the asialoglycoprotein receptor remain to be identified. However, in the chronic study a significant decrease in k_e from 0.24 min^{-1} to 0.18 min^{-1} was already apparent at day 2 when the blood alcohol concentration was around 50 mg (equivalent in vitro to an 'extracellular' concentration of 10 mM). There is therefore a clear distinction between the effects of acute and chronic ethanol administra-

tion at apparent low extracellular concentrations on the endocytotic activity of the hepatocyte. This distinction is lost once the metabolic activity of the cell is compromised.

Acknowledgements

This work was supported by the Medical Research Council. We thank the Department of Chemical Pathology, St. George's Hospital, for blood alcohol analysis.

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