Regulation of vesicular pH in liver macrophages and parenchymal cells by ammonia and anisotonicity as assessed by fluorescein isothiocyanate–dextran fluorescence

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Short-term-cultivated rat hepatocytes and Kupffer cells were allowed to endocytose fluorescein isothiocyanate (FITC)-coupled dextran, in order to study the effects of aniso-osmotic exposure and NH_4Cl on apparent vesicular pH (pH_{ves}) by single-cell fluorescence. Following a 2 h loading period with FITC–dextran in normo-osmotic (305 mosmol/l) medium, the apparent pH_{ves} was 6.01 ± 0.05 ($n = 39$) in parenchymal cells and 4.94 ± 0.04 ($n = 76$) in Kupffer cells. Under these conditions pH_{ves} in parenchymal cells, but not in Kupffer cells, was sensitive to changes in ambient osmolarity. Inhibition of vacuolar H^+ -ATPase by concanamycin A did not affect the osmosensitivity of pH_{ves} in parenchymal cells. However, the effects of anisotonicity on pH_{ves} were largely abolished in the presence of 4,4'-di-isothiocyanato-stilbene-2,2'-disulphonic acid (DIDS) or when extracellular chloride was substituted for gluconate. In neither Kupffer cells, nor liver parenchymal cells did hypo-osmotic cell swelling cause an increase in intracellular Ca^{2+} . With regard to vesicular acidification, the following differences were noted between parenchymal and Kupffer cells. (1) In Kupffer cells endocytosed FITC–dextran reached a strongly acidic compartment with a pH value of approx. 5 within 5 min, whereas it took 4–5 h in parenchymal cells. Modification of pH_{ves} by hypo-osmolarity in

Kupffer cells was only observed in a short-lived 'early' compartment with a pH value of approx. 6. (2) In contrast to pH_{ves} in parenchymal cells, pH_{ves} in Kupffer cells was very sensitive towards alkalinization by NH_4Cl : addition of NH_4Cl at 1 or 10 mM increased apparent pH_{ves} by 0.80 or 1.46 in Kupffer cells, but only by 0.18 or 0.56 in parenchymal cells. The low ammonia sensitivity of pH_{ves} in parenchymal cells was observed not only in the less acidic (pH approx. 6) endocytotic compartment which is reached by FITC–dextran within 2 h, but also in the stronger acidic compartment (pH approx. 5) which is reached after 4–5 h. (3) NH_4Cl had no effect on the osmosensitivity of pH_{ves} in parenchymal cells, whereas in Kupffer cells pH_{ves} became sensitive to anisotonicity when NH_4Cl was present. Osmosensitivity of pH_{ves} in Kupffer cells under these conditions, however, was not affected by genistein, DIDS or colchicine, whereas these compounds abolished the osmosensitivity of pH_{ves} in parenchymal cells. It is suggested that regulation of pH_{ves} by cell volume in liver parenchymal cells involves changes of vesicular chloride conductance. In addition, there are marked differences between Kupffer and parenchymal cells with respect to vesicular ammonia permeability and the kinetics of endocytotic membrane flow and acidification.

INTRODUCTION

Alterations of hepatocellular hydration, which reflect changes of liver cell volume on a short-term time scale, occur within minutes under the influence of hormones, cumulative substrate uptake or oxidative stress (for review see [1]). Such alterations of cell hydration were recently recognized as an independent signal, which modulates metabolic liver function and gene expression [1]. One example is the regulation of vesicular acidification by cell hydration, as shown in studies on Acridine Orange or fluorescein isothiocyanate (FITC)-dextran fluorescence [2–5]. Here, cell swelling increases the pH in acidic vesicular compartments (pH_{ves}), whereas cell shrinkage decreases it. Regulation of pH_{ves} by cell volume occurs in early but not in late endocytotic compartments. Here, 'early' and 'late' compartments were defined by their time-dependent accessibility to added FITC–dextran [5]. The early compartment has a pH value around 6 and is characterized by its accessibility within a 2 h FITC–dextran loading period, whereas the more acidic late compartment (pH

approx. 5) is reached when the 2 h loading period with FITC–dextran is followed by a 5 h chase period with unlabelled dextran. Osmoregulation of pH_{ves} in the early compartment is mediated by a swelling-activated signal transduction pathway which involves a G-protein- and tyrosine kinase-dependent, but Ca^{2+} - and cAMP-independent activation of mitogen-activated protein kinases (MAP kinases) Erk-1 and Erk-2 [5,6]. Interruption of this swelling-activated signal transduction pathway at the level of G-proteins and tyrosine phosphorylation blocks both the hypo-osmolarity-induced MAP kinase activation and the swelling-induced increase in pH_{ves} [5,6]. Although these results suggest that swelling-induced alterations in protein phosphorylation mediate the cell volume sensitivity of pH_{ves} , it is still unclear whether changes in cell hydration affect primarily the activity of the vacuolar H^+ -ATPase or the chloride conductance which is required in order to counterbalance the electrical potential created by the proton pump. Furthermore, it remained unclear whether regulation of pH_{ves} by cell volume also occurs in other hepatic cell types and little is known about the interaction

Abbreviations used: AOA, (amino-oxy)acetic acid hemichloride; $[\text{Ca}^{2+}]_i$, cytosolic free Ca^{2+} concentration; DIDS, 4,4'-di-isothiocyanato-stilbene-2,2'-disulphonic acid; FCS, fetal-calf serum; FITC, fluorescein isothiocyanate; FITC–dextran, fluorescein isothiocyanate–dextran (average molecular mass 70 kDa); KHB, Krebs–Henseleit buffer; MAP kinase, mitogen-activated or microtubuli-associated protein kinase; pH_{ves} , vesicular pH; t-BOOH, t-butyl hydroperoxide.

