Characterization of the swelling-induced alkalinization of endocytotic vesicles in fluorescein isothiocyanate–dextran-loaded rat hepatocytes

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Short-term cultivated rat hepatocytes were allowed to endocytose fluorescein isothiocyanate (FITC)-coupled dextran and the apparent vesicular pH ($pH_{ves.}$) was measured by single-cell fluorescence. After 2 h of exposure to FITC–dextran, the apparent pH in the vesicular compartments accessible to endocytosed FITC–dextran was 6.01 ± 0.05 ($n = 39$) in normo-osmotic media. Hypo-osmotic exposure increased, whereas hyper-osmotic exposure decreased apparent $pH_{ves.}$ by 0.18 ± 0.02 ($n = 26$) and 0.12 ± 0.01 ($n = 23$) respectively. Incubation of the cells with unlabelled dextran for 2h before a 2-h FITC–dextran exposure had no effect on apparent pH_{ves} and its osmosensitivity. When, however, hepatocytes were exposed to unlabelled dextran for 5 h after a 2 h exposure to FITC–dextran, in order to allow transport of endocytosed FITC–dextran to late endocytotic/lysosomal compartments, apparent $pH_{ves.}$ decreased to 5.38 ± 0.04 ($n = 12$) and the apparent pH in the vesicular compartment containing the dye was no longer sensitive to aniso-osmotic exposure. These findings indicate that the osmosensitivity of $pH_{ves.}$ is apparently restricted to early endocytotic compartments. Aniso-osmotic regulation of apparent $pH_{ves.}$ in freshly FITC-loaded hepatocytes was not accompanied by aniso-osmolarity-induced changes of the cytosolic free calcium concentration, and neither vasopressin nor extracellular ATP, which provoked a marked Ca^{2+} signal, affected apparent $pH_{ves.}$. Dibutyryl-cyclic AMP (cAMP) or vanadate (0.5 mmol/l) were without effect on apparent $pH_{ves.}$ and its osmosensitivity. However, pertussis toxin-treatment or genistein (but not daidzein) or the erbstatin analogue methyl 2,5-dihydroxycinnamate fully abolished the osmo-sensitivity of apparent $pH_{ves.}$, but did not affect apparent $pH_{ves.}$. It is concluded that regulation of $pH_{ves.}$ by cell volume occurs in early endocytotic compartments, but probably not in lysosomes, and is mediated by a G-protein and tyrosine kinase-dependent, but Ca^{2+}- and cAMP-independent mechanism.

INTRODUCTION

Alterations of hepatocellular hydration, which reflects 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 another signal transduction pathway being involved in hormone and amino acid action, which influences a variety of metabolic liver functions and gene expression [1]. 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 [1–4]. Recent studies using Acridine Orange or fluorescein isothiocyanate (FITC)–dextran fluorescence have indicated that cell swelling increases, whereas cell shrinkage decreases the pH in vesicular compartments ($pH_{ves.}$) [5–7]. However, it remained open whether the pH in all vesicular compartments or only a subfraction of them is cell volume-sensitive. Likewise, the mechanism of the swelling-induced vesicular alkalinization remained obscure, although evidence was presented for a requirement of intact microtubules, as suggested by the colchicine-sensitivity of $pH_{ves.}$ regulation by cell volume [6,7]. These questions were addressed in the present study, which shows that regulation of $pH_{ves.}$ by cell volume occurs in early but not in late endocytotic compartments and is mediated by a G-protein- and tyrosine kinase-dependent, but Ca^{2+}- and cyclic AMP (cAMP)-independent mechanism.

MATERIAL AND METHODS

Nigericin, ionomycin, cholera toxin, staurosporine, sodium orthovanadate, vasopressin, L-histidine and FITC–dextran (average molecular mass 70 kDa), and Mes were from Sigma (Munich, Germany). Pluronic F-127 and the acetoxymethylester of fura-2 were from Molecular Probes Inc. (Eugene, OR, U.S.A.). Daidzein was from Biomol Res. Lab. Inc. (Hamburg, Germany), genistein and erbstatin analogue (methyl 2,5-dihydroxycinnamate) were from Calbiochem-Novabiochem GmbH (Bad Soden, Germany), pertussis toxin was from Research Biochemicals Incorporated (Natick, MA, U.S.A.), dibutyryl-cAMP, UTP and ATP were from Boehringer (Mannheim, Germany). L-Glutamine was from Merck (Darmstadt, Germany).

