Dependence of Hepatocytic Autophagy on Intracellularly Sequestered Calcium*

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Autophagic sequestration of endogenous lactate dehydrogenase or electroinjected [3H]raffinose in isolated rat hepatocytes was strongly suppressed by the Ca++ chelator EGTA, unless the cells had previously been electroloaded in the presence of high concentrations of Ca++ (1.2 mM). The extracellular Ca++ chelator bis-(o-aminophenoxy)-ethane-N,N,N',N' ‑tetraacetic acid (BAPTA) and the intracellular Ca++ chelator BAPTA/tetra(acetoxymethyl)-ester (BAPTA/AM) both inhibited autophagy to the same extent as did EGTA. Inhibitors of Ca++-activated protein kinases (KN-62, H-7, W-7) had little or no effect on autophagy, indicating that the Ca++ requirement of autophagy was not mediated by such kinases. Agents that elevate cytosolic Ca++ by releasing Ca++ from intracellular stores, like thapsigargin, 2,5-di-( tert-butyl)-1,4-benzoquinone (tBuBHQ) and the ionophores A23187 and ionomycin, inhibited autophagy strongly, implicating depletion of sequestered rather than of cytosolic intracellular Ca++ as a common mechanism of inhibition. Lysosomal (propylamine-sensitive) protein degradation, known to be largely autophagy-dependent, was inhibited by thapsigargin and tBuBHQ. Thapsigargin had no effect on cellular ATP levels, but all agents tested (thapsigargin, tBuBHQ, ionophores) inhibited protein synthesis. Our results suggest that autophagy, like protein synthesis, is dependent on the presence of Ca++ in some intracellular storage compartment.

Materials and Methods

Animals and Cells—Hepatocytes were isolated by two-step collagenase perfusion (24) from 18-h starved, male Wistar rats (250-300 g) maintained on a controlled, cyclic feeding and illumination scheme. The cells were incubated at 37 °C for 2 h as 0.4-ml aliquots in rapidly shaking 15-cm centrifuge tubes at a cell concentration of about 80 mg of wet weight/ml for 3 h as 2-ml aliquots in stationary, albumin-coated 6-cm Petri dishes (25) at a cell concentration of about 8 mg of wet weight/ml. The incubation medium was protein-free suspension buffer (24), supplemented with Mg2+ (2 mM) and pyruvate (20 mM).

Measurement of Autophagy—Autophagy was measured either as the sequestration of [3H]raffinose, diffusion-loaded into electropor-meabilized hepatocytes during a 1-h preincubation at 0 °C (22) or as the sequestration of endogenous lactate dehydrogenase in non-per-meabilized cells incubated in the presence of 0.3 mM leupeptin (23). The fraction of the probe present in sedimentable (i.e. autophagically sequestered) form was measured at the beginning and end of (or during) the incubation, and the difference (net sequestration) was expressed as percent or percent/h.

Measurement of Intracellular Calcium—Hepatocytes were loaded with the fluorescent Ca++ indicator Fura-2 (26) during a 30-min preincubation at 37 °C in the presence of 50 μM Fura-2. After washing three times at 0 °C, the cells were adsorbed onto polylysine-coated quartz coverslips and examined at 37 °C in a ratio microfluorimeter as previously described (27) while the required reagent additions were performed with a micropipette. The intracellular concentration of free Ca++ in single cells was estimated from the 340/380 nm fluorescence ratio.

Other Determinations—Protein degradation was measured as the percent release of acid-soluble radioactivity from hepatocytes labeled in vivo by the intravenous injection of 50 μCi of [14C]valine (28).

Protein synthesis was measured in the presence of a complete, balanced amino acid mixture (6) as the incorporation of [14C]valine into acid-precipitable material. The synthesis rate was calculated on the basis of measured initial cellular wet weight, the known protein/wet weight ratio (24), and the known valine content of hepatocellular protein (25).

Hepatocellular ATP content was measured at the end of incubation in neutralized perchloric acid extracts, using the luciferin/luciferase assay procedure and the luminometer provided by LKB-Wallac Oy, Oulu, Finland.

Reagents—[14C]Valine (250 Ci/mol; 50 μCi/ml) was purchased from Amersham Corp., [14C]raffinose (5 Ci/mol; 1 μCi/ml) from...
FIG. 1. Effect of EGTA on raffinose autophagy in hepatocytes electrically preloaded in the presence of calcium. Hepatocytes were preloaded with [3H]raffinose for 1 h at 0°C in suspension buffer containing 1.2 mM Ca\(^{2+}\), then resealed, washed, and incubated with 0.1 mM EGTA at 37°C for the length of time indicated (A) or for 2 h at 37°C with the concentration of EGTA indicated (B). The net amount of [3H]raffinose sequestered in autophagic vacuoles was measured and expressed as percentage of the total cell-associated radioactivity. Each value is the mean ± S.E. of three to four experiments.

