The formation of plasma membrane blebs in hepatocytes exposed to agents that increase cytosolic Ca^{2+} is mediated by the activation of a non-lysosomal proteolytic system

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Exposure of isolated hepatocytes to extracellular ATP, cystamine or ionophore A23187 was associated with an increase in cytosolic Ca^{2+} concentration, a stimulation of intracellular proteolysis, and the appearance of plasma membrane blebs which preceded the loss of cell viability. Both bleb formation and cell killing were prevented when inhibitors of Ca^{2+}-activated neutral proteases, such as antipain or leupeptin, were included in the incubation medium, whereas inhibitors of lysosomal proteases had no effect. Thus, the activation of a Ca^{2+}-dependent, non-lysosomal proteolytic system appears to be responsible for the plasma membrane blebbing and, ultimately, the cytotoxicity associated with treatment of hepatocytes with agents that disrupt intracellular Ca^{2+} homeostasis.

Cytosolic Ca^{2+} *Blebbing* *Proteolysis* *Hepatotoxicity*

1. INTRODUCTION

Previous studies in our and other laboratories have shown that formation of plasma membrane blebs is an early morphological sign of chemical and ischemic injury to isolated, intact cells [1,2]. The occurrence of plasma membrane blebbing has been described not only in isolated cell systems but also in cells in culture [3] and in tissue sections [4], indicating that this phenomenon is not limited to one experimental model. The formation of plasma membrane blebs has been related to modification of the cytoskeleton, inasmuch as substances like cytochalasin B or D and phalloidin, which directly modify cytoskeletal proteins, cause similar cell surface alterations in isolated hepatocytes [5,6].

The molecular basis of plasma membrane blebbing is presently unknown. However, during recent years the mechanisms by which individual cytoskeletal proteins interact have been extensively investigated, and calcium ions have been shown to play a critical role in the modulation of cytoskeletal structure and function. Through its ability to regulate the activity of cytoskeletal Ca^{2+}-binding proteins, Ca^{2+} can control the assembly of the major constituent proteins of the cytoskeleton, actin and tubulin [7]. Alternatively, changes in cytoskeletal structure may be mediated by catabolic enzymes, some of which are activated by increased cytosolic Ca^{2+} concentration, e.g. Ca^{2+}-dependent proteases [8]. Such proteases have now been isolated from a variety of cells, including hepatocytes [9], and shown to catalyze the degradation of cytoskeletal proteins in platelets [10] and to be involved in the Ca^{2+}-activated proteolysis in muscle associated with pathological processes such as hereditary dystrophies and hypertrophy [11].

We have previously shown that several toxic agents exert their effects on isolated hepatocytes through alterations of intracellular thiol and Ca^{2+} homeostasis, and that an increase in cytosolic Ca^{2+} concentration is associated with the appearance of plasma membrane blebs [12]. More recently, we

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have found that exposure of hepatocytes to the reactive disulfide, cystamine, causes an increase in cytosolic Ca^{2+} concentration which is associated with a stimulation of intracellular proteolysis and loss of cell viability [13]. The finding that pretreatment of the hepatocytes with inhibitors of Ca^{2+}-activated, cytosolic proteases could prevent both the stimulation of proteolysis and the loss of cell viability suggested that Ca^{2+}-activated proteases may be responsible for the toxicity of cystamine in hepatocytes [13].

We now report that plasma membrane blebbing caused by three different agents (cystamine, ATP, and ionophore A23187) in isolated hepatocytes is associated with enhanced rates of protein degradation, and that both surface blebbing and stimulation of proteolysis can be prevented by inhibitors of Ca^{2+}-activated proteases.

2. MATERIALS AND METHODS

Collagenase (grade II) and leupeptin were obtained from Boehringer, Mannheim. Quin 2-tetraacetoxymethyl ester and [^{14}C]valine (spec. act. 280 mCi/mmol) were purchased from Amersham. Cystamine, ATP, antipain, 3-methyladenine, methylamine were from Sigma (St. Louis, MO). All other reagents were of the highest grade of purity available and were obtained from local commercial sources.