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.

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 [8], and plated on collagen-coated glass coverslips (diameter 30 mm) at a density of about 5×10^5 cells/ml. After 2 h cells were washed twice and cultured at 37 °C in a Krebs–Henseleit medium supplemented with glucose (6 mmol/l) (KHB) in a 5 % CO_2 atmosphere for 9 h. Cell viability before use for single-cell fluorescence measurements was more than 95 % as assessed by Trypan Blue exclusion. Primary hepatocytes were then incubated in the above-mentioned KHB with the acetoxy-

Abbreviations used: FITC–dextran, fluorescein isothiocyanate–dextran (average molecular mass 70 kDa); erbstatin analogue, methyl 2,5-dihydroxycinnamate; KHB, Krebs–Henseleit buffer; $[Ca^{2+}]_i$, cytosolic free Ca^{2+} concentration; MAP, mitogen-activated protein.

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methylester of fura-2 (5 μmol/l) and Pluronic (0.02% v/v) for 30 min at 37 °C or with FITC–dextran (5 mg/ml). If not indicated otherwise, the FITC–dextran loading period was 2 h and pH measurements under several conditions were taken within the first 20 min after the loading period. In the case of cholera toxin- and pertussis toxin-pretreatment, cells were exposed simultaneously to cholera toxin or pertussis toxin and FITC–dextran for 3 h. This procedure resulted in an apparent $pH_{ves.}$ of 6.14 ± 0.13 ($n = 7$) and 5.96 ± 0.06 ($n = 11$) in the presence of cholera toxin and pertussis toxin respectively, i.e. the value after 3 h FITC–dextran loading in untreated cells was 6.09 ± 0.06 ($n = 16$) and did not show a significant difference to 2 h FITC–dextran-loaded cells [apparent $pH_{ves.}$ of 6.07 ± 0.02 ($n = 156$)]. In the case of genistein, daidzein and staurosporine pretreatment, cells were exposed to these compounds during the last 30 min of FITC–dextran loading. For erbstatin-analogue pretreatment, cells were exposed to this compound during the last 60 min of FITC–dextran loading. To examine the effect of dibutyryl-cAMP and vanadate, these compounds were added to the cells 2–5 min and 5 min respectively, before pH measurements were made.

Thus, hepatocytes used for the fluorescence measurement 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 the KHB equilibrated with O_2/CO_2 (95:5, v/v), resulting in a pH of 7.4. The temperature was 37 °C. FITC–dextran- and fura-2-loaded hepatocytes were used for assessment of apparent $pH_{ves.}$ and cytosolic calcium ($[Ca^{2+}]_i$), respectively. Measurements of apparent $pH_{ves.}$ and $[Ca^{2+}]_i$ in single cells were performed with an inverted fluorescence microscope set-up 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 the excitation wavelengths of 488/440 nm and 340/380 nm respectively. The emitted light was directed through a 480–520 bandpass filter for FITC–dextran fluorescence and though a 515–565 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). The 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 the autofluorescence obtained as pH-independent signal (excitation at 440 nm) was 20–50%. For both 340 and 380 nm excitation the autofluorescence was low (about 3 and 5% respectively). The autofluorescence ratios (488/440 nm) and (340/380 nm) were not affected by aniso-osmotic exposure and treatments.

Calibration of apparent $pH_{ves.}$ and $[Ca^{2+}]_i$

Apparent $pH_{ves.}$ values were obtained from the corrected ratios of 488/440 nm fluorescence after appropriate calibration according to [9]. In brief, this calibration procedure involved the superfusion of cells with solutions containing KCl (105 mosmol/l)/$MgCl_2$ (1 mmol/l)/Hepes (30 mmol/l; pH varied between 6.6 and 7.4) or Mes (30 mmol/l; pH varied between 5 and 6) and nigericin (5 μmol/l). The fluorescence ratio was linear with $pH_{ves.}$ between 5 and 7.4 [6].

$[Ca^{2+}]_i$ was calculated from the ratio of 340 nm/380 nm fluorescence values (after subtraction of background fluorescence) as described in [10].

