Proc. Natl. Acad. Sci. USA
Vol. 91, pp. 9165–9169, September 1994
Physiology

Involvement of microtubules in the link between cell volume and pH of acidic cellular compartments in rat and human hepatocytes

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Communicated by Gerhard Giebisch, May 31, 1994

ABSTRACT **Cell swelling is shown to induce an increase in acridine orange fluorescence intensity, an effect pointing to the alkalinization of acidic vesicles. Since autophagic hepatic proteolysis is accomplished by pH-sensitive proteinases within acidic lysosomes, this effect may contribute to the well-known inhibitory effect of cell swelling on proteolysis. In the present study, the role of microtubules in volume-dependent alterations of pH in acidic vesicles of rat and human hepatocytes was studied. Colcemid and colchicine were used to depolymerize microtubules and vesicular pH was monitored using two different fluorescent dyes, fluorescein isothiocyanate conjugated-dextran and acridine orange. Colcemid and colchicine, but not the inactive stereoisomer γ-lumicolchicine, blunted the increase of pH during osmotic cell swelling. The alkalinization of acidic vesicles by NH_4Cl was not significantly modified by colcemid or colchicine, indicating that the vesicles were still sensitive to alkalinizing procedures other than cell swelling. Further, colchicine, but not γ-lumicolchicine, inhibited the antiproteolytic action of osmotic cell swelling. The present observations point to an involvement of the microtubule network in the link of cell volume, lysosomal pH, and proteolysis.**

It has recently become clear that cell volume is a major determinant of proteolysis (1, 2). Amino acids such as glutamine and hormones such as insulin have been shown to inhibit proteolysis in hepatocytes by inducing cell swelling. Amino acids elicit cell swelling by their concentrative uptake and cellular accumulation, while insulin exerts its effect by activation of ion uptake via Na^+, K^+, $2Cl^-$ cotransport and Na^+/H^+ exchange (1, 2). The antiproteolytic action of insulin and glutamine is fully mimicked by the respective osmotic alterations of cell volume and is abolished if alterations of cell volume are reversed by appropriate alterations of extracellular osmolarity. Further, inhibition of Na^+, K^+, $2Cl^-$ cotransport by furosemide or bumetanide not only blunts the swelling effect of insulin but also leads to a proportional inhibition of proteolysis. In the presence of both furosemide and amiloride, insulin-induced cell swelling and its antiproteolytic action are completely abolished. The mechanism linking cell volume alterations to proteolysis remained elusive, however, until recently when it was observed that cell swelling leads to an increase in acridine orange fluorescence intensity. This effect indicated an alkalinization of acidic intracellular compartments (3). Since proteolysis resides largely within acidic lysosomes and is accomplished by pH-sensitive lysosomal proteinases (4), the alkalinization of acidic intracellular compartments could indeed couple cell swelling to the inhibition of proteolysis. The mechanism by which cell volume changes are communicated to the acidic cellular compartments remained, however, unknown. In view of the described interaction between microtubules and intracellular vesicles (5, 6), the present study has been performed to examine a possible involvement of the microtubule network. To this end, the effect of cell swelling on fluorescein isothiocyanate (FITC)-dextran or acridine orange fluorescence has been studied both in intact cells and in cells treated with either colchicine or colcemid, drugs known to depolymerize microtubules (7, 8). In addition, a possible effect of γ-lumicolchicine, a stereoisomer of colchicine without an inhibitory effect on microtubules (9), was investigated.

METHODS

Cell Culture. Human hepatocytes were prepared from pieces of liver not appropriate for transplantation. The pieces were perfused through polyethylene catheters inserted into the main portal veins of the cut surface. The subsequent isolation procedure was according to the method of Berry and Friend (10) with some modifications (11). Rat hepatocytes were prepared by collagenase treatment according to a method previously described (12, 13). All cells were cultured in Dulbecco's modified Eagle medium supplemented with 10% fetal calf serum, 100 μg of penicillin/streptomycin per ml, 1 μM dexamethasone, 0.01 mM T_3, 0.01 mM T_4, and 5 μg of insulin per ml and were maintained at 37°C in a humidified atmosphere of 5% CO_2. Human cells were used after 1–2 days in culture and rat hepatocytes were used after 4–5 days in culture.

