Effects of aniso-osmolarity and hydroperoxides on intracellular pH in isolated rat hepatocytes as assessed by (2',7')-bis(carboxyethyl)-5(6)-carboxyfluorescein and fluorescein isothiocyanate–dextran fluorescence

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Freshly isolated rat hepatocytes were plated for 4–6 h and either loaded with (2',7')-bis(carboxyethyl)-5(6)-carboxyfluorescein (BCECF) or allowed to endocytose fluorescein isothiocyanate (FITC)-coupled dextran in order to study the effects of aniso-osmotic exposure and oxidative stress on cytosolic (pH_{cyt}) and apparent vesicular pH (pH_{ves}) by single-cell fluorescence recordings. In the presence of normo-osmotic (305 mosmol/l) medium pH_{cyt} was 7.23 ± 0.03 ($n = 108$), whereas an apparent pH of 6.07 ± 0.02 ($n = 156$) was found in the vesicular compartment accessible to endocytosed FITC–dextran. Substitution of 60 mM NaCl against 120 mM raffinose had no effect on pH_{cyt} or apparent pH_{ves} , whereas addition of NH_4Cl increased both pH_{cyt} and apparent pH_{ves} . Hypo-osmotic cell swelling lowered pH_{cyt} , whereas simultaneously apparent pH_{ves} increased. These effects were rapidly reversible upon re-institution of normo-osmotic media. Similarly, an increase of apparent pH_{ves} was observed when cell swelling was induced by Ba^{2+} , glutamine or histidine. Conversely, hyperosmotic cell shrinkage due to addition of NaCl or raffinose led to a cytosolic alkalization and

a vesicular acidification. Both, H_2O_2 (0.2 mmol/l) and t-butylhydroperoxide (0.2 mmol/l) were without effect on pH_{cyt} , but lowered apparent pH_{ves} by about 0.2 pH units. Ba^{2+} (1 mmol/l) diminished the acidifying effect of the hydroperoxides by about 50%. Pretreatment of the cells with colchicine, but not with lumicolchicine, largely abolished the effects of aniso-osmolarity and hydroperoxides on pH_{ves} . The data suggest that hepatocellular hydration affects the proton gradients built up across the membranes of endocytotic FITC–dextran-accessible compartments in a microtubule-dependent way. They further suggest that hydroperoxides induce vesicular acidification in a colchicine- and Ba^{2+} -sensitive way. Because hydroperoxides induce Ba^{2+} -sensitive cell shrinkage [Hallbrucker, Ritter, Lang, Gerok and Häussinger (1992) Eur. J. Biochem. 211, 449–458], the results are compatible with the view that hydroperoxide-induced cell shrinkage mediates vesicular acidification. It is concluded that modulation of vesicular pH by the hepatocellular hydration state may play a role in triggering some metabolic changes in response to cell swelling/shrinkage.

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 reviews see [1,2]). Such alterations of cell hydration were recently recognized as another signal-transduction pathway of hormone and amino acid action, which influences a variety of metabolic liver functions [1,3]. Intracellular vesicular compartments and cytoskeletal structures participate in some cell volume-sensitive pathways, such as lysosomal proteolysis or the putative swelling-induced insertion/retrieval of bile acid-transporter molecules into/from the canalicular membrane in response to cell swelling/shrinkage [4–7]. Indeed, pretreatment with colchicine largely abolishes the swelling-induced inhibition of proteolysis [7] and stimulation of bile acid secretion in liver [6]. Further, cell swelling was shown to alkalize intracellular acidic compartments when assessed by means of Acridine Orange fluorescence and it was hypothesized that lysosomal alkalization may contribute to the antiproteolytic effect of cell swelling [8]. Unfortunately, owing to inherent limitations of the Acridine Orange fluorescence technique [9] such studies did not allow testing for the reversibility or duration of the effects due to loss of the dye from the acidic compartments following alkalization. We therefore decided to assess the effect