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between cell volume and ammonia in regulating pH_{ves} , although ammonia is long known to impair vesicular acidification due to proton trapping following ammonia diffusion into the vesicular lumen [7,8]. This study shows that volume sensitivity of pH_{ves} in liver parenchymal cells probably resides at the level of vesicular chloride conductance and that there are marked differences in the regulation of pH_{ves} by aniso-osmolarity and ammonia in Kupffer and parenchymal cells.

MATERIALS AND METHODS

Materials

Ionomycin, FITC-dextran (average molecular mass 70 kDa), dextran (average molecular mass 76 kDa), Hepes, Mes, colchicine, lumicolchicine, 4,4'-diisothiocyanato-stilbene-2,2'-disulphonic acid (DIDS) and t-butyl hydroperoxide (t-BOOH) were from Sigma (Munich, Germany). Concanamycin A was from Ciba-Geigy (Basel, Switzerland). Pluronic F-127 and the acetoxymethyl ester of fura-2 were from Molecular Probes Inc. (Eugene, U.S.A.). Genistein and erbstatin analogue (methyl-2,5-dihydroxycinnamate) were from Calbiochem-Novabiochem GmbH (Bad Soden, Germany). ATP was from Boehringer (Mannheim, Germany). (Amino-oxy)acetic acid hemichloride (AOA) was from Kodak (Rochester, U.S.A.). H_2O_2 was from Merck (Darmstadt, Germany). Polybead-dyed microparticles $1\ \mu\text{m}$ (red, 2.5% solid-Latex) were from Polyscience, Inc. (Warrington, U.S.A.). Fetal-calf serum (FCS) was from Biochrom KG (Berlin, Germany).

FITC-dextran was dissolved in Krebs-Henseleit buffer (KHB) at a concentration of 10 mg/ml and dialysed against the same buffer for 48 h in order to remove unbound fluorescein.

Isolation, cultivation and loading of Kupffer cells

Rat Kupffer cells were isolated from livers of male Wistar rats, fed *ad libitum* on stock diet by centrifugal elutriation as described previously [9]. Viability of the cell preparation was higher than 95% as determined by Trypan Blue exclusion. Kupffer cells were cultured at 37 °C in RPMI 1640 medium containing 10% (v/v) heat-inactivated FCS. The cells were incubated in an atmosphere of 95% air and 5% CO_2 ; the medium was changed every 24 h. All experiments were performed with Kupffer cells kept in primary culture for 48–72 h. At that time, the cultures consisted of > 99% Kupffer cells, as demonstrated by their morphological appearance and their phagocytotic activity (Figure 1). For this purpose, 48 h-cultivated Kupffer cells were exposed for 60 min to microparticles (0.025% Latex, diam. $1\ \mu\text{m}$), thereafter they were washed twice and examined by light microscopy (Figure 1). This finding also excluded significant contamination of these cultures with sinusoidal endothelial cells. When sinusoidal endothelial cell cultures were prepared and cultivated for 24 h as described in [9] only 1–2% of the cells took up Latex particles ($1\ \mu\text{m}$ diam.) within 1 h (results not shown).

For single-cell fluorescence measurement Kupffer cells were plated on glass coverslips (diam. 30 mm). Kupffer cells were incubated in Krebs-Henseleit medium (supplemented with 6 mM glucose) (KHB) with the acetoxymethyl ester of fura-2 ($5\ \mu\text{M}$) and Pluronic F-127 (0.02%) for 30 min at 37 °C or with FITC-dextran (5 mg/ml) for 10–120 min or with FITC-dextran (20 mg/ml) for 5 min at 37 °C. In some experiments, Kupffer cells were exposed during the last 60 min of the FITC-dextran loading period to colchicine ($5\ \mu\text{M}$), concanamycin A ($0.1\ \mu\text{M}$), erbstatin analogue ($5\ \mu\text{M}$), DIDS (0.5 mM) or during the last 30 min to genistein ($5\ \mu\text{M}$). Measurements of pH under several

Figure 1 Intracellular localization of Latex and FITC-dextran fluorescence in Kupffer cells

To control purity of Kupffer cell cultures (upper panel, bar = $40\ \mu\text{m}$), Kupffer cells (48 h in culture) were incubated for 60 min with microparticles (0.025% Latex, $1\ \mu\text{m}$) to allow phagocytosis. In cultures of Kupffer cells, all cells phagocytosed Latex (middle panel, bar = $40\ \mu\text{m}$). No phagocytosis occurred in cultures of endothelial cells (results not shown). Loading of isolated Kupffer cells with FITC-dextran for 2 h results in a granular appearance of the fluorescence, suggesting intravesicular localization of the dye (lower panel, bar = $20\ \mu\text{m}$).

conditions were taken within the first 5–20 min after the loading period.