Depolymerization of Microtubules. To obtain depolymerization of microtubules, cells grown on glass coverslips were incubated for 60 min either with 2.5 or 5 μM colcemid (*N*-deacetyl-*N*-methylcolchicine; Serva) or with 5 μM colchicine (Sigma). In additional experiments, cells were treated with 5 μM γ-lumicolchicine for 60 min or with 5 μM colchicine for 5, 30, 60, or 80 min.

To verify the efficacy of the treatment with microtubule-depolymerizing agents, treated cells were fixed in methanol for 6 min at −20°C following the incubation period. Thereafter, hepatocytes were incubated for 45 min at 37°C with monoclonal antibodies against α-tubulin (Amersham). Following a wash with phosphate-buffered saline, a tetramethylrhodamine B isothiocyanate (TRITC)-conjugated goat anti-mouse IgG and IgM (Dianova, Hamburg, Germany) was applied for an additional 60 min at 37°C. For visualization of F-actin, cells were fixed with 3.5% formaldehyde (10 min at room temperature) and permeabilized with 1% Triton X-100 in phosphate-buffered saline (2 min at room temperature). F-actin was stained with 2 μg of TRITC-phalloidin per ml (Sigma) for 10 min at room temperature in the dark. Following a wash in phosphate-buffered saline, all samples were mounted in Mowiol 4-88 (Hoechst Pharmaceuticals). Cells

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Abbreviation: FITC, fluorescein isothiocyanate.

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were examined with a Nikon Optiphot microscope equipped for epifluorescence with appropriate filter sets for detecting TRITC fluorescence. Micrographs were taken with a Nikon Planapo 40/1.0 oil lens on Kodak Tri-X pan 400 film at 1600 ASA rating.

Determination of FITC-Dextran and Acridine Orange Fluorescence. Prior to experiments, hepatocytes were incubated for 2 hr with 70 μM FITC-dextran (Sigma) or for 15 min with 10 μM acridine orange hydrochloride (Sigma). The cells were bathed in extracellular fluid composed of (in mM) 115 NaCl, 21 $NaHCO_3$, 5 KCl, 1.3 $CaCl_2$, 1 $MgCl_2$, and 2 NaH_2PO_4, equilibrated to pH 7.4 by gassing with O_2/CO_2 (19:1) and maintained at 37°C. Hypoosmotic solution was prepared by reducing the content of NaCl by 40 or 60 mM. All chemicals used for the preparation of solutions were obtained from Sigma.

For the determination of FITC-dextran fluorescence, the excitation wavelength was alternated between 480 and 440 nm using a high-speed filter wheel (Nitschke, Fröbe, and Johna, Freiburg, Germany) and the emitted light was directed through a 520-nm cutoff filter into a photon counting tube (Hamamatsu H3460-04, Herrsching, Germany). Raw fluorescence signals were corrected for noise and autofluorescence, the latter being unaffected by anisotonic exposure. Specific vesicular pH was calculated following calibration of the dye (14). Briefly, cells were superfused with solutions containing KCl (105 mM), $MgCl_2$ (1 mM), Hepes (30 mM, with pH ranging from 6.6 to 7.4), or 2-(*N*-morpholino)ethanesulfonic acid (30 mM, with pH ranging from 5 to 6) and nigericin (5 μM). The fluorescence ratios at 480/440 were linear with vesicular pH between 5 and 7.4.