DuPont NEN, and ATP-monitoring reagents from Pharmacia LKB Biotechnology Inc. Thapsigargin was initially donated by S. Bragger Christensen, Copenhagen, Denmark, and later purchased from Life Technologies, Inc. BAPTA/AM\(^1\) and Fura-2 were obtained from Calbiochem, San Diego, CA; 2,5-di-(tert-butyl)-1,4-benzoquinone from Fluka Chemie AG, Buchs, Switzerland; leupeptin from Peptide Institute Inc., Osaka, Japan, and KN-62 from Seikagaku Corp., Chuo-Ku, Tokyo, Japan. Calcium ionophores (ionomycin; tBuBHQ) and other biochemicals were purchased from Sigma.

RESULTS

Effect of the Calcium Chelator EGTA on Autophagy in Hepatocytes Preloaded by Electroporation—The measurement of autophagy as raffinose sequestration includes a preloading step during which electropermeabilized hepatocytes are incubated with [3H]raffinose for 1 h at 0°C in a Ca\(^{2+}\)-containing medium (1.2 mM Ca\(^{2+}\)) (22). Subsequent incubation of hepatocytes at 37°C in a Ca\(^{2+}\)-free medium containing 0.1 mM EGTA had relatively little effect on hepatocytic autophagy (Fig. 1A); only at an EGTA concentration as high as 1 mM was a substantial inhibition (50%) observed (Fig. 1B).

Inhibition of Autophagy by EGTA in Non-preloaded Cells—It would seem likely that permeabilized hepatocytes might become loaded with Ca\(^{2+}\) as well as with raffinose during the preloading step, thereby acquiring some resistance against Ca\(^{2+}\) depletion by EGTA. We therefore measured autophagy by an alternative method that does not involve electroporation, i.e. as the sequestration of an endogenous cytosolic enzyme, lactate dehydrogenase (23). Lactate dehydrogenase autophagy in non-permeabilized cells was highly sensitive to EGTA, strong inhibition being observed at 0.1 mM (Fig. 2A). In contrast, lactate dehydrogenase autophagy in electroporereabilized, preloaded cells displayed the same EGTA-resistance as raffinose autophagy (Fig. 2B). The permeabilization/preloading step thus clearly confers EGTA resistance, most probably as a result of Ca\(^{2+}\) preloading. Without preloading, the chelation of extracellular Ca\(^{2+}\) by EGTA resulted in suppression of autophagic activity. Measurements of intracellular free Ca\(^{2+}\) in Fura-2-loaded hepatocytes revealed that EGTA caused a rapid (within a few minutes) fall in intracellular Ca\(^{2+}\) (results not shown).

Both Extra- and Intracellular Calcium Chelation Causes Inhibition of Autophagy—Another chelator of extracellular Ca\(^{2+}\), BAPTA (30), inhibited autophagy just as effectively as did EGTA (Fig. 3). The acetoxyethyl ester of BAPTA, BAPTA/AM, penetrates the plasma membrane and is cleaved intracellularly to yield free BAPTA (31); this agent can therefore be used as a specific chelator of intracellular Ca\(^{2+}\). Incubation of hepatocytes with BAPTA/AM suppressed autophagy to the same extent as seen with extracellular chelators (Fig. 3). The inhibition would thus seem to be a consequence of intracellular Ca\(^{2+}\) depletion.

Inhibition of Autophagy by Agents Which Deplete Intracellular Calcium Stores—Thapsigargin strongly inhibits Ca\(^{2+}\)-ATPases (calcium pumps) associated with the endoplasmic and sarcoplasmic reticulum, thereby causing a rapid depletion of Ca\(^{2+}\) from these intracellular Ca\(^{2+}\) stores (32, 33). In isolated hepatocytes, thapsigargin induced a transient rise in the intracellular free Ca\(^{2+}\) level (Fig. 4A), as previously observed (32).