Isolation and loading of parenchymal cells

Isolated parenchymal cells were prepared from livers of male Wistar rats, fed *ad libitum* on stock diet, by collagenase perfusion as described previously [10]. They were plated on collagen-coated glass coverslips (diam. 30 mm) at a density of about

5×10^5 cells/ml. After 2 h the cells were washed twice and cultured at 37 °C in KHB in a 5 % CO₂ atmosphere for up to 9 h. Cell viability before use for single-cell fluorescence measurements was more than 95 % as assessed by Trypan Blue exclusion. Parenchymal cells were incubated in KHB with the acetoxymethyl ester of fura-2 (5 μ M) and Pluronic F-127 (0.02 %) for 30 min at 37 °C or with FITC-dextran (5 mg/ml). The FITC-dextran loading period was 2 h and pH measurements were taken within the first 20 min after the loading period. Thus, hepatocytes used for fluorescence measurements were in culture for at least 4 h (including dye-loading periods), i.e. a time period required for the reorganization of microtubules, as evidenced by immunofluorescence (results not shown). In chloride-free incubations, cells were pretreated for 60 min with chloride-free solutions containing (mmol/l) 121 sodium gluconate, 1 MgSO₄, 8 calcium gluconate, 5 glucose, 25 NaHCO₃, 0.4 KH₂PO₄, 1.6 K₂HPO₄ (O₂/CO₂; 95/5, v/v). In NH₄⁺-containing Cl⁻-free solution, NaHCO₃ was replaced with equimolar NH₄HCO₃.

Single-cell fluorescence measurements

For fluorescence recording, the coverslips were mounted in a perfusion chamber and continuously superfused at a rate of 15 ml/min. The superfusate was the KHB being equilibrated with O₂/CO₂ (95/5, v/v) and resulting in a pH value of 7.4. The temperature was 37 °C for parenchymal cells. Kupffer cells were incubated at 30 °C to prevent photo-damage and dye leakage from vesicular compartments following illumination. FITC-dextran- and fura-2-loaded cells were used for assessment of apparent vesicular pH (pH_{ves}) and cytosolic calcium concentration ([Ca²⁺]_i) respectively. Measurements of apparent pH_{ves} and [Ca²⁺]_i in single cells were performed with an inverted fluorescence microscope setup developed by R. Nischke, U. Fröbe and J. Johna (Department of Physiology, University of Freiburg, Freiburg, Germany). This apparatus allows FITC-dextran and fura-2 fluorescence measurements at the single cell level at excitation wavelengths of 488/440 nm and 340/380 nm, respectively. The emitted light was directed through a 515–565 nm bandpass filter for FITC-dextran fluorescence and through a 480–520 nm bandpass filter for fura-2 fluorescence. By use of adjustable pinholes the field of measurement was chosen to be within one single cell. A time resolution of 15 Hz was achieved by use of a high-speed filter wheel and a single photon counting tube (Hamamatsu H3460-04, Herrsching, Germany). Raw fluorescence signals were corrected for noise and autofluorescence. For determination of autofluorescence unloaded cells were monitored at 340/380 nm and 488/440 nm. Autofluorescence was subtracted from the fluorescence signals found in dye-loaded cells undergoing the respective experimental protocol. Autofluorescence on the pH-dependent signal (excitation at 488 nm) was 7–15 % for FITC-dextran fluorescence; the corresponding value for autofluorescence obtained as a pH-independent signal (excitation at 440 nm) was 20–50 %. For both 340 nm and 380 nm excitation the autofluorescence was low (about 3 % and 5 % respectively). Autofluorescence ratios (488/440 nm) and (340/380 nm) were not affected by anisotonic exposure and treatments.

Calibration of apparent pH_{ves} and [Ca²⁺]_i

Apparent pH_{ves} was obtained from the corrected ratios of 488/440 nm after appropriate calibration according to the method of Thomas et al. [11]. In brief, this calibration procedure involved the superfusion of cells with solutions containing KCl (105 mosmol/l), MgCl₂ (1 mM), Hepes (30 mM; pH varied between 6.6 and 7.4) or 2-[N-morpholino]ethanesulphonic acid

(30 mM; pH varied between 5 and 6) and nigericin (5 μ M). The fluorescence ratio was linear with pH_{ves} between 5 and 7.4 [3].

[Ca²⁺]_i was calculated from the ratio of 340/380 nm fluorescence values (after subtraction of background fluorescence) as described in [12].

The formula used for calculation of [Ca²⁺]_i was

$$[\text{Ca}^{2+}]_i = K_d \times (R - R_{\min}) / (R_{\max} - R) \times (\text{Sf}_2/\text{Sb}_2)$$

where R is the observed fluorescence ratio. The values $R_{\max}$, $R_{\min}$ (maximum and minimum ratios) and the constant Sf_2/Sb_2 (fluorescence of free and Ca²⁺-bound fura-2 at 380 nm) were calculated by using 1 μ M ionomycin and 5 mM EGTA to equilibrate intracellular and extracellular Ca²⁺ in intact fura-2-loaded cells. The dissociation constant for the fura-2-Ca²⁺ complex was taken as 224 nM [12].