For the determination of acridine orange fluorescence, light (490 nm) from a monochromator light source (Uhl, Munich, Germany) was directed through grey filters (nominal transmission 0.7% Oriel, Darmstadt, Germany) and was deflected by a dichroic mirror (515 nm, Omega Optical, Brattleboro, VT) into the microscope objective (Plan-Neofluar 40×, Zeiss). Emitted fluorescence was directed through a 530-nm cutoff filter to a photomultiplier tube (213-IP28A, Seefelder Messtechnik, Seefeld, Germany). To decrease the size of the region from which the fluorescence was collected, a plate with a pinhole (diameter 1.6 mm) was placed in the image plan of the phototube. Fluorescence in the absence of acridine orange was <1% of that in the presence of acridine orange. This was not significantly modified by the experimental maneuvers and ruled out any interference by autofluorescence and scattered light. Data acquisition was executed using a computer program (IMG 8, Lindemann & Meiser, Homburg, Germany).

Perfusion of Liver. Livers from male Wistar rats (120–250 g of body weight) fed ad libitum on stock diet (Altromin) were perfused as described (15) in a nonrecirculating manner with bicarbonate-buffered Krebs–Henseleit saline plus L-lactate (2.1 mM), pyruvate (0.3 mM), and leucine (0.1 mM). The influent K^+ concentration was 5.9 mM. The perfusate was gassed with O_2/CO_2 (19:1) at 37°C. In isoosmotic perfusions, the osmolarity was 305 mOsm; hypoosmotic exposure was performed by lowering the NaCl concentration in the perfusion medium by 60 mM.

Determination of Proteolysis. The rate of proteolysis was assessed by measuring the release of ^3H from isolated perfused rat livers after prelabeling of liver proteins *in vivo* by intraperitoneal injection of 200 μCi of L-[4,5-^3H]leucine (1 Ci = 37 GBq) ≈16 hr prior to the perfusion experiment, as described (16). In all experiments, the influent perfusate was supplemented with unlabeled leucine (0.1 mM) in order to prevent reutilization of [^3H]leucine for protein synthesis and to accelerate the washout of preexisting free [^3H]leucine. Chromatographic analysis of the effluent perfusate revealed that ^3H released from the liver was >98% associated with leucine (16). As shown previously, hypoosmotic-induced alterations in [^3H]leucine release also paralleled alterations in leucine release from the liver, as determined by amino acid analysis (16). ^3H release into the perfusate during isoosmotic perfusions amounted to about 500 cpm/min per g of liver and was determined by scintillation spectrometry. Following a preperfusion period of ≈ 80 min, ^3H release from the liver reached a steady state that was maintained for at least an additional 100 min. In control experiments [^3H]leucine release decreased by only 0.06%/min. Radioactivity released under these conditions was observed to be derived from proteolysis.

Statistical Analysis. Data are expressed as arithmetic means ± standard error of the mean (SEM). Statistical analysis was made by paired or unpaired *t* test, where applicable. Statistically significant differences were assumed when $P < 0.05$.

RESULTS

A decrease of extracellular osmolarity (removal of 60 mM NaCl) led to an increase of the FITC-dextran fluorescence ratio, reflecting an increase of pH within FITC-dextran-containing compartments. This increase of fluorescence ratio was significantly blunted following treatment with colchicine for 30, 60, or 80 min but not by a 60-min treatment with γ-lumicolchicine (Table 1). The calibration procedure (14) generated an apparent mean vesicular pH of 6.02 ± 0.33 (n = 22) under isoosmotic conditions. The calculation, however, is biased if the population of FITC-dextran-containing vesicles is not homogenous. Any deviation from homogeneity favors overestimation of mean vesicular pH and underestimation of alterations. Since we cannot rely on homogeneity of the vesicle preparation, the results (Table 1) are expressed in terms of "apparent pH" values. A shift in pH of +0.21 ± 0.04 (n = 14) occurred following exposure of the cells to hypoosmotic extracellular fluid. This shift was blunted to +0.05 ± 0.02 (n = 7) in cells treated for 60 min with colchicine. Colchicine did not, however, significantly modify the NH_4Cl-induced increase of FITC-dextran fluorescence ratio. In colchicine-treated cells the apparent vesicular pH tended to be slightly higher (6.20 ± 0.05, n = 7) than in untreated cells. However, the difference was not significant.

