RESEARCH COMMUNICATION

Effect of cell volume on Acridine Orange fluorescence in hepatocytes

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Hepatic proteolysis is inhibited by cell swelling following a variety of experimental manoeuvres, such as reduction of extracellular osmolarity, concentrative uptake of amino acids, or blockade of K^+ channels by barium. On the other hand, proteolysis is known to be accomplished by pH-sensitive lysosomal proteases. Accordingly, NH_3/NH_4^+ inhibits proteolysis by intralysosomal alkalization. The present study has been performed to test for an effect of cell volume on the pH of acidic intracellular compartments, as assessed by Acridine Orange

fluorescence at > 520 nm ($F_{>520}$). $F_{>520}$ is enhanced by NH_3/NH_4^+ (2 and 20 mmol/l respectively), by glutamine (2 mmol/l), by the K^+ -channel blocker barium (10 mmol/l) and by reduction of extracellular osmolarity (by 20 and 80 mosmol/l respectively). The observations point to release of Acridine Orange from acidic cellular compartments, which is indicative of alkalization of these compartments during cell swelling. This effect may contribute to the regulation of proteolysis.

INTRODUCTION

Amino acids are well known to inhibit hepatic proteolysis (for review see Mortimore and Pösö, 1984, 1987). The intracellular mechanisms mediating this effect, however, have remained elusive until recently, when it has been recognized that cell swelling, as it occurs during the concentrative uptake of amino acids, markedly inhibits proteolysis (Häussinger et al., 1990b, 1991; Häussinger and Lang, 1991a,b, 1992; Hallbrucker et al., 1991a). It has been shown that the anti-proteolytic effect of glutamine, for instance, is fully mimicked by proportionate cell swelling due to reduction of the extracellular osmolarity or inhibition of the action of K^+ channels in the cell membrane (Häussinger et al., 1990b, 1991; Häussinger and Lang, 1991a,b, 1992). These observations raised the question of how alterations of cell volume are linked to proteolysis. Autophagic proteolysis occurs largely within acidic lysosomes and is accomplished by pH-sensitive lysosomal proteases (Dean, 1975; Tager et al., 1988). Ammonia inhibits proteolysis, presumably by producing intralysosomal alkalization (Seglen, 1977). Thus, it appears feasible that cell swelling affects proteolysis by modifying intralysosomal pH.

To test this hypothesis, we have utilized Acridine Orange. This fluorescent weak base accumulates within acidic compartments, where it predominates in the protonized, impermeable species and where its fluorescence is quenched. Any alkalization of these acidic compartments leads to a release of the dye and a corresponding increase of fluorescence (Lake et al., 1987; Sabolic and Burckhardt, 1983; Warnock et al., 1982; Van Dyke et al., 1985).

As a matter of fact, addition of NH_3/NH_4^+ elicited the expected increase of Acridine Orange fluorescence, pointing to alkalization of intravesicular pH. Cell swelling, either by reduction of extracellular osmolarity or by exposure to glutamine, similarly led to the respective increase of fluorescence intensity, pointing to intravesicular alkalization.

EXPERIMENTAL

Isolated hepatocytes were prepared by collagenase treatment according to the method of Meijer and co-workers (Meijer et al.,

1975; Hansen et al., 1986). The cells were plated on sterile cover glasses and incubated at 37 °C with 5 % CO_2 and 95 % humidified air in Dulbecco's modified Eagle's medium with 10 % (v/v) fetal-calf serum, L-glutamine (2 mmol/l), penicillin (100 units/ml), streptomycin (100 µg/ml), dexamethasone (1 µmol/l), 3-I-thyronine (10 µmol/l), thyroxine (10 µmol/l) and bovine insulin (5 mg/l). Medium was replaced every 24 h. The cells were used within 1–3 days of culture. No significant differences of response have been observed between day 1 and day 3 of culture.

During the experiments coverslips with subconfluent layers were exposed for 15 or 20 min respectively to the fluorescent dyes 2',7'-bis-(2-carboxyethyl)-5-(and -6)carboxyfluorescein (BCECF) and Acridine Orange (see below) and mounted into a perfusion chamber allowing for rapid exchange of extracellular fluid (volume 0.1 ml, perfusion rate 4 ml/min). The extracellular solutions (perfusates) used are listed in Table 1. All experiments were performed at 37 °C. The cells were exposed to control 'perfusate' for 10–20 min before experimental solutions were applied.

