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Glycogen autophagy in newborn rat hepatocytes

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Summary. Glycogen autophagy in newborn rat hepatocytes was studied by using enzyme determinations and electron microscopy. Cyclic AMP induced glycogen autophagy in these cells. Glycogen-hydrolyzing acid glucosidase activity increased whereas acid mannose 6-phosphatase activity decreased in the liver of these animals. Parenteral glucose, which prevents postnatal glucagon secretion and tissue cyclic AMP elevation, and propranolol which antagonizes cyclic AMP, inhibited glycogen autophagy. Glucosidase activity decreased and phosphatase activity increased. These findings raise the possibility that cyclic AMP-induced autophagic mechanisms in newborn rat hepatocytes are associated with changes in the activity of acid mannose 6-phosphatase.

Key words: Glycogen autophagy, Acid glucosidase, Acid mannose 6-phosphatase, Cyclic AMP, Newborn rat

Introduction

During the immediate postnatal period there is a precipitous fall in the liver glycogen level of all mammalian species. Phosphorolytic degradation of the glycogen by phosphorylase in the hyaloplasm of newborn hepatocytes plays a key role in this process (Dawkins, 1963; Walker, 1968). Hydrolytic degradation of the polysaccharide by acid glucosidase in the autophagic vacuoles of these cells also becomes important in this period (Rosenfeld, 1964; Kotoulas and Phillips, 1971; Pfeifer, 1971; Kalamidas et al., 1994).

In newborn rats, a burst of autophagic activity has been observed in the hepatocytes four to six hours after birth. At this age, an increase in the lysosomal number, total volume and glycogen-hydrolyzing activity, has been noted. Most of the lysosomes are glycogen-containing autophagic vacuoles. These phenomena appear to be regulated by the cAMP tissue levels (Kotoulas and Phillips, 1971; Kotoulas, 1981, 1986; Kalamidas et al., 1994). In a previous paper, it was suggested that alterations in this autophagic activity may be associated with changes in the activity of the important lysosomal enzyme acid mannose 6-phosphatase (Kalamidas and Kotoulas, 2000). In this paper, our previous studies on glycogen autophagy are further extended to include determinations of this enzyme activity.

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Materials and methods

Chemicals

Adenosine 3',5' cyclic monophosphate (cAMP), Lot 29F-7030; Actinomycin D, Lot 62C-3400; Glycogen, Lot 126F-3846; D-Mannose 6-phosphate disodium salt, Cat. No. M-6876; p-nitrophenyl phosphate di-sodium.6H2O, Lot 08846189; Sodium tartrate dihydrate, Lot 29710123 and the reagents for determining glucose, inorganic phosphorus and cyclic AMP-dependent protein kinase were obtained from Sigma. Propranolol hydrochloride (Inderal), Lot PL29/5062 was from Imperial Chemical Industries. D-glucose was obtained from Serva. Reagents for electron microscopy were obtained as before (Kalamidas et al., 1994).

Experimental design

Newborn Wistar rats were obtained from twelve pregnant females and treated as before (Kalamidas and Kotoulas, 1999). For the biochemical and electron microscopic work, three normal animals were killed at birth and every two hours after birth up to the age of 12 hours. The rest of the animals were divided into four groups according to the agent used. Five animals were injected intraperitoneally with cyclic AMP at birth and 3 hours after birth (a dose of 100 mg/kg each time), propranolol at birth and 2 hours after birth (a dose of 16.7 mg/kg each time), glucose at birth and every two hours thereafter (a dose of 2.33 g/kg each time) or actinomycin at birth and 3 hours after birth (a dose of 0.8 mg/kg each time). An equal number of controls were injected with carrier only, except in the group of propranolol, where normal animals were used as controls. The animals injected with cyclic AMP or propranolol were killed at the age of 6 hours while those injected with glucose or actinomycin were killed at the age of 12 hours.

For the quantitative morphometric work on electron micrographs randomly selected animals were used. Three treated animals and three controls were studied from each group.