In both human and rat hepatocytes, a reduction of extracellular osmolarity (removal of 40 mM NaCl) led to a marked increase of acridine orange fluorescence intensity (Fig. 1). This increase was fully reversible upon reexposure of the cells to isoosmotic extracellular fluid (Fig. 1). As expected, NH_4Cl (2 mM) led to an increase of acridine orange fluorescence intensity, which was also fully reversible (Fig. 1). The

Table 1. Influence of NH_4Cl and of a decrease of extracellular osmolarity by removal of 60 mM NaCl (hypoosmotic) on the apparent pH as calculated by the increase in FITC-dextran fluorescence ratio (480/440) in cells exposed to colchicine (5 μM) and γ-lumicolchicine (5 μM) and under control conditions

Treatment	Increase in apparent pH	
	Hypoosmotic	20 mM NH_4Cl
Control	+0.21 ± 0.04 (14)	+0.32 ± 0.04 (13)
Colchicine		
5 min	+0.19 ± 0.02 (8)	+0.25 ± 0.03 (7)
30 min	+0.05 ± 0.02 (6)	+0.37 ± 0.04 (6)
60 min	+0.05 ± 0.02 (7)	+0.30 ± 0.04 (4)
80 min	+0.06 ± 0.05 (4)	Not tested
γ-Lumicolchicine		
60 min	+0.16 ± 0.03 (6)	+0.04 ± 0.04 (6)

Values are expressed as mean ± SEM [n = number of experiments (in parentheses)]. The increase in apparent pH is calculated following exposure to hypoosmotic solution or NH_4Cl.