of aniso-osmotic cell volume changes on vesicular pH (pH_{ves}) by recording the fluorescence of endocytosed fluorescein isothiocyanate (FITC)-dextran at the single hepatocyte level. FITC–dextran is known to be taken by a fluid-phase endocytosis and has been used in the past repeatedly for assessment of pH_{ves} *in vivo* in a variety of cell types [10–15], including hepatocytes [16]. In addition, we studied the effects of hydroperoxides, which induce liver cell shrinkage due to K^+ -channel opening [17,18], on subcellular pH. Our results suggest that alterations of hepatocellular hydration exert a marked influence on the pH gradient between the cytosol and these FITC–dextran-accessible vesicular compartments. Because several functions of the vacuolar system are known to depend critically upon the acidification of this compartment (for reviews see [19,20]), it is feasible that cell volume alterations might, in part, affect cellular functions by interfering with vacuolar acidification.

MATERIALS AND METHODS

Materials

Nigericin and FITC–dextran (average molecular mass 70 kDa), Hepes, t-butylhydroperoxide (t-BOOH), H_2O_2 , colchicine, lumicolchicine and MES were from Sigma (Munich, Germany); the acetomethyl ester of (2',7')-bis(carboxyethyl)-5(6)-carboxyfluorescein (BCECF) (BCECF-AM) was from Molecular

Abbreviations used: BCECF, (2',7')-bis(carboxyethyl)-5(6)-carboxyfluorescein; BCECF-AM, BCECF acetomethyl ester; FITC, fluorescein isothiocyanate; pH_{cyt} , cytosolic pH; pH_{ves} , vesicular pH; t-BOOH, t-butylhydroperoxide.

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Figure 1 pH calibration curves for FITC dextran (a) and BCECF (b) fluorescence

For experimental details see the Materials and methods section. Data are given as means $\pm$ S.E.M. (*n*).

Figure 2 Intracellular localization of BCECF (a) and FITC-dextran fluorescence in control (b) and colchicine-treated (c) hepatocytes

Loading of isolated hepatocytes with FITC-dextran results in a granular appearance of the fluorescence, suggesting intravesicular localization of the dye. These vesicles are preferentially found in the perinuclear area in control experiments (b), but are randomly distributed after 60 min of colchicine treatment (c). On the other hand, BCECF fluorescence (a) is homogeneously distributed in the cytosol. The length of the white bar corresponds to 20 μ m.

Probes (Eugene, OR, U.S.A.). FITC-dextran was dissolved in Krebs-Henseleit buffer at a concentration of 10 mg/ml and dialysed against the same buffer for 48 h in order to remove unbound fluorescein. L-Glutamine was from Merck (Darmstadt,

Figure 3 Effect of aniso-osmotic exposure, raffinose and NH_4Cl on subcellular pH

Cytosolic pH (pH_{cyt}) was assessed by means of BCECF fluorescence; vesicular pH (pH_{ves}) by fluorescence of endocytosed FITC-dextran. Representative traces from at least five different experiments are shown.

Germany) and α -D-raffinose was from Serva (Heidelberg, Germany).

Isolation and loading of hepatocytes

Isolated hepatocytes were prepared from livers of male Wistar rats, fed *ad libitum* on stock diet, by collagenase perfusion as described previously [21] and plated on collagen-coated glass coverslips (diam. 30 mm) at a density of about 1×10^6 cells/ml. Cells were cultured at 37 °C in a Krebs-Henseleit medium supplemented with glucose (6 mmol/l) (KHB) in a 5% CO_2 atmosphere for 4–6 h. Cell viability before use for single-cell fluorescence measurements was more than 95% as assessed by Trypan Blue exclusion. Primary hepatocytes grown for 2–3 h on the thin coverslips were then washed with the above-mentioned KHB and incubated in the same solution with FITC-dextran (5 mg/ml) for another 2–4 h at 37 °C. In experiments for assessment of cytosolic pH (pH_{cyt}), primary hepatocytes were grown for 3–4 h and, after washing with KHB, were incubated in the same medium containing BCECF-AM (2 $\mu\text{mol/l}$) for another 30 min. In some experiments, the hepatocytes were in addition exposed during the last 60 min of cultivation before the experiment to colchicine or lumicolchicine (5 $\mu\text{mol/l}$, each). Thus hepatocytes used for the fluorescence measurements were in culture for at least 4 h (including the dye-loading periods); i.e. the time period required for the reorganization of microtubules, as evidenced by immunofluorescence (results not shown).