BCECF fluorescence was utilized for determination of cytosolic pH (pH_i). To this end, the hepatocytes were incubated for 20 min with 10 µmol/l BCECF acetoxymethyl ester [2',7'-bis-(2-carboxyethyl)-5-(and -6)carboxyfluorescein, acetoxymethyl ester; Molecular Probes, Eugene, OR, U.S.A.]. Measurements were made under an inverted microscope (Axiovert 35; Zeiss, Germany) equipped for epifluorescence and photometry (Almers and Neher, 1985). Light from a xenon arc lamp (XB 75; Osram, Berlin, Germany) was directed through grey filters (nominal transmission 3 %; Oriel, Darmstadt, Germany), a 488 nm or 440 nm interference filter (half-width 10 nm; Oriel, Darmstadt, Germany) and a diaphragm and was deflected by a dichroic mirror (FT 510; Zeiss, Germany) into the objective (Plan-Neofluar 40×; Zeiss, Germany). The emitted fluorescence was directed through a 520 nm-cut-off filter to a photomultiplier tube (R4829; Hamamatsu, Herrsching, Germany). To reduce the region from which fluorescence was collected, a pin hole was placed in the image plane of the phototube. Thus fluorescence from five to ten cells was collected. Fluorescence values were corrected for cellular autofluorescence, and intracellular pH was

Table 1 Composition of solutions used

Constant constituents were 5 mmol/l KCl, 1.3 mmol/l CaCl_2 , 21 mmol/l NaHCO_3 , 1 mmol/l MgCl_2 , 5 mmol/l glucose, all solutions were equilibrated with 5% CO_2 and 85% O_2

Solution ... Constituent	Concn. of variable constituent (mmol/l)							
	1	2	3	4	5	6	7	8
NaCl	115	95	105	105	75	75	103	115
Na_2HPO_4	2	2	2	2	2	2	—	2
Mannitol	—	—	20	—	80	—	—	80
NH_4Cl	—	20	—	—	—	—	—	—
BaCl_2	—	—	—	—	—	—	10	—

calibrated with the high-potassium/nigericin technique (Ganz et al., 1989). Acridine Orange fluorescence has been utilized as an indicator for alkalization of acidic intracellular compartments. Acridine Orange is a fluorescent dye, which upon excitation with 450–490 nm wavelength light emits light at > 520 nm ($F_{>520}$). The dye is trapped by acidic cellular compartments and the fluorescence quenched in these compartments. Any alkalization of the intracellular acidic compartments leads to a release of Acridine Orange from these compartments and thus to an increase of fluorescence intensity (Warnock et al., 1982; Sabolic and Burckhardt, 1983; Van Dyke et al., 1985; Lake et al., 1987). Other factors modifying Acridine Orange fluorescence are dye concentration and ionic strength, the possible influence of these factors, however, is less than 10% in the present study (Palmgren, 1991). To load the cells, they were incubated for 15 min with 10 $\mu\text{mol/l}$ Acridine Orange hydrochloride (Sigma Chemie, Munich, Germany). For determination of Acridine Orange fluorescence excitation wavelengths of 450–490 nm were used and the emitted light directed through a cut-off filter of 520 nm. In a separate series of experiments, the Acridine Orange fluor-

escence at > 630 nm ($F_{>630}$) was determined. To test for the possible influence of scattered light (Quinlan et al., 1983; Halestrap, 1989) and autofluorescence the emission of light at wavelengths of > 520 nm was determined from unloaded cells, which were otherwise treated identically to loaded cells. The light intensity of these cells at > 520 nm remained, in the absence of Acridine Orange, less than 1% of $F_{>520}$ in the presence of Acridine Orange in control conditions (presence of glutamine or hypotonic extracellular fluid), ruling out the contribution of autofluorescence. Furthermore, no significant increase of fluorescence has been observed following the treatments. Thus, scattered light cannot account for the observed increase of $F_{>520}$ upon cell swelling in the presence of Acridine Orange.

Where applicable, the data are expressed as arithmetic means $\pm$ S.E.M. Where applicable, n indicates the number of culture dishes studied. For each series, cells were prepared from at least four livers. Statistical analysis was made by paired Student's t -test, where applicable; $P < 0.05$ was considered to be statistically significant.

RESULTS

As illustrated in Figure 1 and listed in Table 2, the exposure of the hepatocytes to $\text{NH}_3/\text{NH}_4^+$ (2 or 20 mmol/l respectively) leads to a sharp increase of Acridine Orange fluorescence at wavelengths > 520 nm ($F_{>520}$), which upon return to control solution (removal of $\text{NH}_3/\text{NH}_4^+$) declines below the original value. In addition, monensin (10 $\mu\text{mol/l}$) and chloroquine (1 mmol/l), both substances which alkalize lysosomes (Wiesmann et al., 1975; Sandeaux et al., 1981), enhance $F_{>520}$ (Table 2). Reduction of extracellular osmolarity by omission of mannitol as well as the addition of glutamine (2 mmol/l) to the extracellular fluid led to a similarly marked increase of $F_{>520}$ (Figure 1, Table 2). On the other hand, the Acridine Orange fluorescence at wavelengths > 630 nm ($F_{>630}$) is not significantly modified ($+9.5 \pm 4.1\%$, $n = 8$) after reduction of extracellular osmolarity and is even significantly decreased ($-5.4 \pm 2.0\%$,