Thapsigargin was a potent inhibitor of hepatocytic autophagy, half-maximal inhibition being attained around 1 μM (Fig. 5A). Its effect was fully reversible upon removal (Fig. 5B). Autophagic-lysosomal (propylamine-resistant) protein degradation was likewise inhibited by thapsigargin, as would be expected (Fig. 6). Thapsigargin had no effect on hepatocytic ATP levels, but inhibited protein synthesis (Fig. 7), in agreement with observations made on other cell types (34–37). The suppression of autophagy by thapsigargin was not prevented by calmodulin antagonists (trifluoperazine; calmidazolium; W-7) or by inhibitors of Ca\(^{2+}\)-dependent protein kinases (KN-62; H-7) (38), nor did these agents have much effect on autophagy on their own (data not shown). Activation of Ca\(^{2+}\)/calmodulin-dependent protein kinases as the result of an elevated intracellular Ca\(^{2+}\) level would thus not seem to be involved in autophagy suppression. The transient nature of the Ca\(^{2+}\) elevation (Fig. 4A) would, in any case, render it unlikely to support a sustained suppression of autophagy.

Like thapsigargin, 2,5-di-(tert-butyl)-1,4-benzoquinone (tBuBHQ) depletes intracellular Ca\(^{2+}\) stores in hepatocytes (39) and raises intracellular free Ca\(^{2+}\) levels (Fig. 4B). tBuBHQ inhibited autophagy strongly (Fig. 8A), albeit with less potency (half-maximal effect at 30 μM) than thapsigargin. tBuBHQ also inhibited hepatic protein degradation (Fig. 8B), to approximately the same extent as did propylamine.

1 The abbreviations used are: BAPTA/AM, bis-(o-aminophenoxy)ethane-N,N',N'-tetraacetic acid, tetraacetoxymethyl ester; BAPTA, bis-(o-aminophenoxy)ethane-N,N',N'-tetraacetic acid; ER, endoplasmic reticulum; tBuBHQ, 2,5-di-(tert-butyl)-1,4-benzoquinone.
Fig. 2. Inhibition of lactate dehydrogenase autophagy by EGTA: effect of preloading in the presence of calcium. Hepatocytes were incubated at 37 °C for the length of time indicated with leupeptin (0.3 mM, to protect autophagic lactate dehydrogenase) in the absence (○) or presence (●) of 0.1 mM EGTA. The net amount of cytosolic lactate dehydrogenase sequestered in autophagic vacuoles at each time point was measured and expressed as percentage of total cell-associated lactate dehydrogenase. A, no electropermeabilization or preloading; B, electropermeabilized cells preloaded for 1 h at 0 °C in suspension buffer containing 1.2 mM Ca2+ before the final incubation. Each value is the mean ± S.E. of three to four experiments.

Fig. 3. Inhibition of autophagy by both intra- and extracellular calcium chelators. Hepatocytes were incubated for 2 h at 37 °C with leupeptin (0.3 mM) and the indicated concentration of EGTA (○), BAPTA (●) or BAPTA/AM (●). The rate of net lactate dehydrogenase (LDH) sequestration during the incubation has been expressed as percent/h (relative to total cell-associated lactate dehydrogenase). Each value is the mean of two independent experiments.

Fig. 4. Intracellular calcium release elicited by thapsigargin or 2,5-di-(tert-butyl)-1,4-benzoquinone. Thapsigargin, 5 μM (A) or 2BuBuHQ, 0.1 mM (B) was added to hepatocytes loaded with Fura-2, and the concentration of intracellular free Ca2+ measured fluorimetrically by the 340/380 nm fluorescence ratio method.

Role of Ca2+ in Autophagy

Our results indicate that hepatocytic autophagy is dependent on the presence of sequestered Ca2+ in some intracellular storage compartment that is sensitive to interference by a number of Ca2+-perturbing agents. In hepatocytes, there is evidence for the presence of Ca2+ in the nuclear envelope (45–47), the Golgi/trans-Golgi network (15, 16), the endoplasmic reticulum (ER) (48–51), and the mitochondria (52–54). There are indications that Ca2+-ATPase inhibitors like thapsigargin and 2BuBuHQ (32, 33, 55, 56) may interfere with Ca2+ transport or Ca2+-dependent processes in nuclei (47) and Golgi/trans-Golgi network (37) as well as in the ER (32, 55); it is therefore questionable whether these inhibitors can be used to distinguish between the various Ca2+ pools. Furthermore, the thapsigargin-sensitive "ER stores" are heterogeneous, comprising, e.g. a hormone/inositol 1,4,5-trisphosphate-sensitive, oxalate-inaccessible pool, a GTP-sensitive, oxalate-accessible pool, and a pool insensitive to both hormones/inositol 1,4,5-trisphosphate and GTP (32, 57, 58). The fact that vasopressin, an effective releaser of hepatocytic ER Ca2+ (51, 59), has relatively little effect on autophagy (60), may indicate that (data not shown), suggesting a virtually complete suppression of the autophagic-lysosomal degradation pathway (40). Protein synthesis was strongly inhibited by 2BuBuHQ (Fig. 8C), as previously demonstrated in the perfused liver (41).