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 KHB equilibrated with O_2/CO_2 (95/5; v/v), resulting in pH 7.4. The temperature was 37 °C. BCECF-loaded and FITC-dextran-loaded hepatocytes were

used for assessment of pH_{cyt} and pH_{ves} respectively. Measurements of apparent pH_{ves} and pH_{cyt} in single cells were performed with an inverted fluorescence microscope system developed by R. Nischke, U. Fröbe and R. Johna (Department of Physiology, University of Freiburg, Freiburg, Germany). This apparatus allows BCECF and FITC-dextran fluorescence measurements at the single-cell level at the excitation wavelengths of 488/440 nm. The emitted light was directed through a 520 nm cut-off filter. 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). The raw fluorescence signals were corrected for the noise and autofluorescence. For determination of the autofluorescence, unloaded cells were monitored at 488/440 nm and the fluorescence change in response to effectors (aniso-osmolarity, amino acids, Ba^{2+} , hydroperoxides) were recorded. 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 0.7–1.2% for BCECF and 7–15% for FITC-dextran fluorescence; corresponding values for the autofluorescence obtained as pH-independent signals (excitation at 440 nm) were 3–7% and 20–50% respectively. The autofluorescence ratio (488/440 nm) was not affected by aniso-osmotic exposure, amino acids and hydroperoxides. pH_{cyt} and pH_{ves} were obtained from the corrected ratios of 488/440 nm after appropriate calibration according to [11]. In brief, this calibration procedure involved the superfusion of cells with solutions containing KCl (105 mmol/l), MgCl_2 (1 mmol/l), Hepes (30 mmol/l; pH varied between 6.6 and 7.8) or Mes (30 mmol/l; pH varied between 5 and 6) and nigericin (5 $\mu\text{mol/l}$). The pH of the calibration superfusate was varied between 6.6 and 7.8 in BCECF-loaded cells and between 5 and 7.4 in FITC-dextran-loaded cells. The fluorescence ratios at 488/

440 nm were linear with pH_{cyt} between 6.6 and 7.8 and linear with pH_{ves} between 5 and 7.4. Calibration curves are shown in Figure 1.

The fluorescence signal from a given cell reflects the ratio of protonated versus non-protonated FITC-dextran. Only if the pH in all vesicles containing FITC-dextran were identical can the fluorescence ratio be directly translated into the pH of this homogeneous compartment. However, FITC-dextran is probably accumulated in a vesicle population inhomogeneous with regard to vesicular pH. In this case, the fluorescence ratio does not reflect the arithmetic mean of the pH in these vesicles but the pH corresponding to the respective percentage of protonated versus unprotonated FITC-dextran. As a result, the pH calculated is closer to the pK of the dye as would be the arithmetic mean and, given the pK of FITC-dextran of 6.4, a calculated pH of 6.05 is higher than would be the arithmetic mean. The bias of inhomogeneous distribution is inherent to any fluorescence measurements and is acknowledged here by use of the term 'apparent pH_{ves} '.

Photographs of the cells (Figure 2) were taken during excitation with 440 nm using a Contax 167MT camera and a Tmax film (3200 ASA, Eastman Kodak).

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 livers.

Figure 4 Effects of aniso-osmolarity on pH_{ves} and pH_{cyt} in isolated rat hepatocytes

For experimental details see the Materials and methods section. Aniso-osmotic conditions were achieved by respective modification of the NaCl concentrations in the Krebs-Henseleit buffer used for superfusion of the dye-loaded hepatocytes. Numbers in parentheses refer to the number of experiments. During normo-osmotic (305 mosmol/l) conditions, the pH_{ves} was 6.07 ± 0.02 ($n = 156$) and pH_{cyt} was 7.23 ± 0.03 ($n = 108$).

