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Alkalinization of acidic cellular compartments following cell swelling

Harald Völkl[a], Gillian L. Busch[c], Dieter Häussinger[b], Florian Lang[c,*]

[a]*Institute of Physiology, University of Innsbruck, Innsbruck, Austria*
[b]*Department of Internal Medicine, University of Freiburg, Freiburg, Germany*
[c]*Institute of Physiology, University of Tübingen, Gmelinstr. 5, D 72076 Tübingen, Germany*

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Abstract

Osmotic swelling of rat hepatocytes increases fluorescence of Acridine orange and of fluorescein isothiocyanate (FITC)-dextran, both indicative of alkalinization of acidic intracellular vesicles. Similar to osmotic cell swelling, insulin and glutamine lead to an increase in Acridine orange fluorescence, an effect virtually abolished upon osmotic reversal of glutamine-induced cell swelling. Barium, which blocks K^+ channels in the plasma membrane, similarly leads to cell swelling and increase of Acridine orange fluorescence. Since proteolysis is governed by lysosomal pH, these observations indicate that the anti-proteolytic action of osmotic cell swelling is mediated by lysosomal alkalinization. Thereby, insulin, glutamine and barium probably exert their anti-proteolytic action by cell swelling and subsequent lysosomal alkalinization.

Key words: Insulin; Glutamine; Barium; Cell volume; Proteolysis; Lysosome

1. Introduction

Insulin and amino acids such as glutamine inhibit autophagic proteolysis in hepatocytes, an effect mediated by increase of cell volume [1–4]. Glutamine swells hepatocytes by concentrative uptake and insulin by stimulating electrolyte uptake via parallel activation of Na^+,K^+,$2Cl^-$ co-transport and Na^+/H^+ exchange [5–11]. The anti-proteolytic actions of insulin and glutamine are fully mimicked by osmotic cell swelling and are abolished when cell swelling is reversed by appropriate alterations of extracellular osmolarity. Inhibition of Na^+,K^+,$2Cl^-$ co-transport by either furosemide or bumetanide not only inhibits the swelling effect of insulin but also leads to a proportionate inhibition of proteolysis [9]. Furthermore, in the presence of both furosemide and amiloride, insulin-induced cell swelling and its anti-proteolytic action are completely abolished. Thus, cell volume may be considered as a 'second messenger' in mediating the regulation of proteolysis in hepatocytes [1]. However, the mechanism linking cell volume to proteolysis has remained completely elusive. Because lysosomal proteolysis largely resides within acidic lysosomes and is accomplished by pH-sensitive lysosomal proteinases [12], these studies were designed to investigate the possibility of a direct effect of cell volume on the regulation of pH in acidic compartments of hepatocytes.

2. Materials and methods

Rat hepatocytes were prepared by collagenase treatment, as previously described [13,14]. Prior to experiments, hepatocytes were incubated for 15 min with 10 μM Acridine orange hydrochloride (Sigma, Munich, Germany) or for 2 h with 5 mg/ml FITC-dextran (m.wt. 70,000, Sigma) in bath solution composed of (in mM) 115 NaCl, 21 $NaHCO_3$, 5 KCl, 1.3 $CaCl_2$, 1 $MgCl_2$, 2 NaH_2PO_4, 5 glucose equilibrated with 5% CO_2 and 95% O_2 and maintained at 37°C. Where indicated, osmolarity was reduced by removal of 20 or 75 mM NaCl for 260 mOsm and 150 mOsm solutions, respectively. Replacement of NaCl with isosmotic raffinose did not modify vesicular pH. Osmolarity of solutions was checked utilizing cryoosmometry (Knaur, Germany).

For microspectrofluorimetric measurements, light from a xenon arc lamp (XB 75, Osram, Berlin, Germany) was directed through grey filters (nominal transmission 3%, Oriel, Darmstadt, Germany) into a 450–490 nm interference filter (Oriel) in the case of Acridine orange. For FITC-dextran studies, a monochromator light source (Uhl, Munich, Germany) was set to wavelengths of 440 nm and 490 nm. In the case of both Acridine orange and FITC-dextran fluorescence measurements, light then passed through a diaphragm and was deflected by a dichroic mirror (FT 510, Zeiss, Germany, or 515 nm, Omega Optical, Brattleboro, Vermont, respectively) into the lens (Plan-Neofluar 40 ×, Zeiss). The emitted fluorescence was directed through a 530 nm cut-off filter to a photomultiplier tube (R4829, Hamamatsu, Herrsching, Germany or 213-IP28A, Seefelder Meßtechnik, Seefeld, Germany). In order to reduce the region from which fluorescence was collected, a pinhole was made in the image plane of the phototubus. Fluorescence in the absence of Acridine orange or FITC-dextran was less than 1% of the values in the presence of the dyes and was not significantly modified by the experimental manoeuvres.