Analysis of results

Data are expressed as means $\pm$ S.E.M. (n = number of coverslips studied). For each series, cells were prepared from at least three

Figure 2 Time course of vesicular acidification in Kupffer cells (A) and parenchymal cells (B)

Kupffer cells were incubated for 5 min with 20 mg/ml FITC-dextran to detect changes in the pH in an 'early' endocytotic compartment. The apparent pH_{ves} dropped from 6.43 ± 0.07 ($n = 15$) to 4.96 ± 0.11 ($n = 8$) within 5 min (A). Parenchymal cells were exposed to unlabelled dextran (5 mg/ml) for 0–5 h after a 2 h loading period with FITC-dextran (5 mg/ml). FITC-dextran is transported from an 'early' compartment (pH_{ves} 6.01 ± 0.05 , $n = 39$) to a 'late' acidic compartment (pH_{ves} 5.38 ± 0.04 , $n = 12$) within 5 h (B). $t = 0$ denotes the time points, when the FITC-dextran loading period was finished.

different livers. Within each series, no difference was found between liver preparations. Results were compared by analysis of variance (ANOVA). $P < 0.05$ was considered to be statistically significant.

RESULTS

Acidification of endocytotic vesicles in Kupffer cells

Exposure of cultured rat Kupffer cells for 2 h to FITC-dextran resulted in a granular fluorescent staining of the cells, suggestive of the presence of the dye in endocytotic vesicles (Figure 1). The apparent pH_{ves} in these vesicles was found to be 4.94 ± 0.04 ($n = 76$). Similar pH values, i.e. 4.98 ± 0.06 ($n = 4$) and 4.96 ± 0.02 ($n = 4$), were found when the time period allowed to endocytose FITC-dextran was shortened to 1 h or 30 min respectively. Apparently, in liver macrophages endocytosed FITC-dextran is rapidly transported to a strongly acidic, presumably lysosomal compartment. In order to detect changes in pH in very early endocytotic compartments, Kupffer cells were exposed to FITC-dextran for only 5 min and were thereafter immediately used for single-cell fluorescence recordings. The fluorescent vesicles generated during the 5 min exposure were mainly located in the cell periphery and to a lesser extent distributed throughout the cytosol (results not shown). As shown in Figure 2(A), pH_{ves} in this early endocytotic compartment was 6.43 ± 0.07 ($n = 15$) but declined within 5 min to 4.96 ± 0.11 ($n = 8$). These findings suggest that endocytosed FITC-dextran is rapidly transported to a strongly acidic compartment and that the half-life of the 'early', less acidic endocytotic compartment (pH approx. 6) is short, i.e. about 2 min, in Kupffer cells. This is in contrast to liver parenchymal cells, in which the transport of endocytosed FITC-dextran from an 'early', i.e. less acidic compartment (pH approx. 6), to a 'late', more acidic (pH approx. 5) compartment takes about 4–5 h (Figure 2B).

Regulation of pH_{ves} in rat Kupffer cells by ammonia and anisotonicity

Kupffer cells were exposed for 2 h to FITC-dextran and the effect of aniso-osmotic exposure on apparent pH_{ves} was studied. As shown in Table 1, neither hypo-osmotic nor hyperosmotic exposure had any effect on apparent pH_{ves} . However, a marked alkalization was found in cells pretreated for 60 min with concanamycin A, a potent inhibitor of vacuolar-type H^+ -ATPases [13,14]. Also in the presence of concanamycin A, no osmosensitivity of apparent pH_{ves} was observed (Table 1). Apparent pH_{ves} was very sensitive to NH_4Cl (Figure 3A), with most effective regulation occurring at NH_4Cl concentrations below 2 mM. In the presence of NH_4Cl , but not in its absence, apparent pH_{ves} was sensitive to aniso-osmotic exposure: apparent pH_{ves} increased following hypo-osmotic cell swelling and decreased following hyperosmotic cell shrinkage (Table 1). These effects of anisotonicity in the presence of NH_4Cl on apparent pH_{ves} were rapidly reversible upon reinstitution of normo-osmotic superfusion medium (results not shown). Sensitivity of apparent pH_{ves} to aniso-osmotic exposure was only slightly affected when the NH_4Cl concentration was increased from 0.1 to 10 mM and was not affected by colchicine, DIDS or genistein. This is different to the osmosensitivity of pH_{ves} in liver parenchymal cells, which is largely abolished by these inhibitors ([3–5] and below). Concanamycin A reduced the effect of anisotonicity in the presence of NH_4Cl (Table 1). Regardless of whether NH_4Cl (0.3 mM) was present or not, hypo-osmotic exposure had no effect on $[\text{Ca}^{2+}]_i$ (Figure 4).

Only a 5 min FITC-dextran loading period was used in order to study the effect of aniso-osmolarity on apparent pH_{ves} in early endocytotic vesicles in Kupffer cells. Institution of hypo-osmotic conditions within the first 5 min of fluorescence recording, i.e. during the time period when apparent pH_{ves} rapidly dropped to values of about 5 in control experiments (compare Figure 2A), led to a significant retardation of progressive acidification in this

Table 1 Effects of anisotonicity and NH_4Cl on apparent pH_{ves} in Kupffer cells

Effects of aniso-osmolarity and NH_4Cl on apparent vesicular pH (pH_{ves}) were determined by means of FITC-dextran fluorescence in control cells or cells pretreated for 60 min with concanamycin A (con A, 0.1 μM), 60 min with colchicine (5 μM), 30 min with genistein (5 μM) or 30 min with DIDS (0.5 mM). The effector-induced change in apparent pH_{ves} was determined in individual Kupffer cells. When NH_4Cl was added, extracellular osmolarity was kept constant by lowering the NaCl concentration. Data are given as means $\pm$ S.E.M. (n). (–) and (+) denote decrease or increase in pH_{ves} ; * $P < 0.05$ by ANOVA. Abbreviation: n.d., not determined.