RESULTS

Effects of aniso-osmolarity on apparent pH_{ves}

Loading of primary hepatocytes with BCECF for 30 min resulted in a homogeneous fluorescent staining throughout the cytoplasm (Figure 2a), whereas a 2 h-long exposure to FITC-dextran produced granular staining of the cells, consistent with the presence of the dye in vesicles (Figure 2b). In line with previous morphometric studies, these vesicles were distributed throughout the cytoplasm, with some enrichment found in the perinuclear area [16]. Disruption of microtubules by treatment of the cells for 60 min with colchicine did not abolish the appearance of the dye in intracellular vesicles; however, it yielded a more random distribution of the dye-containing vesicles throughout the cell (Figure 2c). FITC-dextran is known to be taken up into hepatocytes by fluid-phase endocytosis and to be stored in acidic cellular vesicles without being degraded in this compartment [11,16,22]. Single-cell fluorescence recordings from FITC-dextran-loaded cells being superfused with normotonic (305 mosmol/l) medium yielded an intravesicular apparent pH (pH_{ves}) of 6.07 ± 0.02 ($n = 156$) in the vesicular compartment being defined by its accessibility to the dye. Hypo-osmotic or hyper-osmotic exposure, due to a corresponding decrease or increase of the NaCl concentration of the superfusion medium, led to a rapid increase or decrease of apparent pH_{ves} respectively (Figure 3). These effects of aniso-osmolarity on apparent pH_{ves} were rapidly reversible upon restoration of normo-osmotic superfusion conditions (Figure 3) and the extent of the pH_{ves} change was dependent upon the extent of the aniso-osmotic challenge (Figure 4). The aniso-osmolarity-induced shift of apparent pH_{ves} persisted as long as the aniso-osmotic conditions were maintained, i.e. up to 20 min of exposure which was the longest time period tested, and were also reversible under these conditions upon normo-osmotic re-exposure (results not shown). Because volume-regulatory responses of the hepatocyte, either being isolated in suspension or in the intact rat liver *in situ*, are known to be largely completed within 10 min of the onset of aniso-osmotic exposure [23–25], these observations suggest that vesicular alkalization/acidification in response to hypo-/hyperosmotic exposure is not a transient phenomenon just accompanying regulatory volume decrease/increase, but rather it persists as long as the cells are exposed to aniso-osmotic medium.

Substitution of 60 mmol/l NaCl by 120 mmol/l raffinose (thereby maintaining normo-osmotic conditions) in the superfusion medium did not affect apparent pH_{ves} , whereas sudden removal of the raffinose (thereby creating hypo-osmotic conditions of 185 mosmol/l) produced vesicular alkalization (Table 1 and Figure 3), as it was observed when hypotonic conditions were instituted by removal of NaCl (Figures 3 and 4, Table 1). Further, the decrease of apparent pH_{ves} in response to hyperosmolarity was of similar magnitude when hyperosmotic conditions were instituted by addition of 60 mM NaCl or 120 mM raffinose (Table 1). These results suggest that the observed effects of aniso-osmolarity on apparent pH_{ves} are not explained by alterations of the extracellular Na^+ or Cl^- activity, but are due to changes of extracellular osmolarity or cellular volume.

Effects of aniso-osmolarity on pH_{cyt}

Interestingly, pH_{cyt} , as assessed by BCECF fluorescence, exhibited opposite changes in response to aniso-osmolarity when compared with the effects on pH_{ves} (Figures 3 and 4 and Table 1). In normo-osmotic medium pH_{cyt} was 7.23 ± 0.03 ($n = 108$) and was shifted to more acidic or more alkaline values following hypo-

Table 1 Effects of anisotonicity, glutamine and NH_3 on cytosolic and apparent vesicular pH in isolated rat hepatocytes