The formula used for calculation of $[Ca^{2+}]_i$ was

$$[Ca^{2+}]_i = K_d(R - R_{min.})/(R_{max.} - R)(Sf_2/Sb_2)$$

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 Ca^{2+}-bound fura-2 at 380 nm) were calculated by using 1 μmol/l ionomycin and 5 mmol/l EGTA to equilibrate intracellular and extracellular Ca^{2+} in intact fura-2-loaded cells. The dissociation constant for the fura-2·Ca^{2+} complex was taken as 224 nmol/l [10].

Analysis of results

Data are expressed as means ± S.E.M. (n = number of coverslips studied). For each series, cells were prepared from at least three different livers. Results were compared using the Student's t-test; $P < 0.05$ was considered statistically significant.

RESULTS

Heterogeneity of endocytotic compartments with respect to cell volume-sensitive acidification

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 [11,12]. As shown recently [6,7], loading of primary hepatocytes with FITC–dextran for 2 h produced a granular staining of the cells, consistent with the presence of the dye in endocytotic vesicles. These vesicles were distributed throughout the cytoplasm, with some enrichment found in the perinuclear area [6,13]. Single-cell fluorescence recordings obtained from cells within 20 min after the 2 h FITC–dextran loading period and being superfused with normo-osmotic (305 mosmol/l) medium yielded an apparent $pH_{ves.}$ of 6.01 ± 0.05 ($n = 39$) in the vesicular compartment, being defined by its accessibility to the dye within about 2 h. Hypo-osmotic or hyper-osmotic exposure due to a corresponding decrease or increase of the NaCl concentration in the superfusion medium led to a rapid increase or decrease of apparent $pH_{ves.}$ respectively (Table 1), in line with previous studies [6]. The effects of aniso-osmolarity on apparent vesicular pH were rapidly reversible upon restoration of normo-osmotic superfusion conditions (results not shown, but see [6]). Exposure of the cells to unlabelled dextran for 5 h before the 2 h loading period with FITC–dextran had no effect on apparent $pH_{ves.}$ (6.01 ± 0.05 versus 6.11 ± 0.05 without and with 5 h pre-exposure to dextran) or the osmosensitivity of apparent $pH_{ves.}$ (Table 1). When, however, the cells were exposed to unlabelled dextran for 1–5 h after the 2 h loading period with FITC–dextran, the apparent $pH_{ves.}$ progressively decreased to 5.38 ± 0.04 ($n = 12$) and the effect of aniso-osmolarity on apparent $pH_{ves.}$ gradually disappeared (Table 1). These findings suggest that endocytosed FITC–dextran first enters a less acidic endocytotic compartment ('early endocytotic vesicles') which exhibits a cell volume-sensitive acidification, and is then transported during the next 5 h to a more acidic compartment ('late endocytotic vesicles'), whose acidification is no longer cell volume-sensitive. A similar conclusion

Table 1 Heterogeneity of endocytotic compartments with respect to cell volume-sensitive acidification

Hepatocytes were exposed to unlabelled dextran (5 mg/ml) for 0–5 h after a 2 h loading period with FITC–dextran (5 mg/ml). For control, cells were first exposed for 3 or 5 h to unlabelled dextran before the 2 h loading period with FITC–dextran. The effect of aniso-osmolarity, amino acids and t-butylhydroperoxide (t-BOOH) on apparent $pH_{ves.}$ were determined in the individual hepatocytes (for details see the Materials and methods section). When NH_4Cl (20 mmol/l) was added, extracellular osmolarity was kept constant by lowering the NaCl concentration by 20 mmol/l. Data are given as means ± S.E.M. (*n*). (−) and (+) denote decrease or increase of apparent $pH_{ves.}$. *$P < 0.05$ by Student's *t* test; n.d. = not determined.