Conditions	Initial apparent pH_{ves} without NH_4Cl	Apparent pH_{ves} with NH_4Cl	Change of pH_{ves} induced by		
			Hypo-osmotic exposure (185 mosmol/l)	Hyperosmotic exposure (425 mosmol/l)	NH_4Cl (20 mM)
Control	4.94 ± 0.04 (76)	–	0.00 ± 0.00 (11)	0.00 ± 0.00 (9)	$+1.97 \pm 0.02$ (8)
NH_4Cl (0.1 mM)	4.75 ± 0.06 (10)	4.92 ± 0.05 (10)*	$+0.09 \pm 0.02$ (9)*	-0.08 ± 0.02 (7)*	n.d.
NH_4Cl (0.3 mM)	4.80 ± 0.07 (8)	5.21 ± 0.11 (8)*	$+0.20 \pm 0.02$ (7)*	-0.12 ± 0.02 (7)*	n.d.
NH_4Cl (1 mM)	4.85 ± 0.08 (5)	5.73 ± 0.08 (5)*	$+0.21 \pm 0.03$ (5)*	-0.16 ± 0.03 (5)*	n.d.
NH_4Cl (10 mM)	4.75 ± 0.04 (5)	6.40 ± 0.06 (5)*	$+0.17 \pm 0.02$ (5)*	-0.16 ± 0.01 (5)*	n.d.
Pretreated with con A	6.48 ± 0.09 (13)*	–	0.00 ± 0.00 (7)	0.00 ± 0.00 (5)	$+0.17 \pm 0.05$ (14)*
NH_4Cl (0.3 mM)	6.60 ± 0.11 (7)*	6.62 ± 0.11 (7)	$+0.01 \pm 0.01$ (6)	-0.02 ± 0.02 (7)	n.d.
NH_4Cl (10 mM)	6.62 ± 0.08 (9)*	6.73 ± 0.07 (9)	$+0.05 \pm 0.01$ (9)*	-0.05 ± 0.01 (9)*	n.d.
Pretreated with colchicine					
NH_4Cl (0.3 mM)	4.86 ± 0.09 (4)	5.20 ± 0.10 (4)*	$+0.17 \pm 0.02$ (4)*	-0.11 ± 0.02 (4)*	n.d.
NH_4Cl (10 mM)	4.81 ± 0.08 (4)	6.29 ± 0.04 (4)*	$+0.17 \pm 0.01$ (4)*	-0.16 ± 0.01 (4)*	n.d.
Pretreated with genistein					
NH_4Cl (0.3 mM)	4.57 ± 0.03 (7)*	5.18 ± 0.04 (7)*	$+0.21 \pm 0.02$ (6)*	-0.16 ± 0.03 (7)*	n.d.
NH_4Cl (10 mM)	4.77 ± 0.08 (5)	6.10 ± 0.08 (5)*	$+0.16 \pm 0.01$ (5)*	-0.18 ± 0.02 (5)*	n.d.
Pretreated with DIDS					
NH_4Cl (0.3 mM)	5.29 ± 0.14 (9)*	5.57 ± 0.13 (9)	$+0.14 \pm 0.02$ (8)*	-0.14 ± 0.02 (8)*	n.d.

Figure 3 Effect of NH_4Cl on apparent pH_{ves} in Kupffer cells (A) and parenchymal cells (B)

The effect of different NH_4Cl concentrations on apparent pH_{ves} was determined in single Kupffer cells (A) or in 'early' or 'late' vesicular compartments of parenchymal cells (B). Apparent pH_{ves} values in Kupffer cells were very sensitive to NH_4Cl , whereas a low NH_4Cl sensitivity of apparent pH_{ves} in parenchymal cells was observed in both 'early' and 'late' endocytotic compartments. Extracellular osmolality was kept constant by lowering the NaCl concentration. Results are given as means $\pm$ S.E.M. (*n*).

'early' endocytotic compartment (Figure 5B). Thereafter, however, (i.e. when the baseline pH of approx. 5 was reached) apparent pH_{ves} was clearly insensitive to hypo-osmotic challenges (Figure 5A). These findings indicate that regulation of apparent pH_{ves} by anisotonicity may occur in the very short-lived 'early' endocytotic compartment in Kupffer cells, but not in more acidic 'late' vesicles.

Regulation of apparent pH_{ves} in rat liver parenchymal cells by ammonia and anisotonicity

In line with previous reports [3–5], in liver parenchymal cells a 2 h FITC-dextran loading period resulted in an apparent pH_{ves} of 6.01 ± 0.05 (*n* = 39), which was sensitive to anisotonic exposure (Table 2). As shown previously, regulation of apparent pH_{ves} by cell volume was abolished following pretreatment with colchicine [3], genistein, erbstatin, pertussis or cholera toxin [5]. Concanamycin A led to a slight vesicular alkalization, but had no

Figure 4 Effect of aniso-osmolality on $[\text{Ca}^{2+}]_i$ in the absence (A) or in the presence of 0.3 mM NH_4Cl (B) in Kupffer cells

$[\text{Ca}^{2+}]_i$ was assessed by means of fura-2 fluorescence (for details see the Materials and methods section). During normo-osmotic superfusion conditions, the $[\text{Ca}^{2+}]_i$ was 34 ± 2 nM (*n* = 49). Hypo-osmotic exposure had no effect on $[\text{Ca}^{2+}]_i$ in both the presence and absence of NH_4Cl . Representative tracings from at least five different experiments are shown.

effect on the anisotonicity- or hydroperoxide-induced shifts of apparent pH_{ves} (Table 2). These, however, were abolished by erbstatin, an inhibitor of tyrosine kinases and were blunted by colchicine. Addition of the anion-channel blocker DIDS or incubation with Cl^- -free media had no effect on apparent pH_{ves} , but largely abolished the cell volume dependence of apparent pH_{ves} (Table 2).