Cytosolic (pH_{cyt}) and apparent vesicular pH (pH_{ves}) were determined by means of BCECF and FITC-dextran fluorescence respectively (for details see the Materials and methods section). The effector-induced change in subcellular pH was determined in the individual hepatocyte. In the presence of normotonic Krebs-Henseleit medium with no effectors present, pH_{cyt} was 7.23 ± 0.03 ($n = 108$) and the apparent pH_{ves} was 6.07 ± 0.02 ($n = 156$). When NH_4Cl (20 mM) was added, extracellular osmolarity was kept constant by lowering the NaCl concentration by 20 mM. Data are given as means $\pm$ S.E.M. (n). (–) and (+) denote decrease or increase of respective pH values.

Condition	Osmolarity (mosmol/l)	Change in subcellular pH	
		pH_{cyt}	Apparent pH_{ves}
Normotonic control	305	–	–
Hypotonic (– 60 mM NaCl)	185	-0.10 ± 0.01 (6)	$+0.20 \pm 0.05$ (9)
Hypertonic (+ 60 mM NaCl)	425	$+0.05 \pm 0.01$ (11)	-0.15 ± 0.03 (7)
Hypertonic (+ 120 mM raffinose)	425	$+0.06 \pm 0.01$ (9)	-0.12 ± 0.02 (6)
Glutamine (3 mM)	308	$+0.08 \pm 0.01$ (5)	$+0.04 \pm 0.01$ (11)
Histidine (3 mM)	308	$+0.02 \pm 0.02$ (4)	$+0.05 \pm 0.01$ (7)
Proline (3 mM)	308	$+0.03 \pm 0.01$ (12)	$+0.01 \pm 0.01$ (15)
Leucine (3 mM)	308	0.00 ± 0.00 (8)	$+0.01 \pm 0.01$ (7)
BaCl_2 (1 mmol/l)	308	$+0.02 \pm 0.02$ (6)	$+0.08 \pm 0.01$ (9)
NH_4Cl (1 mM)	305	$+0.05 \pm 0.01$ (5)	$+0.06 \pm 0.02$ (7)
NH_4Cl (20 mM)	305	$+0.36 \pm 0.02$ (23)	$+0.48 \pm 0.05$ (27)
Normotonic (substitution of 60 mM NaCl against 120 mM raffinose)	305	0.00 ± 0.00 (9)	0.00 ± 0.00 (6)
Hypotonic (induced by omission of raffinose from the above normotonic medium)	185	-0.09 ± 0.01 (9)	$+0.26 \pm 0.07$ (6)

Figure 5 Effect of H_2O_2 (0.2 mmol/l), t-BOOH (0.2 mmol/l) and NH_4Cl on subcellular pH

Cytosolic pH (pH_{cyt}) was assessed by means of BCECF fluorescence; apparent vesicular pH (pH_{ves}) by fluorescence of endocytosed FITC-dextran. Representative traces from at least five different experiments are shown.

osmotic cell swelling or hyperosmotic cell shrinkage respectively. Such a behaviour of pH_{cyt} in response to aniso-osmolarity has already been described by the others in isolated hepatocytes [8,26] and other cell types, such as osteosarcoma cell lines [27].

These changes of pH_{cyt} may be explained by an activation/inhibition of the Na^+/H^+ antiporter during regulatory volume increase/decrease which is initiated by hyperosmotic/hypo-osmotic exposure [24,25,28]. Both pH_{cyt} and apparent pH_{ves} ,

Table 2 Effects of hydroperoxides in the absence and presence of Ba²⁺ on cytosolic and vesicular pH in isolated rat hepatocytes

Cytosolic (pH_{cyt}) and apparent vesicular pH (pH_{ves}) were determined by means of BCECF and FITC-dextran fluorescence respectively (for details see the Materials and methods section). The effector-induced change in subcellular pH was determined in the individual hepatocyte. In the presence of normo-osmotic Krebs-Henseleit medium with no effectors present, the pH_{cyt} was 7.23 ± 0.03 ($n = 108$) and the apparent pH_{ves} was 6.07 ± 0.02 ($n = 156$). Data are given as means $\pm$ S.E.M. (n). (–) and (+) denote decrease or increase of respective pH values.