		Change of $pH_{ves.}$ induced by					
No pre-exposure to dextran Time after FITC–dextran loading (h)	Apparent $pH_{ves.}$	Hypo-osmotic exposure (185 mosmol/l)	Hyper-osmotic exposure (425 mosmol/l)	L-Glutamine (3 mmol/l) (308 mosmol/l)	L-Histidine (3 mmol/l) (308 mosmol/l)	t-BOOH (0.2 mmol/l) (305 mosmol/l)	NH_4Cl (20 mmol/l) (305 mosmol/l)
0	6.01 ± 0.05 (39)	+ 0.18 ± 0.02 (26)	− 0.12 ± 0.01 (23)	+ 0.06 ± 0.01 (15)	+ 0.06 ± 0.01 (12)	− 0.19 ± 0.02 (14)	+ 0.32 ± 0.03 (25)
1	6.0 ± 0.09 (7)	+ 0.18 ± 0.02 (6)	− 0.12 ± 0.03 (5)	n.d.	n.d.	n.d.	+ 0.54 ± 0.14 (4)*
2	5.86 ± 0.07 (13)	+ 0.17 ± 0.06 (5)	− 0.06 ± 0.02 (6)*	n.d.	n.d.	n.d.	+ 0.62 ± 0.14 (5)*
3	5.59 ± 0.03 (22)*	+ 0.05 ± 0.02 (8)*	− 0.05 ± 0.02 (6)*	n.d.	n.d.	n.d.	+ 0.87 ± 0.09 (9)*
5	5.38 ± 0.04 (12)*	+ 0.02 ± 0.01 (7)*	− 0.01 ± 0.01 (7)*	0.00 ± 00 (8)*	0.00 ± 0.00 (8)*	− 0.02 ± 0.01 (7)*	+ 0.75 ± 0.08 (15)*
Pre-exposure to dextran Time of exposure before the FITC–dextran loading period (h)							
3	6.02 ± 0.09 (10)	+ 0.17 ± 0.02 (10)	− 0.11 ± 0.01 (9)	n.d.	n.d.	n.d.	+ 0.36 ± 0.05 (9)
5	6.11 ± 0.05 (19)	+ 0.18 ± 0.01 (9)	− 0.13 ± 0.01 (7)	n.d.	n.d.	n.d.	+ 0.35 ± 0.08 (14)

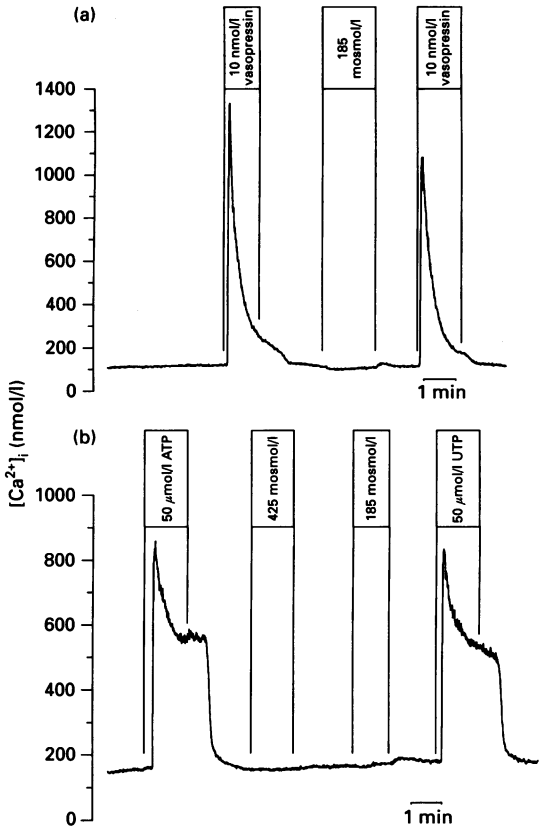

Figure 1 Effect of aniso-osmolarity on $[Ca^{2+}]_i$

$[Ca^{2+}]_i$ was assessed by means of fura-2 fluorescence (for details see the Materials and methods section). During normo-osmotic superfusion, $[Ca^{2+}]_i$ was 188 ± 6 nmol/l ($n = 83$). Representative tracings from at least five different experiments are shown in (**a**) and (**b**).

was reached when the effect of iso-osmotic cell volume modulators on pH was studied in 'early and late' endocytotic vesicles. Hydroperoxides were shown to induce K^+ channel opening [14] and the accompanying cell shrinkage to produce vesicular acidification [6]. Similarly, glutamine and histidine, which induce cell swelling, led to vesicular alkalinization [6]. As shown in Table 1, the effects of t-butylhydroperoxide, glutamine or histidine on apparent $pH_{ves.}$ were only observed in 'early', but not in 'late' endocytotic vesicles. As also shown in Table 1, the 'early' and 'late' compartment also differed with regard to the alkalinizing effect of added NH_4Cl, which was more pronounced in 'late' endocytotic vesicles.