3. Results and discussion

In order to test for an effect of cell volume on lysosomal pH, two fluorescent dyes have been utilized;

*Corresponding author.

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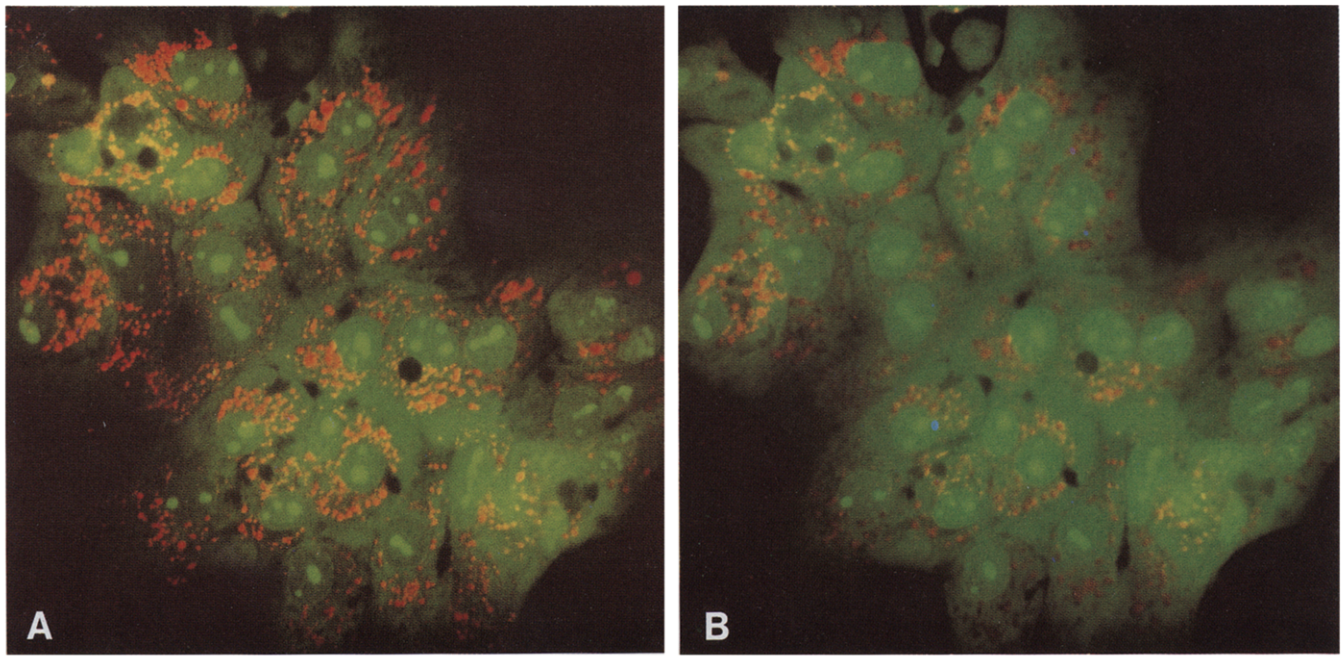
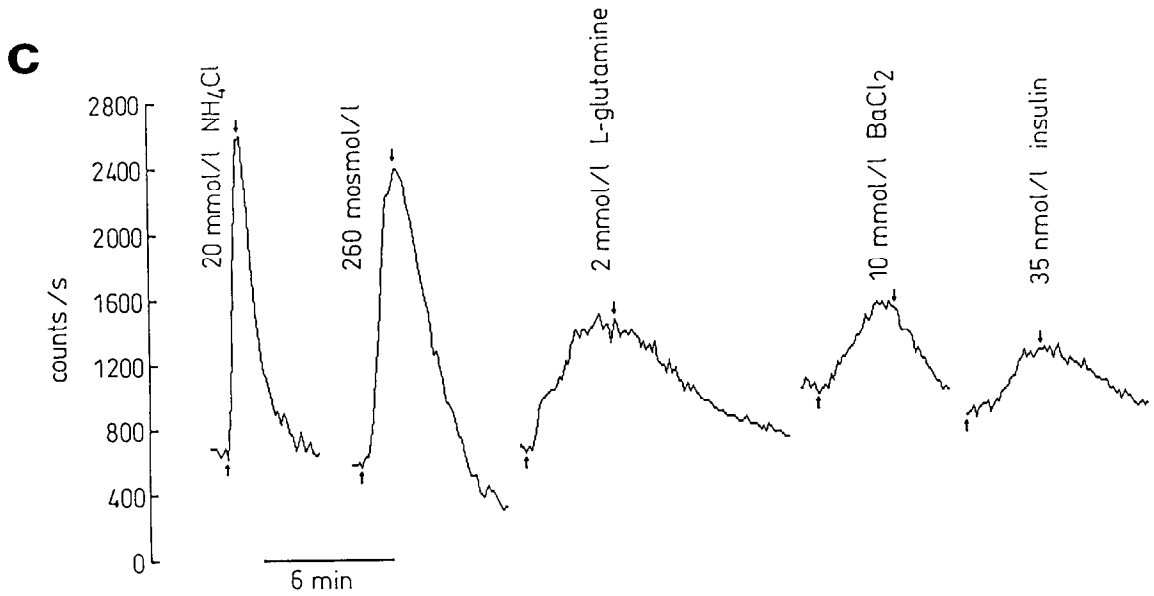
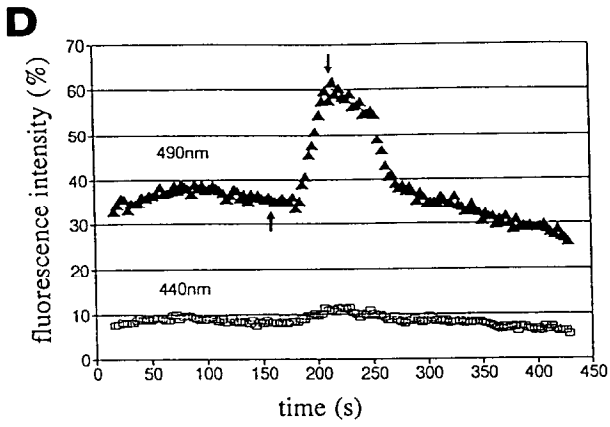
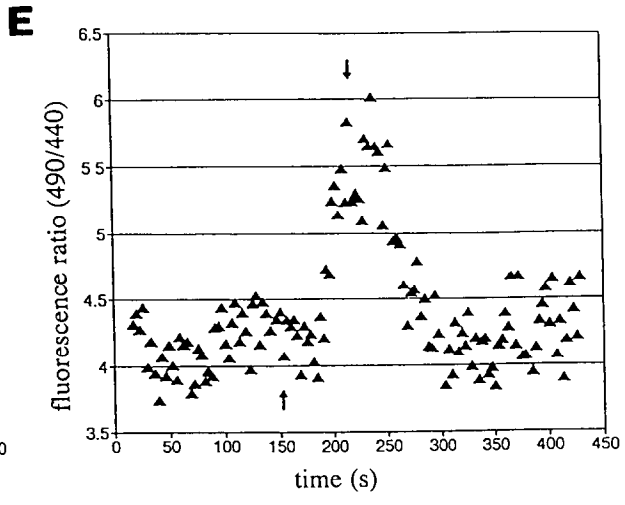