Calcium ionophores like ionomycin and A23187 have been found to deplete intracellular Ca2+ stores in a variety of cell types, including hepatocytes (42–44). As indicated in Table I, the ionophore A23187 inhibited autophagy as well as protein synthesis in isolated hepatocytes, with half-maximal inhibition below 10 μM. There was no evident cytotoxicity (lactate dehydrogenase leakage) except at 100 μM in the presence of extracellular Ca2+. Inhibitory effects of 10 μM A23187 were observed both in high Ca2+, low Ca2+ and EGTA-containing medium, supporting the contention that suppression of autophagy is independent of extracellular Ca2+ and probably due to depletion of Ca2+ from intracellular stores. Ionomycin, on the other hand, had no detectable effects up to 10 μM, but was markedly toxic at 100 μM, especially in the presence of Ca2+ when it induced a massive leakage of cytosolic enzyme (Table I). Ionomycin concentrations between 10 and 100 μM were not tested; it therefore remains possible that intermediate concentrations of ionomycin may have effects similar to those of A23187.

Discussion

Our results indicate that hepatocytic autophagy is dependent on the presence of sequestered Ca2+ in some intracellular storage compartment that is sensitive to interference by a number of Ca2+-perturbing agents. In hepatocytes, there is evidence for the presence of Ca2+ in the nuclear envelope (45–47), the Golgi/trans-Golgi network (15, 16), the endoplasmic reticulum (ER) (48–51), and the mitochondria (52–54). There are indications that Ca2+-ATPase inhibitors like thapsigargin and 2BuBuHQ (32, 33, 55, 56) may interfere with Ca2+ transport or Ca2+-dependent processes in nuclei (47) and Golgi/trans-Golgi network (37) as well as in the ER (32, 55); it is therefore questionable whether these inhibitors can be used to distinguish between the various Ca2+ pools. Furthermore, the thapsigargin-sensitive "ER stores" are heterogeneous, comprising, e.g. a hormone/inositol 1,4,5-trisphosphate-sensitive, oxalate-inaccessible pool, a GTP-sensitive, oxalate-accessible pool, and a pool insensitive to both hormones/inositol 1,4,5-trisphosphate and GTP (32, 57, 58). The fact that vasopressin, an effective releaser of hepatocytic ER Ca2+ (51, 59), has relatively little effect on autophagy (60), may indicate that...
FIG. 5. Inhibition of autophagy by thapsigargin. A, raffinose-loaded hepatocytes were incubated for 3 h at 37 °C with thapsigargin at the concentration indicated. B, raffinose-loaded hepatocytes were preincubated for 20 min at 37 °C without (circles) or with (triangles) 3 µM thapsigargin; then reincubated with (filled symbols) or without (open symbols) thapsigargin. The net amount of [3H]raffinose sequestered in autophagic vacuoles was measured and expressed as percentage of the total cell-associated radioactivity. Values represent single experiments, or the means ± range/S.E. of two to six experiments.

FIG. 6. Inhibition of lysosomal protein degradation by thapsigargin. Rats were given an intravenous injection of [14C]valine (50 µCi in 1 ml) 24 h prior to sacrifice. Hepatocytes were incubated for 3 h at 37 °C with 5 mM valine in the absence (O) or presence (●) of 10 mM propylamine and the concentration of thapsigargin indicated. The net release of [14C]valine during the incubation was measured and expressed as percentage of the total cell-associated radioactivity. Each value represents the mean ± S.E. of three experiments (without propylamine) or a single experiment (with propylamine).

FIG. 7. Effect of thapsigargin on protein synthesis and cellular ATP levels. Hepatocytes were incubated for 3 h at 37 °C with an amino acid mixture (6) containing 5 mM [14C]valine (361 µCi/liter). The net incorporation of radioactivity into cellular protein during the incubation was measured, and the rate of protein synthesis (●) calculated as percent/h on the basis of initial cellular wet weight (29). The cellular ATP content (○) was measured at the end of incubation (3 h). Each value is the mean ± range/S.E. of two to three experiments.

the autophagy-associated Ca²⁺ store is different from the hormone-sensitive stores. A similar conclusion was suggested for the Ca²⁺ store controlling protein synthesis in HeLa cells (35). However, in light of the more persistent cellular effects of thapsigargin as compared to hormones (34, 61, 62), long term studies of Ca²⁺ kinetics are needed to resolve this question unequivocally.