Biochemical methods

The glycogen-hydrolyzing activity of acid glucosidase (acid alpha 1,4 exopolyglucosidase, acid amyloglucosidase) was assayed in homogenates of liver tissue in a 10% dilution with ice-cold distilled water. Usually, 200 μl of homogenate were used for the assay. The total enzyme activity was determined with glycogen (1%) as substrate as described before (Hers, 1963; Lejeune et al., 1963; Lundquist, 1985; Kalamidas and Kotoulas, 1999). Enzyme activity was expressed as micromoles of glucose produced per hour per mg of protein. The cyclic AMP-dependent protein kinase activity was assayed in the homogenates according to Laks and Jungmann (1980) and Laks et al. (1981) in the presence of 10^{-6} M cyclic AMP with protamine sulfate as substrate. Phosphorus bound to substrate was hydrolyzed by the use of 20% NaOH in a boiling water bath, and assayed as inogranic phosphorus (Fiske and Subbarow, 1925; Kalamidas and Kotoulas, 1999). The acid mannose 6-phosphatase activity was assayed in the homogenates essentially according to Arion and Nordlie (1964) and Nordlie and Arion (1964, 1965) with mannose 6-phosphate (7mM) as substrate. Incubation was carried out for 60 min in 0.1M sodium acetate buffer with 0.05% Triton X-100, at pH 5.2 and at 37 °C. The reaction was stopped by 10% trichloroacetic acid (VandeWerve, 1989; Einstein and Gabel, 1991; Kalamidas and Kotoulas, 2000). Enzyme activity was expressed as micromoles of inorganic phosphorus produced per hour per mg of protein. The total and tartrate–resistant, p-nitro-phenyl phosphate-hydrolyzing acid phosphatase activities were determined according to Griffiths et al. (1990) and Hayman and Cox (1994) using 10 mM of the substrate with or without 100 mM of the inhibitor. Enzyme activities were expressed as micromoles of p-nitro-phenyl phosphate hydrolyzed per hour per mg of protein. Formyl-methionyl-leucyl-phenylalanine (FMLP)-inhibitable Ca^{2+}-ATPase activity was determined in the homogenates according to Klempner (1985). Incubation of the reaction mixture was carried out in the presence and absence of 10^{-7} M FMLP (Caroni and Carafoli, 1981; Lagast et al., 1984; Klempner, 1985).

Electron microscopy and morphometric analysis

Liver tissue was fixed for 1 hour in 1% osmium tetroxide. For the quantitative morphometric work on electron micrographs, five blocks were prepared from each liver. From each block two randomly-taken micrographs were used. The pictures were enlarged to a final magnification of 41600. The data of the micrographs from the same block were combined and therefore the means and standard errors were calculated from these combined data. The fractional volume of lysosomes was determined by the use of a lattice composed of horizontal and vertical lines superimposed on the prints (Weibel, 1969, 1979; Kotoulas et al., 1971; Kalamidas and Kotoulas, 1999).

Results

Biochemical results

Glycogen-hydrolyzing acid glucosidase and acid mannose 6-phosphatase activities during the first 12 hours after birth, are shown in Table 1. Glucosidase activity was low at birth but showed an increase at the age of 4 hours. This activity returned to a lower level at the age of 12 hours. Mannose 6-phosphatase activity was also low at birth but showed a virtually continuous rise during this postnatal period. A transient fall was observed at the age of 6 hours. Actinomycin given at birth suppressed both enzymes (glucosidase: controls, 0.172±0.050 μmoles/hr/mg protein, actinomycin-treated animals, 0.098±0.036 μmoles/hr/mg protein, $p<0.05$; mannose 6-phosphatase: controls, 16.3±2.7 μmoles/hr/mg protein, actinomycin-treated animals, 7.2±0.9 μmoles/hr/mg protein, $p<0.05$).

The results of cyclic AMP administration are shown in Table 2. This administration resulted in increased activity of glycogen-hydrolyzing acid glucosidase and decreased activity of acid mannose 6-phosphatase. The opposite results were produced by the parenteral administration of glucose: i.e., decreased activity of glucosidase and increased activity of mannose 6-phosphatase (Table 3). Results similar to those of glucose were produced by propranolol (glucosidase: controls, 0.205±0.077 μmoles/hr/mg protein, propranolol-treated animals, 0.118±0.053 μmoles/hr/mg

Table 1. Liver acid glucosidase and acid mannose 6-phosphatase activities during the first 12 hours after birth. Results are means±standard deviations. Each value includes 3 observations.

	AGE (hours after birth)						
	0	2	4	6	8	10	12
Acid glucosidase (μmoles glucose/ hr/mg protein)	0.084±0.036	0.084±0.042	0.217±0.071	0.201±0.081	0.180±0.064	0.173±0.059	0.175±0.059
Mannose 6-phosphatase (μmoles phosphorus/hr/mg protein)	5.3±1.6	7.9±1.4	8.7±1.5	8.2±1.5	10.5±1.7	14.3±2.3	20.0±2.9