Hepatocytes were isolated from male Sprague–Dawley rats (180–200 g, allowed food and water ad libitum) by collagenase perfusion of the liver as described [14]. Hepatocytes were incubated at a concentration of 1×10^6 cells/ml in a Krebs-Henseleit buffer, supplemented with 12.5 mM Hepes, pH 7.4, and equilibrated with 95% O_2:5% CO_2, at 37°C. Cell viability was assessed by counting the percentage of cells excluding trypan blue. Cytosolic Ca^{2+} concentration was assessed by the fluorometric measurement of the Ca^{2+}-quin 2 complex according to Cooper et al. [15], as described in detail in [13], using a Sigma ZW II spectrophotometer equipped for fluorescence measurements. The rate of intracellular proteolysis was assessed according to Seglen et al. [16]. The labelling of proteins in vivo was achieved by intraperitoneal injection of 0.5 ml [^{14}C]valine (50 μCi), dissolved in 0.9% NaCl, 16 h prior to hepatocyte isolation. The freshly isolated hepatocytes were washed in Krebs-Henseleit buffer and subsequently incubated in the same buffer at 37°C at a concentration of 1×10^6 cells/ml in the presence or absence of the substrate tested. In the experiments with the inhibitors, these agents were added at the time indicated in the text and figure legends. Samples (0.4 ml) were taken at different times and processed as reported in [16]. The rate of intracellular proteolysis is expressed as percentage of $HClO_4$-soluble ^{14}C radioactivity, at any given time point, vs total radioactivity at the beginning of the incubation.

Scanning electron microscopy of hepatocytes was performed according to standard procedures involving glutaraldehyde and osmium fixation, followed by critical point drying and visualization in a Jeol scanning electron microscope (model HSM 35).

3. RESULTS AND DISCUSSION

The agents employed in this study have all been found to alter the balance between influx and efflux of Ca^{2+} in hepatocytes. Thus, the incubation of hepatocytes with cystamine or extracellular ATP inhibits Ca^{2+} efflux, resulting in intracellular Ca^{2+} accumulation and elevation of cytosolic Ca^{2+} concentration. The mechanism by which cystamine inhibits Ca^{2+} efflux is related to its ability to form mixed disulfides with plasma membrane protein thiols, thereby affecting thiol-dependent plasma membrane Ca^{2+} translocase activity [13]. The exposure of hepatocytes to extracellular ATP is also associated with the inhibition of Ca^{2+} efflux [17], although the mechanism of the ATP effect is less clear [18]. The third agent employed in this study, ionophore A23187, mediates a concentration-dependent movement of Ca^{2+} across cellular membranes. In the presence of physiological levels of Ca^{2+} in the incubation medium, A23187 therefore induces rapid entry of Ca^{2+} into the cells and a marked increase in cytosolic Ca^{2+} concentration.

As shown in fig.1, all three agents (cystamine, ATP and A23187) caused plasma membrane blebbing in hepatocytes, the appearance of which was associated with an increased rate of proteolysis (fig.2). The addition of inhibitors of Ca^{2+}-activated proteases to the medium prior to incubation of hepatocytes with the three agents prevented the onset of cell blebbing (fig.1) and