Figure 1 Effects of various conditions on Acridine Orange fluorescence in hepatocytes

Effect of 2 and 20 mmol/l NH_4Cl (solutions 1 and 2), of hypotonic extracellular fluid (220 mosmol/l), of monensin (10 $\mu\text{mol/l}$), chloroquine (1 mmol/l), barium (10 mmol/l) and glutamine (2 mmol/l) with and without increase of extracellular osmolarity on Acridine Orange fluorescence in hepatocytes. Original tracings from different cell preparations, representative of 4–15 separate cell preparations for each series. Upward-directed arrows indicate addition of experimental solution, downward-directed arrows indicate re-addition of control solution.

Table 2 Effect of various experimental conditions on Acridine Orange fluorescence

Numbers in parentheses represent number of observations. *shows statistical significant difference from control. The solutions used are defined in Table 1.

Experimental condition	Maximal increase of Acridine Orange fluorescence (%)	Solutions
2 mmol/l NH_4Cl	138 ± 29 (6)*	1
20 mmol/l NH_4Cl	319 ± 17 (8)*	1 → 2
10 $\mu\text{mol/l}$ Monensin	202 ± 40 (5)*	1
1 mmol/l Chloroquine	357 ± 59 (5)*	1
280 mosmol/l	18 ± 6 (4)*	3 → 4
220 mosmol/l	185 ± 34 (14)*	5 → 6
10 mmol/l Barium	55 ± 18 (5)*	1 → 7
0.5 mmol/l L-Glutamine	22 ± 3 (5)*	1
1.0 mmol/l L-Glutamine	58 ± 11 (4)*	1
2 mmol/l L-Glutamine	114 ± 36 (6)*	1
2 mmol/l L-Glutamine + 380 mosmol/l	17 ± 9 (4)	1 → 8

$n = 7$) after addition of glutamine. The effect of glutamine on $F_{>520}$ is almost abolished when glutamine-induced cell swelling is reversed by a simultaneous increase of extracellular osmolarity (Figure 1, Table 2). The increase of $F_{>520}$ following the addition of $\text{NH}_3/\text{NH}_4^+$ is not significantly modified by a simultaneous increase of extracellular osmolarity (results not shown).

Barium (10 mmol/l), a blocker of K^+ channels, again increases $F_{>520}$ (Table 2), albeit to a lesser extent than $\text{NH}_3/\text{NH}_4^+$ or glutamine.

While $\text{NH}_3/\text{NH}_4^+$, glutamine and barium all increase $F_{>520}$, they have partially opposing effects on cytosolic pH. As indicated by the BCECF fluorescence, the exposure of hepatocytes to 20 mmol/l $\text{NH}_3/\text{NH}_4^+$ leads to a marked cytosolic alkalization: the calculated cytosolic pH (pH_i) increases transiently from 7.11 ± 0.05 up to 8.29 ± 0.13 ($n = 6$) after addition of 20 mmol/l $\text{NH}_3/\text{NH}_4^+$ to the extracellular fluid. Subsequent removal of $\text{NH}_3/\text{NH}_4^+$ is followed by rapid cytosolic acidification to a pH_i value of 6.79 ± 0.06 ($n = 6$).

Reduction of extracellular osmolarity by 80 mosmol/l leads to a slight cytosolic acidification from 7.06 ± 0.04 to 6.89 ± 0.05 ($n = 5$), whereas an increase of extracellular osmolarity leads to a slight cytosolic alkalization from 7.06 ± 0.04 to 7.21 ± 0.05 ($n = 5$). Addition of 2 mmol/l glutamine to the extracellular fluid is also followed by a slight cytosolic alkalization from 6.97 ± 0.04 to 7.16 ± 0.03 ($n = 6$).