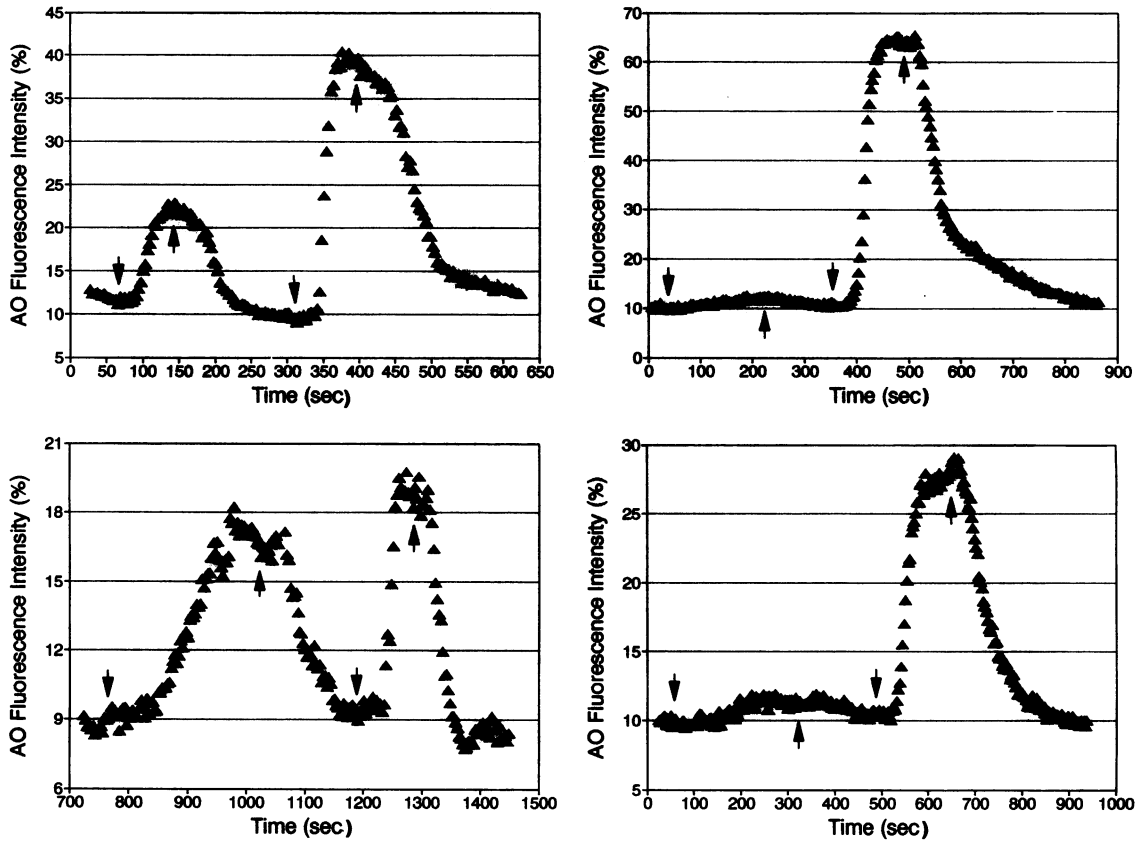

FIG. 1. Influence of a reduction of extracellular osmolarity (removal of 40 mM NaCl) as well as of addition of 2 mM NH_4Cl on acridine orange (AO) fluorescence intensity in human (*Upper*) and rat (*Lower*) hepatocytes. Controls are on the left; cells exposed to colcemid (5 μM for 60 min at 37°C) are on the right. Arrows facing downward indicate treatment with hypoosmotic solution (first) and with 2 mM NH_4Cl (second); arrows facing upward indicate return to isoosmotic solution.

effect of NH_4Cl was more marked than the effect of hypoosmotic extracellular fluid.

A 60-min treatment of the cells with 2.5 μM and 5 μM colcemid depolymerized microtubules in rat hepatocytes (Fig. 2 *A* and *B*) but had no effect on the state or organization of F-actin-containing stress fibers (Fig. 2 *C* and *D*). Similar effects on the microtubules were observed following colchicine treatment, whereas γ-lumicolchicine treatment did not cause a depolymerization of microtubules (not shown).

Following a 60-min treatment of the cells with colcemid, the stimulatory effect of hypoosmotic solutions on acridine orange fluorescence was blunted (Fig. 1, Table 2). However, colcemid treatment did not affect the ability of NH_4Cl to increase acridine orange fluorescence (Fig. 1, Table 2). Further, colcemid treatment alone did not affect the basal level of acridine orange fluorescence intensity.

Similar to treatment with colcemid, a 60-min treatment with colchicine blunted the increase in acridine orange fluorescence intensity following exposure to hypoosmotic extracellular fluid (Table 2). In contrast, γ-lumicolchicine, which did not depolymerize microtubules, did not significantly modify the acridine orange fluorescence following osmotic cell swelling.

Exposure of perfused rat liver to hypoosmotic perfusate (removal of 60 mM NaCl) decreased autophagic proteolysis as evidenced by [^{3}H]leucine release (Fig. 3). Colchicine (5 μM), but not γ-lumicolchicine (5 μM), virtually abolished the antiproteolytic action of reduced osmolarity.