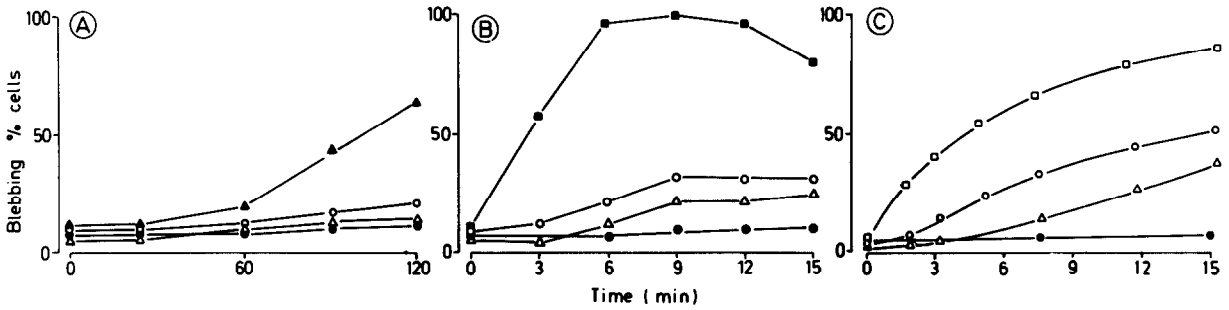


Fig.1. Onset of plasma membrane blebbing in hepatocytes treated with agents that increase cytosolic Ca^{2+} concentration, and protective effects of protease inhibitors. Hepatocytes were preincubated for 30 min in the absence or presence of protease inhibitors (leupeptin, 100 μg/ml; antipain, 50 μM). At 30 min the different agents were added and, when indicated, the percentage of cells exhibiting surface blebs was counted using a light microscope. Symbols. Panel A: control (●); 1 mM cystamine (▲); 1 mM cystamine + leupeptin (△); 1 mM cystamine + antipain (○). Panel B: control (●); 1 mM ATP (■); 1 mM ATP + antipain (○); 1 mM ATP + leupeptin (△). Pancel C: control (●); 15 μM A23187 (□); 15 μM A23187 + antipain (○); 15 μM A23187 + leupeptin (△).

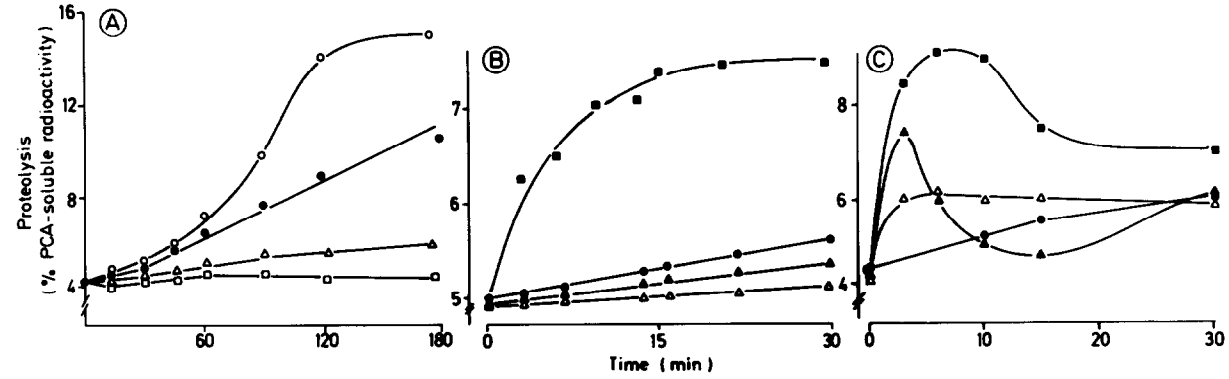
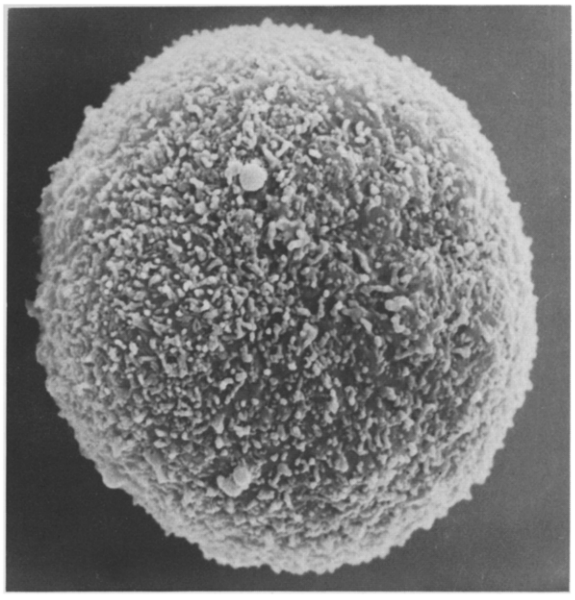