Mechanism of swelling-induced alkalinization of early endocytotic vesicles

The question was addressed whether anisotonicity-induced changes in $[Ca^{2+}]_i$ could explain the cell volume-sensitivity of acidification in the early FITC–dextran-accessible endocytotic compartment. Therefore, the effect of anisotonicity on fura-2 fluorescence was studied. During normo-osmotic superfusion conditions, the intracellular calcium concentration was 188 ± 6 nmol/l ($n = 83$). As shown in Figure 1, neither hypo-osmotic nor hyper-osmotic exposure significantly affected $[Ca^{2+}]_i$, whereas vasopressin or extracellular ATP or UTP (Figure 1) induced marked Ca^{2+} transients. These data suggest that changes in intracellular Ca^{2+} are probably not involved in the regulation

Table 2 Effects of cAMP and inhibitors of signal transduction on the aniso-osmolarity- and NH_4Cl-induced change of apparent $pH_{ves.}$

The effects of aniso-osmolarity on apparent $pH_{ves.}$ were determined by means of FITC–dextran fluorescence in control cells or cells pretreated for 3 h with pertussis toxin (100 ng/ml) or choleratoxin (10 μg/ml), for 30 min with genistein (5 μmol/l), daidzein (5 μmol/l) or staurosporine (100 nmol/l), for 60 min with erbstatin-analogue (5 μmol/l), for 5 min with vanadate (500 μM) or for 2–5 min with dibutyryl-cAMP (50 μmol/l). The effect of ATP (50 μmol/l) and vasopressin (1 nmol/l) on apparent $pH_{ves.}$ were determined (for details see the Materials and methods section). The effector-induced change in apparent $pH_{ves.}$ was determined in individual hepatocytes. The FITC–dextran loading period was 2 h, in the case of cholera toxin and pertussis toxin pretreatment, the loading period was 3 h. When NH_4Cl (20 mmol/l) was added, extracellular osmolarity was kept constant by lowering the NaCl concentration by 20 mmol/l. Data are given as means ± S.E.M. (*n*). (−) and (+) denote decrease or increase of $pH_{ves.}$. *$P < 0.05$ by Student's *t*-test; n.d. = not determined.

Conditions	Apparent $pH_{ves.}$	Change of $pH_{ves.}$ induced by		
		Hypo-osmotic exposure (185 mosmol/l)	Hyper-osmotic exposure (425 mosmol/l)	NH_4Cl (20 mmol/l)
Control (2 h FITC–dextran loading)	6.07 ± 0.02 (156)	+0.18 ± 0.02 (26)	−0.12 ± 0.01 (23)	+0.32 ± 0.03 (25)
Daidzein	6.05 ± 0.07 (9)	+0.17 ± 0.02 (9)	−0.12 ± 0.01 (7)	+0.48 ± 0.07 (9)
Genistein	6.09 ± 0.07 (12)	+0.05 ± 0.02 (11)*	−0.04 ± 0.01 (10)*	+0.44 ± 0.06 (10)
Erbstatin-analogue	6.22 ± 0.09 (6)	+0.01 ± 0.02 (6)*	+0.02 ± 0.02 (6)*	+0.32 ± 0.05 (6)
Staurosporine	6.19 ± 0.08 (7)	+0.20 ± 0.02 (7)	−0.13 ± 0.03 (6)	+0.36 ± 0.02 (6)
Dibutyryl-cAMP	6.00 ± 0.12 (6)	+0.22 ± 0.03 (6)	−0.14 ± 0.02 (6)	+0.33 ± 0.05 (6)
Vanadate	5.98 ± 0.05 (6)	+0.19 ± 0.02 (6)	−0.13 ± 0.02 (6)	+0.34 ± 0.04 (6)
ATP	5.95 ± 0.11 (7)	n.d.	n.d.	n.d.
Vasopressin	5.99 ± 0.11 (8)	n.d.	n.d.	n.d.
Control (3 h FITC–dextran loading)	6.09 ± 0.06 (16)	+0.17 ± 0.02 (14)	−0.11 ± 0.01 (11)	+0.39 ± 0.11 (7)
Pertussis toxin	5.96 ± 0.06 (11)	+0.03 ± 0.01 (8)*	−0.01 ± 0.01 (8)*	+0.46 ± 0.05 (6)
Cholera toxin	6.14 ± 0.13 (7)	+0.02 ± 0.02 (6)*	−0.01 ± 0.01 (6)*	+0.44 ± 0.14 (7)

of vesicular acidification by cell volume. This view is further augmented by the finding that neither extracellular ATP nor vasopressin, which elicited marked transients of $[Ca^{2+}]_i$, had any detectable effect on $pH_{ves.}$ in 2h FITC–dextran-loaded hepatocytes. Also, addition of dibutyryl cAMP (50 μmol/l) had no effect on $pH_{ves.}$ and its osmo-sensitivity (Table 2), suggesting that anisotonicity-induced changes of the cellular cAMP level are not involved in the regulation of $pH_{ves.}$ by cell volume.