DISCUSSION

The present observations confirm previous studies demonstrating that osmotic cell swelling increases the apparent vesicular pH as calculated using FITC-dextran and triggers release of acridine orange from acidic vesicles (3, 17, 18). Alkalinization of acidic vesicles is expected to increase the fluorescence ratio of FITC-dextran, which remains trapped within vesicular compartments, including endosomes and lysosomes, after being endocytosed (14). This fluorescent dye has a pK_a of 6.4 and may be used as a pH indicator for acidic vesicles due to its changing spectral properties in the range of pH 4–7 (19). The dye is resistant to degradation once endocytosed and its spectral properties are not seriously affected by dye concentration, ionic strength, or protein concentration. Acridine orange diffuses in the nonionized, but not the ionized, form across membranes readily (20–23). The nonionized form, which prevails in alkaline fluid, emits green fluorescence upon excitation at 490 nm. The ionized form, which prevails in acid fluid, emits a less intense orange fluorescence upon excitation at 490 nm. Within cells, the dye is trapped in acidic vesicles, where it binds H^+, thus becoming impermeable. In these vesicles, the acridine orange fluorescence is largely quenched. Thus, any alkalinization of the intracellular acidic vesicles is expected to lead, at least transiently, to an increase in fluorescence intensity. Acridine orange and FITC-dextran do not necessarily accumulate within the same acidic vesicles in equal proportion. Nevertheless, as shown in Tables 1 and 2, NH_3/NH_4^+ exerts the expected effect with both dyes—i.e., an increase in the apparent pH as calculated by an increase of the FITC-dextran fluorescence ratio (480/440 nm) and an increase in the acridine orange fluorescence intensity measured by excitation at 490 nm. As such, the preferential entry of lipid-soluble NH_3 into the lysosomes leads to subsequent H^+ trapping (i.e., alkalinization) by luminal formation of NH_4^+ (24).

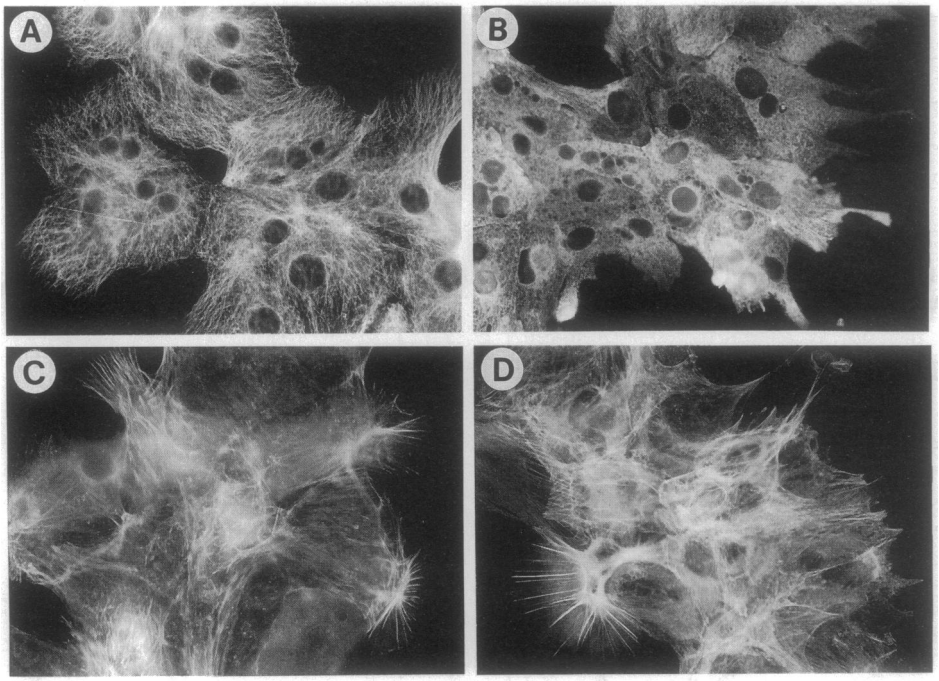

FIG. 2. Staining of microtubules (*A* and *B*) and stress fibers (*C* and *D*) of rat hepatocytes without (*A* and *C*) and after treatment with 2.5 μM colcemid for 60 min at 37°C (*B* and *D*). Note the complete depolymerization of microtubules in *B*. The use of 5 μM colcemid or 5 μM colchicine produced similar results, whereas 5 μM γ-lumicolchicine did not affect the organization of microtubules. (Epifluorescence microscopy; ×225).