Fig.2. Stimulation of intracellular proteolysis by agents that increase cytosolic Ca^{2+} concetration and effects of protease inhibitors. Hepatocytes, isolated 16 h following i.p. injection of 50 μCi [^{14}C]valine, were preincubated at 37°C (1×10^6 cells/ml) for 30 min in the absence or presence of protease inhibitors. After 30 min, the various agents were added and the incubation continued. When indicated, 0.4 ml samples were removed from the incubation, and the rate of intracellular proteolysis was assessed as outlined in section 2. Symbols. Panel A: control (●); 1 mM cystamine (○); 1 mM cystamine + 100 μg/ml leupeptin (□); 1 mM cystamine + 50 μM antipain (△). Panel B: control (●); 1 mM ATP (■); 1 mM ATP + 100 μg/ml leupeptin (△); 1 mM ATP + 50 μM antipain (▲). Panel C: control (●); 15 μM A23187 (■); 15 μM A23187 + 50 μM antipain (▲); 15 μM A23187 + 100 μg/ml leupeptin (△).

markedly decreased the rate of proteolysis (fig.2). The effect of one of these inhibitors, leupeptin, on ATP-mediated hepatocyte blebbing is shown in fig.3.

To determine if several proteolytic systems were involved in the observed stimulation of protein degradation in hepatocytes exposed to cystamine, ATP, or A23187 different classes of inhibitors were tested. Lysosomotropic agents, such as methylamine and chloroquine, as well as the inhibitor of autophagy, 3-methyladenine, and serine protease inhibitors, i.e. trypsin inhibitor, were all unable to prevent the onset of plasma membrane blebbing. In contrast, leupeptin and antipain, which also inhibit non-lysosomal Ca^{2+}-dependent proteases, prevented both the stimulation of proteolysis and the formation of plasma membrane blebs caused by exposure of hepatocytes to cystamine, ATP, or ionophore A231287. Moreover, the latter inhibitors also prevented the



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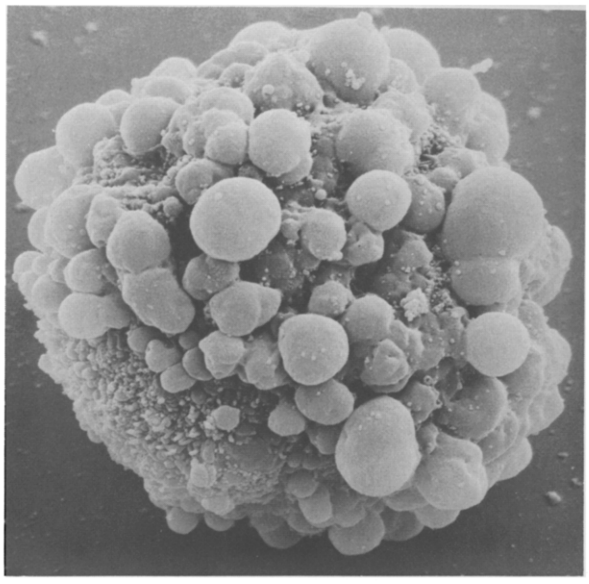

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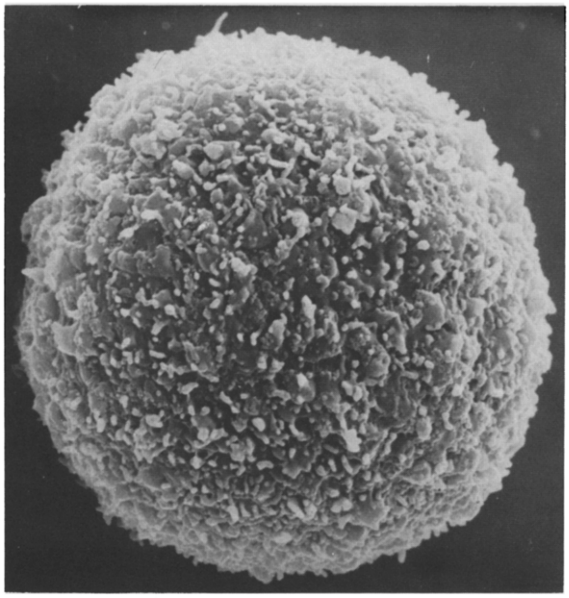