Neither genistein (5 μmol/l), an inhibitor of tyrosine kinases, nor its inactive analogue daidzein (5 μmol/l) affected apparent $pH_{ves.}$ under normo-osmotic conditions (Table 2). Interestingly, however, in the presence of genistein, but not daidzein, the effects of aniso-osmotic exposure on apparent $pH_{ves.}$ were almost completely abolished (Table 2). Also the erbstatin analogue, methyl 2,5-dihydroxycinnamate, which acts as a tyrosine kinase inhibitor, completely abolished the volume sensitivity of apparent $pH_{ves.}$ (Table 2). On the other hand, vanadate, which is known to act as an unspecific inhibitor of phosphate transferring enzymes and various ATPases [13,15], and staurosporine were without effect on $pH_{ves.}$ and its osmosensitivity (Table 2).

Pretreatment of the cells with pertussis or cholera toxin had no effect on the apparent $pH_{ves.}$, yet caused the sensitivity of vesicular acidification to aniso-osmotic cell volume changes to disappear (Table 2). None of the inhibitors tested had a significant effect on NH_4Cl-induced alkalinization (Table 2).

DISCUSSION

Vesicle heterogeneity with respect to volume-dependent acidification

FITC–dextran is taken up into hepatocytes by fluid-phase endocytosis [11] and the vesicular compartment reached by the dye is heterogeneous and dependent upon the length of exposure to the dye [11,16,17]. Lysosomes and endosomes exhibit an acidic pH, which is dependent upon the activity of both a proton ATPase and Cl^- conductance, which is required to dissipate the membrane potential generated by the proton pump [16–23]. Acidification is known to increase from early to late endosomes and to lysosomes, in which the lowest $pH_{ves.}$ (5.0–5.5) is reached [22,23]. The mechanisms underlying these differences in steady-state acidification of endosomes and lysosomes respectively, may be due to a functional heterogeneity between H^+-ATPases in different organelles, different proton leak rates and ion conductances [17]. Endocytosed FITC–dextran is transported in a time-dependent manner from an early endocytotic compartment to the lysosomal compartment [17]. This is also suggested by the experiments depicted in Table 1, indicating that endocytosed FITC–dextran is transported from an 'early' endocytotic compartment, which exhibits a pH of about 6 to a compartment which is more acidic (pH 5.4) and exhibits a pH close to that found in lysosomes. These compartments differ not only in the apparent $pH_{ves.}$, but also with respect to the osmosensitivity of $pH_{ves.}$. Apparently, $pH_{ves.}$ regulation by cell volume is restricted to the 'early' endocytotic compartment, suggesting that lysosomal pH is not affected by cell volume. Thus, the previously hypothesized role of vesicular pH in the mediation of proteolysis control by cell volume [24,25] probably does not involve lysosomes (as was discussed in [7,24,25]), but may largely be exerted at the level of a prelysosomal compartment. Here, it is of interest to note that evidence has been given recently to the acidity of prelysosomal/endocytic vacuoles (amphisomes), i.e. a fusion compartment of endocytotic vesicles with vacuoles formed by autophagic sequestration [26]. It should be noted that the terms 'early' and 'late' endocytotic vesicles used in the present paper are defined by the experimental protocol, i.e. the duration of exposure of cells to FITC–dextran and unlabelled dextran. Since the shortest exposure time was 2 h, it cannot be excluded that some FITC–dextran-containing lysosomes are also picked up fluorimetrically in the 'early' endocytotic compartment. Thus, the aniso-osmolarity-induced pH shifts in some vesicular subfractions of the 'early' endocytotic compartment may be higher than the apparent pH changes shown in the Tables suggest, because they were calculated from the ratio of protonated versus unprotonated FITC ('apparent $pH_{ves.}$').