As shown previously, the effectiveness of cell swelling in eliciting vesicular alkalinization is not restricted to osmotic cell swelling—that is, swelling resulting from concentrative uptake of glutamine, inhibition of K^+ channels with Ba^{2+}, and electrolyte uptake stimulated by insulin also proved effective in enhancing acridine orange fluorescence intensity (18). Thus, pH in acidic vesicles, like autophagic proteolysis, correlates with cell volume rather than ambient osmolarity or electrolyte composition. The extent of apparent pH change reflected by FITC-dextran fluorescence is small. However, the likely heterogeneous distribution of the dye in various stages of maturing endosomes and lysosomes may lead to an underestimation of the pH changes in lysosomes. Whether or not the pH changes in the relevant vesicles are sufficient to significantly modify proteolysis cannot be answered with certainty from the present results. However, the alkalinization induced by cell swelling is well in the range of the alkalinization triggered by NH_3, which is well known to inhibit proteolysis by alkalinization of lysosomes (25). In any case, the alkalinization of acidic vesicles may contribute to, but does not necessarily completely account for, the antiproteolytic effect of cell swelling.

Table 2. Influence of NH_4Cl and of a decrease of extracellular osmolarity by removal of 40 mM NaCl (hypoosmotic) on the acridine orange fluorescence intensity in cells exposed to colcemid (5 μM), colchicine (5 μM), and γ-lumicolchicine (5 μM) and under control conditions

	% increase in fluorescence intensity	
Treatment	Hypoosmotic	2 mM NH_4Cl
Human hepatocytes		
Control	+68.8 ± 2.9 (9)	+253 ± 24 (4)
Colcemid	+11.2 ± 1.0 (12)	+310 ± 12 (12)
Rat hepatocytes		
Control	+39.1 ± 0.6 (36)	+146 ± 13 (6)
Colcemid	+17.1 ± 0.5 (35)	+136 ± 4.5 (11)
Colchicine	+12.7 ± 0.5 (26)	Not tested
γ-Lumicolchicine	+38.8 ± 6.7 (4)	Not tested

Values are expressed as mean ± SEM [*n* = number of experiments (in parentheses)]. The increase in fluorescence intensity is expressed as % of the intensity prior to exposure to hypoosmotic solution or NH_4Cl.

The prior experiments raised the question of how cell volume could modify the pH in acidic vesicles. The present observations demonstrate that the swelling-induced alterations of FITC-dextran and acridine orange fluorescence are

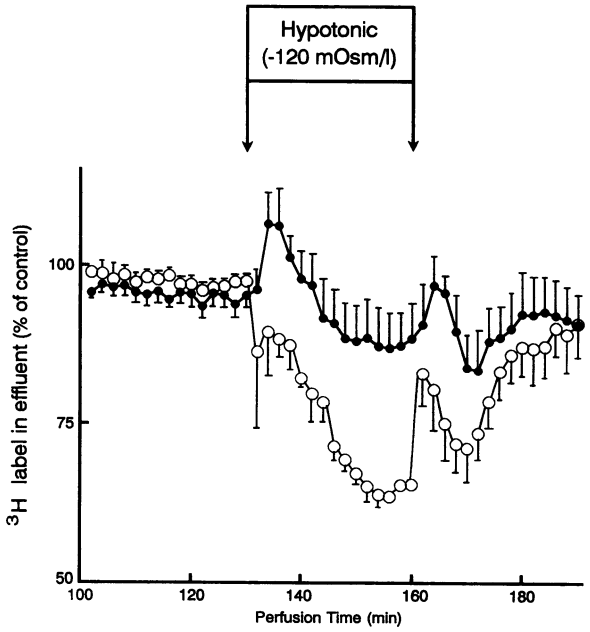

FIG. 3. Effect of osmotic cell swelling on autophagic proteolysis in perfused rat liver. [^{3}H]Leucine release from perfused rat liver is plotted versus time. In the presence of colchicine (5 μM; ●), but not in the presence of γ-lumicolchicine (5 μM; ○), a decrease of extracellular osmolarity inhibited [^{3}H]leucine release. Values are expressed as mean ± SEM (*n* = 4).

sensitive to colcemid and colchicine and that the antiproteolytic action of osmotic cell swelling is similarly sensitive to colchicine. Since, for technical reasons, proteolysis has been examined in perfused liver and fluorescence measurements have been performed using isolated hepatocytes, the data are not fully comparable. Nevertheless, the sensitivity of both processes to colchicine treatment supports the view that the two phenomena are indeed related.