C

Fig.3. Scanning electron micrographs of hepatocytes incubated in the absence (A) or presence (B,C) of 1 mM ATP: in (C) the hepatocytes were preincubated with 100 μg/ml leupeptin for 30 min prior to addition of 1 mM ATP. Samples were taken 6 min after the addition of ATP. Magnifications: (A,C) $\times$4000 and (B) $\times$2500.

loss of viability of hepatocytes exposed to cystamine or ATP and markedly diminished the cytotoxic effect of A23186 (table 1).

Similar to their lack of effect on surface blebbing, methylamine and 3-methyladenine were unable to prevent the cytotoxicity induced by the three agents, further supporting the assumption that lysosomal proteases were not involved in the proteolysis caused by the rise in cytosolic Ca^{2+}.

Moreover, leupeptin and antipain did not exert their protective effects through modification of cytosolic Ca^{2+}, inasmuch as they did not prevent the rise in cytosolic Ca^{2+} caused by the three agents. Fig.4 shows an experiment in which pretreatment of hepatocytes with leupeptin had no apparent effect on the increase in cytosolic Ca^{2+} in hepatocytes exposed to extracellular ATP, although this treatment protected the hepatocytes

Table 1

Cytotoxicity in hepatocytes treated with agents that increase cytosolic Ca^{2+} concnetration, and effects of various protease inhibitors

Agents	Incubation time	Trypan blue permeability, percentage of cells				
		Control	Leupeptin	Antipain	Methylamine	3-Methyladenine
Control	30 min	9±3	10±1	7±3	12±1	11±2
ATP, 1 mM	30 min	50±6	12±6	12±4	52±2	52±7
A23187, 15 μM	30 min	65±2	25±2	28±2	66±5	60±3
Control	180 min	19±6	16±5	12±2	22±1	24±3
Cystamine, 1 mM	180 min	58±7	16±5	18±4	55±4	62±8

Hepatocytes were preincubated for 20 min in the absence or presence of protease inhibitors (leupeptin, 100 μg/ml; antipain, 50 μM; methylamine, 10 mM; 3-methyladenine, 5 mM). At 20 min the various agents were added and, when indicated, the percentage of cells permeable to trypan blue was counted in a light microscope. Values are expressed as means ± SD of 5 different experiments.

from both surface blebbing and loss of viability.

It is not yet known which proteins are substrates for Ca^{2+}-activated proteolysis. However, our observation that inhibitors of Ca^{2+}-activated proteases prevent the onset of blebbing, suggests that cytoskeletal proteins may be targets for the proteolytic activity. Furthermore, our results support the assumption that plasma membrane blebbing is an early sign of toxicity in hepatocytes and suggest that Ca^{2+}-activated proteases may play an important role in the development of cytotoxicity resulting from disruption of intracellular Ca^{2+} homeostasis. They also provide a possible link between the inhibition of plasma membrane Ca^{2+} translocase activity, suggested to be a critical biochemical lesion of several hepatotoxins [13,19], and cytotoxicity. Although we do not exclude that other mechanisms may be equally important in causing toxic damage to hepatocytes, experimental evidence for their nature and role in hepatocyte blebbing and toxicity is presently lacking.