DISCUSSION

The present observations yield the expected cytosolic alkalization after exposure to $\text{NH}_3/\text{NH}_4^+$: the preferential entry of NH_3 into the cell leads to subsequent H^+ trapping by cytosolic formation of NH_4^+ (Roos and Boron, 1981). The rapid removal of extracellular $\text{NH}_3/\text{NH}_4^+$ is then followed by the respective cytosolic acidification. Similarly, NH_3 may be expected to preferentially enter acidic compartments within the cell with subsequent intracompartamental H^+ trapping and NH_4^+ formation. The present observations support this assumption. As suggested by the alterations of Acridine Orange fluorescence, similar changes occur during exposure of the cells to hypotonic

extracellular fluid. Accordingly, the observations would indicate that cell swelling similarly leads to alkalization of acidic intracellular compartments. The shift of Acridine Orange fluorescence is not just due to cytosolic alkalization: cell swelling rather leads to cytosolic acidification (see Results section), in part due to conductive bicarbonate loss (Völkl and Lang, 1988a,b; Weiss and Lang, 1992).

Glutamine, in a similar manner to the reduction of extracellular osmolarity, leads to an increase of Acridine Orange fluorescence, pointing to alkalization of acidic intracellular compartments. This effect of glutamine could well result from cell swelling, due to concentrative uptake of the amino acid into the cell (Häussinger et al., 1990b). Accordingly, reversal of glutamine-induced cell swelling by a simultaneous increase in the volume of extracellular fluid almost abolishes the effect of glutamine. The effect of $\text{NH}_3/\text{NH}_4^+$, on the other hand, which probably does not depend on cell swelling, is almost unaffected by the simultaneous increase of extracellular osmolarity. An effect similar to those of glutamine and hypotonic extracellular fluid on Acridine Orange fluorescence is observed after the application of barium. The K^+ -channel blocker leads to depolarization of the cell membrane, fostering anion accumulation in the cells (Lang et al., 1990). Accordingly, barium has been shown previously to mimic the effects of hypotonic extracellular fluid and glutamine on both cell volume and proteolysis (Häussinger et al., 1991; Hallbrucker et al., 1991b).

The effect of different glutamine concentrations parallels dose-dependent cell swelling and anti-proteolytic activity (Wettstein et al., 1990; Hallbrucker et al., 1991b). However, a close linear correlation between Acridine Orange fluorescence and cell volume cannot automatically be expected and the present observations do not allow any reliable quantitative estimate of vesicular alkalization. Furthermore, the mechanism, i.e. how cell volume could affect intravesicular pH, remains completely elusive. Cell swelling could either interfere with acid transport into the vesicles, i.e. with H^+ ATPase (Schneider, 1983; Lake et al., 1987) or Cl^- channels (Schmid et al., 1988, 1989), or it could somehow lead to a base shunt across the cell membrane, similar to the action of $\text{NH}_3/\text{NH}_4^+$. Interestingly, net formation of $\text{NH}_3/\text{NH}_4^+$ from amino acids is enhanced in hepatocytes exposed to hypotonic extracellular fluid (Häussinger and Lang, 1990; Häussinger et al., 1990b). However, whether or not $\text{NH}_3/\text{NH}_4^+$ formation is sufficient to significantly interfere with intravesicular pH cannot be answered at present.

Hypertonic extracellular fluid leads to a slight decrease of Acridine Orange fluorescence, pointing to intravesicular acidification during cell shrinkage.

As autophagic proteolysis is strongly dependent on lysosomal pH, the intravesicular alkalization observed here could well participate in the anti-proteolytic effects of cell swelling after treatment with glutamine, barium and reduction of extracellular osmolarity (Häussinger et al., 1990a, 1991; Hallbrucker et al., 1991a,b; Häussinger and Lang, 1991a,b). Accordingly, glutamine could exert its anti-proteolytic action, at least in part, by swelling the cells, which in turn impedes proteolysis by lysosomal alkalization. But again, the present observations do not allow any reliable estimate of the quantitative importance of vesicular alkalization to the anti-proteolytic action of cell swelling.

Obviously, the Acridine Orange technique cannot discriminate between different types of acidic intracellular compartments, and the question arises whether the alkalization observed upon cell swelling indeed reflects alkalization of lysosomes, which could account for proteolysis. However, hypotonic cell swelling is as potent as $\text{NH}_3/\text{NH}_4^+$, which certainly does not discriminate between different acidic compartments. Thus, it is likely that the

alkalinization occurring after cell swelling is not restricted to a minor portion of acidic compartments. As lysosomes constitute a major portion of acidic intracellular compartments (Tager et al., 1988), it appears very unlikely that intravesicular alkalization following cell swelling does not include lysosomes.

In conclusion, evidence is presented for alkalization of acidic intracellular compartments following cell swelling, which is induced by decreased extracellular osmolarity, blockade of K^+ channels and concentrative uptake of glutamine. Since proteolysis in acidic intracellular lysosomes is accomplished by pH-sensitive proteases, the observed alkalization could account, at least in part, for the inhibitory effect of cell swelling on proteolysis. Thus, the present observations point to another element of the intracellular cascade linking amino acids to the control of proteolysis.

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