Interestingly, the alkalinizing effect of NH_4Cl was more

pronounced in 'late' than in 'early' endocytotic vesicles accessible to FITC–dextran (Table 1). One interpretation for this observation could be a heterogeneity of endocytotic vesicles with respect to NH_3/NH_4^+ permeability.

Signalling events

Table 2 shows that both the swelling-induced alkalinization and the shrinkage-induced acidification of 'early' endocytotic vesicles is sensitive to genistein (but not daidzein), erbstatin and pertussis and cholera toxins. This tempts one to the assumption that regulation of vesicular acidification by cell volume involves G-proteins and a not yet identified tyrosine kinase. Tyrosine kinase inhibitors are known to be unspecific and to inhibit several metabolic pathways, especially at high concentrations [27–29]. However, comparatively low concentrations of inhibitor were used in the present study and the specificity of the genistein-effect is underlined by the finding that neither daidzein, a structural analogue of genistein without inhibitory action on tyrosine kinases, nor vanadate or staurosporine abolished the cell volume-sensitivity of $pH_{ves.}$ regulation. Furthermore, erbstatin, a potent tyrosine kinase inhibitor which is structurally not related to genistein, gave a similarly inhibitory effect as genistein. From these findings, it is concluded that inhibition of the volume-sensitivity of $pH_{ves.}$ regulation by genistein and erbstatin can indeed be ascribed to tyrosine kinase inhibition. Alterations of protein phosphorylation are known to occur in response to osmotic water shifts across the plasma membrane (for review see [1,30]), and the recently recognized association between tubulin and G-proteins, including a role of the cytoskeleton in signal transduction in brain [31], may explain the recently described colchicine sensitivity of $pH_{ves.}$ regulation by cell volume [6,7]. The findings in Table 2 also rule out the possibility that regulation of $pH_{ves.}$ by cell volume is due to alterations of intracellular Cl^-, which is required to dissipate the membrane potential generated by the H^+-pump in order to augment the acidification process. Evidence for a role of protein kinase A-dependent protein phosphorylation in the regulation of chloride channel activity and acidification in endosomes prepared from calf brain or rabbit proximal tubule has been given [32,33] and phosphoproteins in the endosomal membrane have been described recently [34]. However, protein kinase A is apparently not involved in the regulation of $pH_{ves.}$ in liver, as suggested by the lack of effect of cAMP. Recent studies carried out in non-hepatic cell types have indicated that hyperosmotic shock leads to the activation of protein kinases, which are related to the mitogen-activated protein (MAP) kinase family, but which are distinct from Erk-1 and Erk-2 [35–37]. The relevance of these findings for our present investigation is unclear. However, in rat hepatoma cells, a swelling-induced signal-transduction pathway has been identified which leads within 1 min to a G-protein- and tyrosine kinase-dependent activation of MAP kinases, Erk-1 and Erk-2 [38]. These characteristics of swelling-induced MAP kinase activation closely resemble those of the volume-dependent regulation of apparent $pH_{ves.}$ in 'early endocytotic vesicles' described here, and accordingly, it is suggested that Erk-1 and Erk-2 activation may play an important role in the link between cell volume and the apparent $pH_{ves.}$

Cell volume-dependent regulation of $pH_{ves.}$ probably does not involve alterations of $[Ca^{2+}]_i$, because Ca^{2+}-mobilizing hormones, such as extra-cellular ATP or vasopressin, had no effect on apparent $pH_{ves.}$ and because aniso-osmotic exposure of the hepatocytes had no effect on intracellular Ca^{2+}. This latter finding is at variance with a previous report [39]. The reason for this discrepancy is unknown, but may relate to the fact that cultured hepatocytes were used in the present study, whereas freshly isolated liver cells were used in [39].

Potential physiological relevance

At present little is known about the physiological regulators of endosomal acidification in liver, except that a decrease in acidification follows ethinyloestradiol administration [40] and that it is regulated by cell volume [6,7]. However, there is broad evidence that the acidification of endocytotic vesicles is essential for the regulation of sorting and uncoupling of receptor–ligand complexes and intracellular membrane flow [18,22], which may play a role in some disease states such as cystic fibrosis [41]. Because cell volume alterations can occur under a variety of physiological and pathophysiological conditions (for review see [1]), the interesting question arises as to what extent processes, such as receptor ligand sorting, are modified by cell volume-triggered changes of $pH_{ves.}$

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