Condition	Change of subcellular pH	
	pH _{cyt}	Apparent pH _{ves}
t-Butylhydroperoxide (10 μ mol/l)	n.d.	-0.09 ± 0.02 (5)
t-Butylhydroperoxide (50 μ mol/l)	n.d.	-0.12 ± 0.02 (6)
t-Butylhydroperoxide (200 μ mol/l)	0.00 ± 0.00 (9)	-0.20 ± 0.02 (10)
t-Butanol (200 μ mol/l)	0.00 ± 0.00 (5)	0.00 ± 0.00 (5)
H ₂ O ₂ (200 μ mol/l)	0.00 ± 0.00 (5)	-0.16 ± 0.02 (13)
t-Butylhydroperoxide (200 μ mol/l) in the presence of BaCl ₂ (1 mmol/l)	n.d.	-0.08 ± 0.01 (10)
H ₂ O ₂ (200 μ mol/l) in the presence of BaCl ₂ (1 mmol/l)	n.d.	-0.09 ± 0.01 (7)

Table 3 Effects of colchicine and lumicolchicine on the aniso-osmolarity- and hydroperoxide-induced change of apparent pH_{ves}

The effects of aniso-osmolarity and hydroperoxides on apparent vesicular pH (pH_{ves}) were determined by means of FITC-dextran fluorescence in control cells or cells pretreated for 1 h with colchicine (5 μ mol/l) or lumicolchicine (5 μ mol/l) (for details see the Materials and methods section). The effector-induced change in apparent pH_{ves} was determined in the individual hepatocyte. In the presence of normo-osmotic Krebs-Henseleit medium with no effectors present, the apparent pH_{ves} was 6.07 ± 0.02 ($n = 156$) and was not significantly affected by colchicine [6.20 ± 0.05 (7)] or lumicolchicine [6.20 ± 0.06 (6)]. Data are given as means $\pm$ S.E.M. (n). (–) and (+) denote decrease or increase of apparent pH_{ves}.

Condition	Change of the pH _{ves}		
	Control	Colchicine	Lumicolchicine
Hypotonic (185 mmol/l)	0.20 ± 0.05 (9)	0.05 ± 0.02 (7)	0.16 ± 0.03 (6)
Hypertonic (425 mmol/l)	-0.15 ± 0.03 (7)	-0.04 ± 0.01 (8)	-0.09 ± 0.01 (6)
t-Butylhydroperoxide (0.2 mmol/l)	-0.20 ± 0.02 (10)	-0.09 ± 0.01 (8)	-0.20 ± 0.02 (7)
H ₂ O ₂ (0.2 mmol/l)	-0.16 ± 0.02 (13)	-0.08 ± 0.01 (5)	-0.19 ± 0.01 (7)
NH ₄ Cl (20 mmol/l)	0.48 ± 0.05 (27)	0.37 ± 0.04 (6)	0.40 ± 0.04 (6)

however, are shifted to more alkaline values in response to NH₄Cl, which is known to enter cellular compartments as NH₃ by non-ionic diffusion and to induce proton trapping (Figure 3).

Effect of amino acids on apparent pH_{ves}

As shown in Table 1, addition of glutamine (3 mmol/l), which is known to induce rapid hepatocyte swelling [1], also led to an increase of apparent pH_{ves}. This was also found upon addition of histidine (3 mmol/l), but not in response to leucine (3 mmol/l) or proline (3 mmol/l). Glutamine, histidine and proline led to a slight increase of pH_{cyt}, whereas leucine was ineffective.