A
B


C
counts / s
2800
2400
2000
1600
1200
800
400
0
20 mmol/l NH4Cl
260 mosmol/l
2 mmol/l L-glutamine
10 mmol/l BaCl2
35 nmol/l insulin
6 min


D
fluorescence intensity (%)
70
60
50
40
30
20
10
0
490nm
440nm
0
50
100
150
200
250
300
350
400
450
time (s)


E
fluorescence ratio (490/440)
6.5
6
5.5
5
4.5
4
3.5
0
50
100
150
200
250
300
350
400
450
time (s)

Acridine orange [15–18] and FITC-dextran [19–22]. Acridine orange is a weak base and the non-ionized, but not the ionized, form of this fluorescent dye diffuses across membranes readily. The non-ionized form, which prevails in alkaline fluid, emits green fluorescence upon excitation at a wavelength of 470 nm. The ionized form, which prevails in acid fluid, emits orange fluorescence upon excitation at 470 nm. Within cells, the dye is trapped by acidic compartments where it binds H^+, thus becoming impermeable. In these vesicles, the Acridine orange fluorescence is largely quenched. Thus, any alkalinization of the intracellular acidic compartments is expected to lead, at least transiently, to an increase in fluorescence intensity due to the release of dye from acidic compartments and its accumulation in the cytoplasm. NH_3/NH_4^+ exerts the expected increase in Acridine orange fluorescence (Fig. 1C, Table 1). A similar increase in fluorescence intensity follows cell swelling induced by reduction of extracellular osmolarity, as well as by addition of glutamine or insulin (Fig. 1C, Table 1). As illustrated in response to glutamine treatment, the orange fluorescence of the vesicles is simultaneously decreased (Fig. 1A,B). If the effect of glutamine and insulin on Acridine orange fluorescence is indeed a consequence of cell swelling, the change in fluorescence intensity should be reversed by increase of extracellular osmolarity. As shown for glutamine, this is indeed the case (Table 1). The effect of NH_3/NH_4^+, however, which does not depend on cell swelling, is only weakly affected by simultaneous increase in extracellular osmolarity (Table 1). Similar to the effect of glutamine, insulin and hypotonic extracellular fluid, barium treatment results in an increase in cell volume and an inhibition of proteolysis. The K^+ channel blocker leads to depolarization of the cell membrane, fostering anion accumulation in the cells [23]. Barium similarly increases Acridine orange fluorescence (Fig. 1C, Table 1).

Thus, all four manoeuvres which increase cell volume (reduction of extracellular osmolarity and addition of glutamine, insulin and barium) stimulate the release of Acridine orange from acidic compartments. This effect can only be explained by an alkalinization of acidic cellular compartments. Indeed, ammonia (NH_3/NH_4^+) is known to inhibit proteolysis by lysosomal alkalinization [24]; the preferential entry of lipid-soluble NH_3 into the lysosomes leads to subsequent H^+ trapping by lysosomal formation of NH_4^+ [25]. Fluorescence studies using BCECF (results not shown) indicate that the manoeuvres elicit only small, and partially opposite, effects on cytosolic pH, i.e. slight alkalinization (glutamine) or acidification (reduced osmolarity).

Table 1
Effect of various experimental manoeuvres on Acridine orange fluorescence (AOF^{470})

Experimental condition	Increase in AOF^{470} (%)	
1 mM NH_4Cl	123 ± 10*	(n = 8)
260 mOsm	247 ± 25*	(n = 7)
2 mM L-glutamine	106 ± 23*	(n = 6)
40 nM insulin	38 ± 7*	(n = 6)
2 mM barium	51 ± 17*	(n = 5)
380 mOsm + 2 mM glutamine	15 ± 2*	(n = 4)
380 mOsm + 1 mM NH_4Cl	126 ± 19*	(n = 4)

Mean values ± S.E.M. *Indicates significant ($P < 0.05$) effect as evaluated by paired comparison. Original tracings are illustrated in Fig. 1.

The effect of cell volume on pH of intracellular compartments is further supported by experiments utilizing FITC-dextran. This fluorescent dye is internalized into endocytic compartments where it remains in a macromolecular form and can be visualized by fluorescence microscopy in a typical lysosomal pattern [19]. Because the excitation spectrum for FITC differs at various pHs, any change of vesicular pH is reflected by a change of the fluorescence ratio. Following osmotic swelling this ratio increased significantly (30 ± 7%, n = 7, $P < 0.05$, Fig. 1D,E), reflecting an increase in pH.

In conclusion, glutamine, insulin and barium exert their anti-proteolytic effect by swelling the cells, which in turn impedes proteolysis by lysosomal alkalinization. Thus, the present observations point to another element of the intracellular cascade regulating control of autophagic proteolysis.

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Fig. 1. Photographs of Acridine orange-loaded hepatocytes before (A) and 5 min after (B) addition of 2 mM glutamine. Note the disappearance of the orange intracellular vesicles, demonstrating loss of Acridine orange from these vesicles. Influence of NH_4Cl, hypotonic fluid, glutamine, barium and insulin on Acridine orange fluorescence at > 530 nm in isolated hepatocytes (C). Influence of osmotic cell swelling on FITC-dextran fluorescence during excitation with 440 nm and 490 nm (D) as well as the fluorescence ratio (E). Upward arrows indicate addition of hypotonic solution (150 mOsm), downward arrows readdition of control solution. Representative experiments. Mean values ± S.E.M. see Table 1.

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