Interestingly, NH_4Cl produced only a slight alkalinizing effect on apparent pH_{ves} in parenchymal cells without affecting cell volume sensitivity of apparent pH_{ves} (Figure 3B, Table 2). Inhibition of urea synthesis due to inhibition of aminotransferases by AOA [15] did not affect apparent pH_{ves} or its sensitivity to NH_4Cl (results not shown).

In line with previous data [5], apparent pH_{ves} decreased from 6.01 ± 0.05 (*n* = 39) to 5.38 ± 0.03 (*n* = 37) when the 2 h FITC-dextran loading period was followed by a 5 h exposure to unlabelled dextran ('late' endocytotic compartment; Table 3). This approach has been used previously [5] to label a late, more acidic vesicular compartment with the fluorescent dye. The data in Figure 2(B) indicate that endocytosed FITC-dextran was transported within 5 h to a more acidic 'late' endocytotic compartment, which lacks apparent pH_{ves} regulation by anisotonicity (Table 3) [5]. As shown in Figure 3(B), sensitivity of apparent pH_{ves} to NH_4Cl was similar in the 'late' and the 'early' endocytotic compartment in parenchymal cells and much less

Figure 5 Inhibition of vesicular acidification by cell swelling in Kupffer cells

Kupffer cells were incubated for 5 min with 20 mg/ml FITC–dextran to detect changes in pH in an early endocytotic compartment. The apparent pH_{ves} dropped from 6.43 ± 0.07 ($n = 15$) to 4.96 ± 0.11 ($n = 8$) within 5 min. Thereafter hypo-osmotic exposure has no effect on apparent pH_{ves} (A). Hypo-osmotic exposure during the fall of apparent pH_{ves} within 5 min, significantly slows down acidification of apparent pH_{ves} (B). Representative tracings from at least four different experiments are shown.

pronounced than in Kupffer cells (compare Figures 3A and 3B). Thus, the difference in the resting apparent pH_{ves} in ‘early’ and ‘late’ endocytotic vesicles does not explain the low ammonia sensitivity of apparent pH_{ves} in parenchymal cells.

DISCUSSION

Cell volume sensitivity of apparent pH_{ves} in Kupffer and parenchymal cells

Previous studies have indicated a cell volume-sensitive regulation of pH in endocytotic vesicles in rat liver parenchymal cells, which are accessible to FITC–dextran during a 2 h loading period (‘early’ compartment). Evidence has been presented that the alkalinizing effect of cell swelling is transmitted by a Ca^{2+} - and cAMP-independent, but G-protein- and tyrosine kinase-dependent mechanism [5], which also leads to a swelling-induced activation of MAP kinases Erk-1 and Erk-2 [6]. The recently recognized association between tubulin and G-proteins including a role of the cytoskeleton in signal transduction [16], as well as

Table 2 Effects of anisotonicity, oxidative stress and NH_4Cl on apparent pH_{ves} in early endocytotic compartments of parenchymal cells

Parenchymal cells were loaded for 2 h with FITC–dextran, in order to stain a less acidic endocytotic compartment (early endocytotic compartment). The effects of aniso-osmolality and NH_4^+ on apparent pH_{ves} were determined by means of FITC–dextran fluorescence in control cells or cells pretreated for 90 min with concanamycin A (0.1 μM), 60 min with erbstatin analogue (5 μM), 60 min with colchicine (5 μM), lumichlorhicine (5 μM) or 30 min with DIDS (0.5 mM). In the case of Cl^- -free conditions, cells were incubated for 60 min in Cl^- -free solution after a 2 h loading period. The effector-induced change in apparent pH_{ves} was determined in single cells. When NH_4Cl or NH_4HCO_3 was added, extracellular osmolality was kept constant by lowering the NaCl or NaHCO_3 concentration. Data are given as means $\pm$ S.E.M. (n). (–) and (+) denote decrease or increase of pH_{ves} ; * $P < 0.05$ by ANOVA. Abbreviation: n.d., not determined.