The membranous organelles responsible for autophagic sequestration, the phagophores (2), are possibly derived from the ER cisternae (3, 4, 63); their function could therefore well be dependent on intracisternal Ca²⁺. The possibility should also be considered, however, that intracellular Ca²⁺ depletion may affect autophagy indirectly. All the Ca²⁺-depleting agents tested seem to inhibit autophagy and protein synthesis in parallel, and although hepatocytic autophagy is basically protein synthesis-independent (63, 64), a common signal mechanism may be involved. It has been suggested that both the inhibition of protein synthesis and the induction of gene expression (of GRP78, an ER protein probably involved in Ca²⁺-dependent protein folding) by Ca²⁺-depleting agents may be mediated by a secondary signal resulting in protein kinase activation (36). It is interesting in this connection to note that thapsigargin and Ca²⁺ ionophores have been shown to cause activation of MAP kinase (65), a protein kinase involved in the regulation of gene expression (66). Studies using inhibitors of protein kinases and protein phosphatases indicate that protein kinases may be involved both in positive and negative control of autophagy (38). The lack of effect of the inhibitor KN-62 in the present experiments suggests that the effect of Ca²⁺ depletion is not due to inhibition of the Ca²⁺/calmodulin-dependent protein kinase II (38), but the possibility should be considered that the Ca²⁺ dependence of au-
assistance provided by Mona Birkeland.

Inhibition of autophagy, lysosomal protein degradation and protein synthesis by 2,5-di-(tert-butyl)-1,4-benzoquinone. A, raffinose-loaded hepatocytes were incubated for 3 h at 37 °C with BuBHQ at the concentration indicated, and the net amount of [3H]raffinose sequestered in autophagic vacuoles was measured and expressed as percentage of the total cell-associated radioactivity. B, hepatocytes prelabeled with [3H]valine by in vivo injection, 24 h before sacrifice, were incubated for 3 h with 5 mM valine, and the rate of protein degradation was measured as the release of acid-soluble [3H]valine, expressed as percent/h. C, hepatocytes were incubated for 3 h at 37 °C with an amino acid mixture containing 5 mM [3H]valine, and protein synthesis was measured as the net incorporation of radioactivity into cellular protein, expressed as percent/h. Values represent the mean ± range of two experiments (A) or single experiments (B, C).

Table 1
Effect of calcium ionophores on hepatic autophagy and protein synthesis

<table>
<thead>
<tr>
<th>Ionophore concentration (µM)</th>
<th>Autophagy</th>
<th>Protein synthesis</th>
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<tbody>
<tr>
<td></td>
<td>High Ca⁺⁺</td>
<td>Low Ca⁺⁺ + EGTA</td>
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<tr>
<td></td>
<td>(2 mM)</td>
<td>(100 µM)</td>
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<tr>
<td>% LDH sequestered/h</td>
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<tr>
<td>Ionomycin</td>
<td>100 µM</td>
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<tr>
<td>0</td>
<td>0.61 ± 0.05</td>
<td>0.60 ± 0.03</td>
</tr>
<tr>
<td>1</td>
<td>0.70 ± 0.10</td>
<td>0.61 ± 0.04</td>
</tr>
<tr>
<td>10</td>
<td>0.71 ± 0.05</td>
<td>0.56 ± 0.05</td>
</tr>
<tr>
<td>100</td>
<td>0.73 ± 0.09</td>
<td>0.53 ± 0.03</td>
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<tr>
<td>A23187</td>
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<tr>
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<tr>
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<tr>
<td>100</td>
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* Highly toxic; >90% loss of cytosolic protein (lactate dehydrogenase, LDH) in 2 h.

† Toxic; loss of cytosolic protein (LDH) in 2 h twice as high as from control cells. Cells incubated without ionophores lost 5% LDH in 2 h in the presence of Ca⁺⁺, and 10–15% in the absence of Ca⁺⁺ or with EGTA. Autophagy was measured as the net sequestration of endogenous LDH during a 2-h incubation in the presence of leupeptin (300 µM) and expressed as %/h relative to total cellular LDH; each value is the mean ± S.E. of three experiments. Protein synthesis was measured as the net incorporation of [3H]valine during a 2-h incubation in the presence of leupeptin and a complete, balanced amino acid mixture and expressed as percent/h relative to total cellular protein; each value is the mean ± range of two experiments.

tophagy may reflect an autophagy-regulatory function of other Ca⁺⁺-sensitive protein kinase.

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REFERENCES