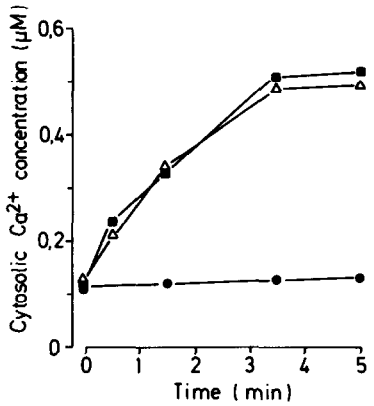

Fig.4. Increase in cytosolic Ca^{2+} level in control and leupeptin-treated hepatocytes after addition of 1 mM ATP. Quin 2-loaded hepatocytes were incubated in a cuvette maintained at 37°C, where the hepatocytes were continuously stirred under an atmosphere of 95% O_2:5% CO_2. Cytosolic Ca^{2+} measurements were performed as indicated in section 2. Leupeptin-treated hepatocytes were preincubated in the presence of the protease inhibitor for 10 min prior to loading with quin 2 AM. Symbols: control (●); 1 mM ATP (■); 100 μg/ml leupeptin + 1 mM ATP (△).

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REFERENCES

[1] Jewell, S.A., Bellomo, G., Thor, H., Orrenius, S. and Smith, M.T. (1982) Science 217, 1257.

[2] Trump, B.F., Berezesky, I.K., Laiho, K.U., Osornio, A.R., Mergner, W.J. and Smith, M.W. (1980) in: Scanning Electron Microscopy (O'Hare, A.M.F. ed.) vol.2, pp. 437–462, SEM Inc., Chicago.

[3] Hyslop, P.A., Hinshaw, D.B., Schraufstatter, I.V., Skylar, L.A., Spragg, R.G. and Cochrane, C.G. (1986) J. Physiol., in press.

[4] Trump, B.F. et al., (1984) J. Cell Biol. 99, 180a.

[5] Prentki, M., Chaponnier, C., Jeanreneaud, B. and Gabbiani, G. (1979) J. Cell Biol. 81, 592.

[6] Smith, M.T. and Orrenius, S. (1984) in. Drug Metabolism and Drug Toxicity (Mitchell, J.R. and Horning, M.G. eds) pp. 71–98, Raven Press, New York.

[7] Weeds, A. (1982) Nature 296, 811.

[8] Sugita, H., Ishiura, S., Kamakura, K., Nakase, H., Hagiwara, H., Nonaka, I. and Tomomatsu, K. (1984) in: Calcium Regulation in Biological Systems (Ebashi, S. et al. eds) pp. 243–256, Academic Press, Tokyo.

[9] De Martino, G. (1981) Arch. Biochem. Biophys. 211, 253.

[10] Collier, M.C. and Wang, K. (1982) J. Biol. Chem. 257, 6937.

[11] Kameyama, T. and Etlinger, J.D. (1979) Nature 279, 344.

[12] Smith, M.T., Thor, H., Jewell, S.A., Bellomo, G., Sandy, M.S. and Orrenius, S. (1984) in: Free Radicals in Molecular Biology, Aging and Disease (Armstrong, D. et al. eds) pp. 103–117, Raven Press, New York.

[13] Nicotera, P., Hartzell, P., Baldi, C. Svensson, S.-Å., Bellomo, G. and Orrenius, S. (1986) J. Biol. Chem., in press.

[14] Moldéus, P., Högberg, J. and Orrenius, S. (1978) Methods Enzymol. 52, 50.

[15] Cooper, R.H., Coll, K.E., Williamson, J.R. (1985) J. Biol. Chem. 260, 3281.

[16] Seglen, P.O., Grinde, B. and Solheim, A.E. (1979) Eur. J. Biochem. 95, 215.

[17] Bellomo, G., Nicotera, P. and Orrenius, S. (1984) Eur. J. Biochem. 144, 19.

[18] Mirabelli, F., Bellomo, G., Nicotera, P., Moore, M. and Orrenius, S. (1986) J. Biochem. Toxicol. 1, 29.

[19] Tsokos, J.D., Todol, E.L., McMillin-Wood, J.B. and Mitchell, J.R. (1985) Mol. Pharmacol. 28, 56.