Conditions	Change of pH_{ves} induced by				
	Apparent pH_{ves}	Hypo-osmotic (185 mosmol/l)	Hypertonic (425 mosmol/l)	H_2O_2 (0.2 mM)	NH_4^+ (20 mM)
Control (2 h FITC–dextran)	6.01 ± 0.05 (39)	$+0.18 \pm 0.02$ (26)	-0.12 ± 0.01 (23)	-0.16 ± 0.01 (19)	$+0.32 \pm 0.03$ (25)
Pretreated with lumichlorhicine	6.20 ± 0.06 (6)	$+0.16 \pm 0.03$ (6)	-0.09 ± 0.01 (6)	-0.19 ± 0.01 (7)	$+0.40 \pm 0.04$ (6)
Pretreated with colchicine	6.20 ± 0.05 (7)	$+0.05 \pm 0.02$ (7)*	-0.04 ± 0.01 (8)*	-0.20 ± 0.02 (7)	$+0.37 \pm 0.04$ (6)
Pretreated with erbstatin	6.22 ± 0.09 (6)	$+0.01 \pm 0.02$ (6)*	$+0.02 \pm 0.02$ (6)*	n.d.	$+0.32 \pm 0.05$ (6)
Concanamycin A	6.35 ± 0.07 (12)*	$+0.14 \pm 0.02$ (13)	-0.11 ± 0.01 (13)	n.d.	$+0.22 \pm 0.02$ (13)*
Pretreated with colchicine	6.38 ± 0.08 (6)*	$+0.08 \pm 0.01$ (6)*	-0.06 ± 0.02 (6)*	n.d.	$+0.15 \pm 0.03$ (6)*
Pretreated with erbstatin	6.33 ± 0.11 (9)*	$+0.02 \pm 0.01$ (9)*	-0.02 ± 0.01 (9)*	n.d.	$+0.13 \pm 0.01$ (8)*
DIDS	5.87 ± 0.04 (23)	$+0.05 \pm 0.01$ (10)*	-0.04 ± 0.01 (10)*	-0.02 ± 0.01 (7)*	$+0.14 \pm 0.03$ (18)*
NH_4Cl (0.3 mM)	5.97 ± 0.15 (7)	$+0.15 \pm 0.01$ (7)	-0.09 ± 0.01 (7)	n.d.	n.d.
NH_4Cl (1 mM)	6.19 ± 0.12 (7)	$+0.15 \pm 0.02$ (7)	-0.09 ± 0.01 (7)	n.d.	n.d.
NH_4Cl (10 mM)	6.57 ± 0.10 (7)*	$+0.17 \pm 0.03$ (7)	-0.12 ± 0.01 (6)	n.d.	n.d.
Control (2 h FITC–dextran, 1 h KHB)	6.04 ± 0.06 (21)	$+0.17 \pm 0.02$ (9)	-0.12 ± 0.02 (8)	-0.17 ± 0.01 (9)	$+0.43 \pm 0.04$ (15)
Chloride-free (2 h FITC–dextran, 1 h Cl^- -free)	6.11 ± 0.08 (24)	$+0.05 \pm 0.01$ (14)*	-0.05 ± 0.01 (14)*	-0.02 ± 0.01 (6)*	$+0.19 \pm 0.03$ (20)*

Table 3 Effects of anisotonicity and NH_4Cl on apparent pH_{ves} in a late endocytotic compartment of parenchymal cells

Parenchymal cells were loaded for 5 h with unlabelled dextran following the 2 h loading period to stain a more acidic compartment (late endocytotic compartment). The effects of aniso-osmolality and NH_4Cl on apparent pH_{ves} were determined by means of FITC–dextran fluorescence in control cells or cells pretreated for 90 min with concanamycin A (0.1 μM) or 5 min with AOA (0.2 mM). The effector-induced change in apparent pH_{ves} was determined in single cells. When NH_4Cl was added, extracellular osmolality was kept constant by lowering the NaCl concentration. Data are given as means $\pm$ S.E.M. (*n*). (–) and (+) denote decrease or increase of pH_{ves} ; * $P < 0.05$ by ANOVA. Abbreviation: n.d., not determined.

Conditions	Apparent pH_{ves}	Change of pH_{ves} induced by		
		Hypo-osmotic exposure (185 mosmol/l)	Hyperosmotic exposure (425 mosmol/l)	NH_4Cl (20 mM)
Control	5.38 ± 0.03 (37)	$+0.02 \pm 0.01$ (7)	-0.01 ± 0.01 (7)	$+0.67 \pm 0.07$ (21)
Concanamycin A	6.51 ± 0.08 (11)*	0.00 ± 0.00 (6)	-0.01 ± 0.01 (6)	$+0.16 \pm 0.02$ (11)*
NH_4Cl (10 mM)	5.91 ± 0.08 (12)*	$+0.08 \pm 0.01$ (7)*	-0.10 ± 0.01 (7)*	n.d.
Pretreated with AOA				
NH_4Cl (0.3 mM)	5.44 ± 0.06 (6)	$+0.07 \pm 0.01$ (6)*	-0.04 ± 0.01 (6)*	n.d.
NH_4Cl (10 mM)	5.83 ± 0.08 (6)*	$+0.10 \pm 0.01$ (6)*	-0.07 ± 0.01 (6)*	n.d.

the regulation of microtubule-associated proteins by MAP-kinases, may explain the previously described colchicine sensitivity of apparent pH_{ves} regulation by cell volume [3,4]. As shown in this study, cell volume sensitivity of apparent pH_{ves} in this 'early' endocytotic compartment was not affected by concanamycin A, an inhibitor of vacuolar-type H^+ -ATPases [13,14]. However, it was largely abolished in the presence of the anion-channel blocker DIDS [17,18] or in Cl^- -free media (Table 2). Since neither DIDS nor Cl^- removal affected the resting apparent pH_{ves} and because concanamycin A entered the cells, demonstrated by its alkalinizing effect (Tables 2 and 3), these findings suggest that regulation of apparent pH_{ves} by cell volume could involve changes in the vesicular Cl^- conductance, rather than of H^+ -ATPase activity. This Cl^- conductance is required for vesicular acidification by counteracting the electrogenicity of the H^+ pump [19]. It is conceivable, albeit speculative, that the swelling-induced activation of MAP-kinases might regulate Cl^- conductance by phosphorylation. In line with such a proposal, evidence has been given in other cell types for a regulation of Cl^- channel activity and acidification in endosomes by cAMP-dependent phosphorylation [20,21] and phosphoproteins in the endosomal membrane have been described recently [22].