The inhibitory effect of colcemid and colchicine on the fluorescence response of FITC-dextran and acridine orange to hypotonic swelling cannot be attributed to a nonspecific effect of the drugs themselves on the two dyes or their photodynamic properties as NH_4Cl was still effective in inducing vesicular alkalinization in the presence of the two substances. Colchicine and colcemid are known to induce depolymerization of microtubules (7, 8). In this study, it is shown that the effect of colcemid and colchicine is indeed restricted to the microtubule network. While microtubules were effectively depolymerized by a 60-min treatment with both drugs, the stress fiber network and overall cell shape were observed to be completely unaltered by this treatment (Fig. 2). This result is in agreement with the known colchicine/colcemid sensitivity of microtubules and the known insensitivity of F-actin-containing stress fibers (26–28). The inactive analogue γ-lumicolchicine did not affect volume-induced sensitivity of cell swelling, further pointing to the specificity of the effect. Thus, the data do suggest that microtubules play a role in transducing the effect of vesicular alkalinization during cell swelling. Indeed, cell swelling has been shown to stabilize the microtubular network and stimulate tubulin mRNA synthesis in isolated rat hepatocytes (29). Because ammonia directly alkalinizes acidic compartments, its effect would not be expected to be altered by disturbances of the cytoskeleton. Indeed, colcemid and colchicine did not inhibit the effects of NH_4Cl on FITC-dextran or acridine orange fluorescence.

The present observations do not allow for interpretation of how the microtubule network interferes with the pH in acidic vesicles. Possible mechanisms include inhibition of H^+ pumping, stimulation of H^+ leak, intravesicular buffering of H^+, and modification of vesicle trafficking—i.e., interference of the fusion of acidic vesicles with more alkaline vesicles.

On average, colcemid and colchicine did not completely abolish the effect of osmotic cell swelling on FITC-dextran or acridine orange fluorescence. In fact, the effect of both substances was variable, completely abolishing the effect of cell swelling in some cells and exerting very weak effects in others. This suggests that either the swelling-induced alkalinization is maintained under conditions in which the microtubule network is not completely disintegrated or a fraction of acidic vesicles is alkalinized by mechanisms independent from microtubules (with this fraction varying from cell to cell). Colchicine does not lead to sustained alterations of cell volume (unpublished observations) nor does it interfere with volume-regulatory K^+ fluxes or regulation by cell volume of other metabolic pathways such as glycine oxidation and the pentose phosphate pathway (9). On the other hand, treatment with colchicine inhibits the swelling-induced stimulation of bile acid excretion (9). Thus, the microtubule network is indeed a very likely element linking cell volume changes to bile acid transport and some metabolic functions such as proteolysis but does not trigger all volume-dependent functions of the cell. Clearly, additional mechanisms do operate to adjust cellular function in response to altered cell volume (30, 31).

These studies would not have been possible without the expert assistance of Uwe Schüler in establishing our microspectrofluorimetry system and the generous gift of the imaging computer program from Bernd Lindemann and Willy Meiser. We thank Irina Öttl and Ute Emmerich for their skilled technical assistance and Simone Wärntges for preparation of the manuscript. The preparation and provision of human hepatocytes by Drs. Werner Lauchart and Richard Viebahn of the University of Tübingen Surgical Clinic are greatly appreciated. G.L.B. is the recipient of a fellowship of the Medical Research Council of Canada. This study was supported by the Deutsche Forschungsgemeinschaft, Grant La 315/4-1 and Grant SFB 154, and the Leibnitz program and by the Österreichische Fonds zur Förderung der wissenschaftlichen Forschung, Grant P 8294 MED.

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