Effect of hydroperoxides on subcellular pH

H₂O₂ and t-BOOH were recently shown to induce hepatocellular shrinkage due to opening of Ba²⁺-sensitive K⁺ channels in the

plasma membrane [17,18]. When added at a concentration of 0.2 mmol/l to perfused rat liver, these hydroperoxides lowered cell water content by 14 % and 10 % respectively; an effect which diminished by about 60 % in presence of the K⁺-channel blocker Ba²⁺ (1 mmol/l) [17,18]. As shown in Figure 5 and Table 2, both, H₂O₂ (0.2 mmol/l) and t-BOOH (0.2 mmol/l) were without effect on pH_{cyt}, but led to a decrease of apparent pH_{ves}, which was fully reversible upon removal of the hydroperoxides from the superfusion medium. On the other hand t-butanol, the product of t-BOOH metabolism by glutathione peroxidase had no effect on subcellular pH (Table 2). A decrease of apparent pH_{ves} in response to t-BOOH occurred already at concentrations as low as 10 μ mol/l (Table 2). Ba²⁺ (1 mmol/l), which produces slight cell swelling in perfused rat liver [4], led to an increase of apparent pH_{ves} (Table 1) and diminished the hydroperoxide-induced acidification of FITC-dextran-accessible vesicles by about 50 % (Table 2). This suggests that the effect of hydroperoxides on apparent pH_{ves} is probably not due to the hydroperoxide itself, but may relate to its cell volume effects.

Colchicine sensitivity of the hydroperoxide and aniso-osmolarity effects on apparent pH_{ves}

Pretreatment of the hepatocytes for 60 min with colchicine (5 μ mol/l) slightly increased the apparent pH found in FITC-dextran-accessible vesicles from 6.07 ± 0.02 ($n = 156$) to 6.20 ± 0.05 ($n = 7$); the effect, however, is not significant. In colchicine-pretreated hepatocytes the effects of hypo- and hyper-osmotic exposure, as well as of hydroperoxides, were diminished by about 70–80 %, whereas the alkalinizing effect of NH₄Cl was preserved (Table 3). On the other hand, in the presence of lumicolchicine (5 μ mol/l), a stereoisomer of colchicine which has no effect on microtubules [29,30] produced an apparent pH_{ves} similar to colchicine [6.20 ± 0.06 ($n = 6$) versus 6.20 ± 0.05 ($n = 7$)], however, in the presence of lumicolchicine the effects of aniso-osmolarity on apparent pH_{ves} were largely preserved (Table 3).

DISCUSSION

Aniso-osmotic exposure and pH_{ves}

The data show a rapid and reversible alkalinization of intracellular endocytotic compartments and a simultaneous slight acidification of the cytosol in response to hypo-osmotic cell swelling; opposite pH changes occur in response to hyperosmotic cell shrinkage. Similar observations were made recently with respect to Acridine Orange fluorescence [8,31,32]. As shown here with FITC-dextran, the effects are not transient, but they are fully reversible upon normotonic re-exposure. Thus the aniso-tonicity effects on apparent pH_{ves} indicate that the cell is left in an altered functional state even after completion of volume regulatory events, as it was shown for a variety of metabolic pathways, which are controlled by hepatocellular hydration (for review see [1]). Taking into account previous data on aniso-osmolarity-induced liver cell volume changes [1,33,34], aniso-osmotic modulation of cell volume by about ± 15 % is expected to modulate apparent pH_{ves} by 0.2–0.3 pH unit (Figure 4). It is at present speculative whether such small pH shifts in the acidic FITC-dextran-accessible vacuolar compartment are sufficient to explain cell volume effects on some metabolic pathways such as proteolysis [4] or the putative insertion/retrieval of bile salt transporter molecules in the canalicular membrane [6]. Here, one