Interestingly, in rat liver macrophages anisotonicity had no effect on apparent pH_{ves} when the FITC–dextran loading period was 2 h. One possible explanation for this could be that freshly endocytosed FITC–dextran is transported in Kupffer cells much more rapidly than in parenchymal cells into the 'late' stronger acidic compartment, which is cell-volume-insensitive in parenchymal cells also (Tables 1 and 3). In line with this, endocytosed FITC–dextran reaches a 'late', i.e. strongly acidic (pH approx. 5), vesicular compartment in Kupffer cells within 5 min, whereas 4–5 h are required in liver parenchymal cells (Figure 2). Thus, the regulation of apparent pH_{ves} by cell volume in Kupffer cells has probably escaped detection due to a very short half-life of this compartment. When the short-lived early endocytotic compartment was studied following a 5 min FITC loading period, hypo-osmotic exposure indeed produced alkalinization, indicating that this cell-volume-sensitive compartment may exist, but has a very short half-life.

Ammonia sensitivity of apparent pH_{ves} in Kupffer and parenchymal cells

Another interesting difference between vesicular acidification in parenchymal and Kupffer cells was the NH_4Cl sensitivity. Whereas apparent pH_{ves} in Kupffer cells was regulated very

sensitively by ammonia, NH_4Cl exerted comparatively small effects on apparent pH_{ves} in parenchymal cells (Figure 3). This difference was also found when endocytosed FITC–dextran was allowed to reach the strongly acidic 'late' compartment and was not explained by differences in the ureogenic capacity between parenchymal and Kupffer cells, as suggested by the experiments with AOA (Table 3). One possible explanation could be that the vesicle populations accessible to FITC–dextran in parenchymal cells exhibit a restricted ammonia permeability.

Ammonia had no effect on the volume sensitivity of apparent pH_{ves} in 'early' endocytotic vesicles of parenchymal cells (Table 2), but a slight volume sensitivity of apparent pH_{ves} reappeared in 'late' endocytotic vesicles (2 h FITC–dextran followed by a 5 h loading period with unlabelled dextran) when ammonia was present (Table 3). Also in Kupffer cells, addition of ammonia resulted in the appearance of an osmosensitive regulation of apparent pH_{ves} . Under these conditions, vesicular alkalinization (acidification) in response to hypo-osmotic (hyperosmotic) exposure in Kupffer cells strongly differed from the osmosensitive regulation of apparent pH_{ves} in 'early' endocytotic vesicles of parenchymal cells in that it was almost unaffected by genistein or colchicine. These compounds, however, abolished the volume sensitivity of apparent pH_{ves} in parenchymal cells [5]. The following explanation for these findings is proposed: in Kupffer cells, hypo-osmotic exposure will lead to vesicular swelling and the corresponding increase in the vesicular water space will favour NH_3 entry until Donnan's equilibrium is restored. Ammonia trapping (NH_4^+ formation) will give rise to vesicular alkalinization (as long as net proton pumping by H^+ -ATPase is slower than NH_3 entry). Likewise, hyperosmotic exposure will result in vesicular acidification. This apparently does not happen in the vesicular compartments in parenchymal cells and points to a comparatively low ammonia permeability of the vesicle membranes in parenchymal cells. In line with such a difference in ammonia permeability is also the finding that apparent pH_{ves} in Kupffer cells increased from 5.0 to 7.0 upon addition of 20 mM NH_4Cl , but only from 5.4 to 6.0 in parenchymal cells when late endocytotic vesicles were studied (see Tables 1 and 3).

Taken together, our results suggest that the differences in apparent pH_{ves} regulation in Kupffer and parenchymal cells by cell volume and ammonia are predominantly due to both (a) a much more rapid transfer of endocytosed FITC–dextran from early endocytotic vesicles to a strongly acidic, presumably lysosomal compartment in Kupffer cells, and (b) differences in the ammonia permeability of the vesicular compartments in both cell types.

Possible physiological significance

The ammonia concentration in portal venous blood ranges between 0.2 and 0.7 mM [23,24]. Depending on the nutritional condition, the rate of hepatic amino acid breakdown, urea synthesis and intestinal ammonia production, the ammonia concentration may rise to 1 mM or more inside the liver. Vesicular acidification in parenchymal cells seems to be better protected against such fluctuations of the ammonia concentration, than in Kupffer cells. Here, modulation of ambient ammonia between 0 and 1 mM can increase apparent pH_{ves} by about 0.8. Given the important role of vesicular acidification for the phagocytosis process, it is conceivable that hyperammonaemic states such as liver cirrhosis could impair liver macrophage function, thereby allowing the escape of gut-derived endotoxin into the systemic circulation. This could provide a basis for the understanding of an interaction between hyperammonaemia, endotoxaemia and circulatory abnormalities [25] in liver cirrhosis.

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