has to keep in mind that for reasons outlined in the Materials and methods section the changes of apparent pH_{ves} may underestimate the pH changes occurring in individual vesicles. In line with this, NH_4Cl , at concentrations known to inhibit strongly lysosomal proteolysis due to lysosomal alkalization [35], also led to a comparatively small increase of apparent pH_{ves} by 0.48 pH unit (Table 1). Thus cell volume effects on vesicular acidification may contribute to the regulation of proteolysis by cellular hydration; however, the possibility of other mechanisms being equally or more important is not ruled out. Clearly, the FITC-dextran-accessible acidic vesicular compartment is as yet ill-defined, but lysosomes probably make up less than 50% of this compartment in hepatocytes [16]. Furthermore, the possibility also cannot be ruled out that with our experimental setup only a subfraction of lysosomes is reached by the endocytosed FITC-dextran and that we are most likely dealing preferentially with prelysosomal endocytotic compartments. Indeed, only about 40% of these FITC-dextran-accessible vesicles were recently identified as lysosomes, with the remainder constituting a so-called endocytotic storage compartment [16]. This may explain why apparent pH_{ves} found in our study was about 6, whereas intralysosomal pH was reported to be 5.0–5.5 (for review see [20]). Recent flow-cytometry studies [36] suggested the existence of three distinct classes of endocytotic vesicles involved in fluid-phase endocytosis of FITC-dextran. Although there is some evidence that vacuolar acidification probably starts rather early in the maturation of endocytotic vesicles [20], one may speculate that the extent of the acidification in these different endocytotic vesicle populations is different and less compared with lysosomes. Since intravesicular pH is considered to be essential for the regulation of sorting and the uncoupling of receptor-ligand complexes [19,20], the interesting question arises as to what extent such processes are modified by alterations of cellular hydration.

The finding that the effects of aniso-osmolarity on apparent pH_{ves} were sensitive to colchicine, but not to lumicolchicine, suggests that intact microtubules are required for the regulation of apparent pH_{ves} by the cellular hydration state. However, it remains to be established whether the colchicine-sensitivity of other cell volume effects, e.g. on proteolysis [7] and bile acid excretion [6], are causally related to the colchicine-sensitivity of volume effects on vesicular acidification.

Oxidative stress and vesicular pH

Interestingly, hydroperoxides produce vesicular acidification opening another interesting perspective on the understanding of the functional consequences associated with oxidative stress (for reviews see [37]). Several lines of evidence suggest an involvement of hydroperoxide-induced cell shrinkage in triggering the hydroperoxide-induced vesicular acidification. (i) Both hyperosmotic exposure and hydroperoxides produce hepatocellular shrinkage and lower apparent pH_{ves} . (ii) Both, the effect of hydroperoxide-induced cell shrinkage and vesicular acidification are diminished in the presence of Ba^{2+} by 50–70%. (iii) Both, the hyperosmolarity- and hydroperoxide-induced vesicular acidification are sensitive to colchicine.

Amino acids and pH_{ves}

The data on aniso-osmotic-, Ba^{2+} - and hydroperoxide-induced changes of apparent pH_{ves} are compatible with the view that cell hydration may be the common denominator triggering the pH

shifts in the vesicular compartments. In line with this glutamine and histidine, which induce cell swelling [38,39], led to vesicular alkalization, whereas leucine, which had no effect on cell volume [38], was ineffective. However, the possibility cannot be ruled out that glutamine-induced alkalization of the FITC-dextran-accessible compartment is in part due to NH_3 formation by glutaminase. A major role for glutamine-derived NH_3 , however, is considered unlikely in view of the lag-phase of glutaminase, the use of an NH_3 -free non-recirculating superfusion system and the essential requirement of glutaminase for its activator NH_3 (for review see [40]). On the other hand, proline, which also induces cell swelling [38], had no detectable effect on vesicular pH. The reason for this is unclear.

In the course of our studies we noted that the effects of aniso-osmotic exposure, NH_4Cl , or of hydroperoxides on pH_{ves} were found in all liver cells examined, whereas the apparent pH_{ves} did not respond to amino acid addition in all hepatocytes studied (this was not taken into account in the mean values for apparent pH_{ves} shown in Table 1, where the data from all recordings were acknowledged). For example upon addition of glutamine or histidine only about 70% of the cells examined responded with an increase of apparent pH_{ves} . The most likely explanation for this may be hepatocyte heterogeneity in concentrative amino acid transport across the plasma membrane (for a review see [41]), the activity of which is expected to determine the extent of amino-acid-induced cell swelling.

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