protein, p<0.05; mannose 6-phosphatase: controls 9.3±1.5 μmoles/hr/mg protein, propranolol-treated animals, 13.6±1.8 μmoles/hr/mg protein, p<0.05). No statistically significant changes in the total and tartrate-resistant activities of p-nitro-phenyl phosphate-hydrolyzing acid phosphatase were found after cyclic AMP treatment (total: controls, 6.9±1.3 μmoles/hr/mg protein, cyclic AMP-treated animals, 8.1±1.7 μmoles/hr/ mg protein, p> 0.05; tartrate-resistant: controls, 4.1±0.8 μmoles/hr/mg protein, cyclic AMP-treated animals, 4.7±1.1 μmoles/hr/mg protein, p>0.05) or parenteral glucose treatment (total: controls, 9.2±1.7 μmoles/hr/mg protein, glucose-treated animals, 8.3±1.4 μmoles/hr/mg protein, p>0.05; tartrate-resistant: controls, 3.2±0.7 μmoles/hr/mg protein, glucose-treated animals, 2.8±0.7 μmoles/hr/mg protein, p>0.05). Glucose-treated animals showed decreased activity of cyclic AMP-dependent protein kinase (controls, 45.1±12.3 μmoles/hr/mg protein, glucose-treated animals 19.7±6.6 μmoles/hr/mg protein, p<0.05).

Morphological results

Both qualitative and quantitative changes were studied. The term lysosomes refers to lysosomes and related particles including autophagic vacuoles (DeDuve and Wattiaux, 1966; Dunn, 1990a,b). The appearance of normal rat hepatocytes during the first 12 hours after birth has been described before (Kotoulas and Phillips, 1971; Kotoulas et al., 1971; Kalamidas et al., 1994; Kalamidas and Kotoulas, 1999). Control animals in no respect differed from normal animals at the same age.

At birth, vast areas of hyaloplasmic glycogen were present. Lysosomes were small and rare. At 4-6 hours the hyaloplasmic glycogen was markedly reduced. Numerous large lysosomes appeared and at least 85% of their total volume represented autophagic vacuoles. They were usually located at the margins of hyaloplasmic glycogen areas and most of them contained various amounts of glycogen. At 10-12 hours the hyaloplasmic glycogen was totally depleted. The lysosomes were greatly increased in number and size. They usually represented huge, rather elongated and often multilobular organelles containing amorphous residual material in the form of serially-disposed, roughly spherical aggregates. Such a lysosomal morphology at the age of 12 hours has been found before (Kotoulas et al., 1971; Kalamidas et al., 1994). Lysosomal glycogen was thoroughly degraded and only negligible quantities of undigested polysaccharide were seen inside the lysosomes. These organelles usually had the form of residual bodies (Fig. 1). Actinomycin prevented the development of lysosomes and the degradation of glycogen inside the autophagic vacuoles. Thus, in the actinomycin-treated animals the autophagic vacuoles were relatively small and filled with undigested glycogen, as described before (Kotoulas, 1988).

The administration of cyclic AMP resulted in increased number and size of lysosomes at the age of 6 hours (total lysosomal volume: controls, 1.33±0.18% of cytoplasm, cAMP-treated animals, 2.36±0.28% of cytoplasm, p<0.05). At least 85% of their total volume represented autophagic vacuoles. Most of them contained various quantities of glycogen. Some of the lysosomes already advanced to the stage of large residual bodies containing practically no recognizable material, something that normally occurs at the age of 12 hours (Kalamidas and Kotoulas, 1999). The administration of glucose produced opposite results at the age of 12 hours, i.e., decreased number and size of lysosomes (total lysosomal volume: controls, 5.05±0.80% of cytoplasm, glucose-treated animals, 0.30±0.17% of cytoplasm, p<0.05). At least 85% of their total volume represented autophagic vacuoles. Most of them were filled with undigested glycogen. Residual bodies were absent from the hepatocytes of glucose-treated animals (Fig. 2). Results similar to glucose were obtained with propranolol at the age of 6 hours. Autophagic vacuoles

Table 2. Liver acid glucosidase and acid mannose 6-phosphatase activities at the age of 6 hours after cyclic AMP treatment of newborn rats. Results are means±standard deviations. Each value includes 5 observations.

	Acid glucosidase (μmoles glucose/hr/mg protein)	Acid mannose 6-phosphatase (μmoles phosphorus/hr/mg protein)
Control	0.212±0.081	7.9±1.5
CAMP	0.390±0.100	6.1±1.5
P	< 0.05	< 0.05

Table 3. Liver acid glucosidase and acid mannose 6-phosphatase activities at the age of 12 hours after parenteral glucose treatment of newborn rats. Results are means±standard deviations. Each value includes 5 observations.

	Acid glucosidase (μmoles glucose/hr/mg protein)	Acid mannose 6-phosphatase (μmoles phosphorus/hr/mg protein)
Control	0.169±0.050	17.6±4.2
Glucose	0.120±0.035	22.1±5.0
P	< 0.05	< 0.05

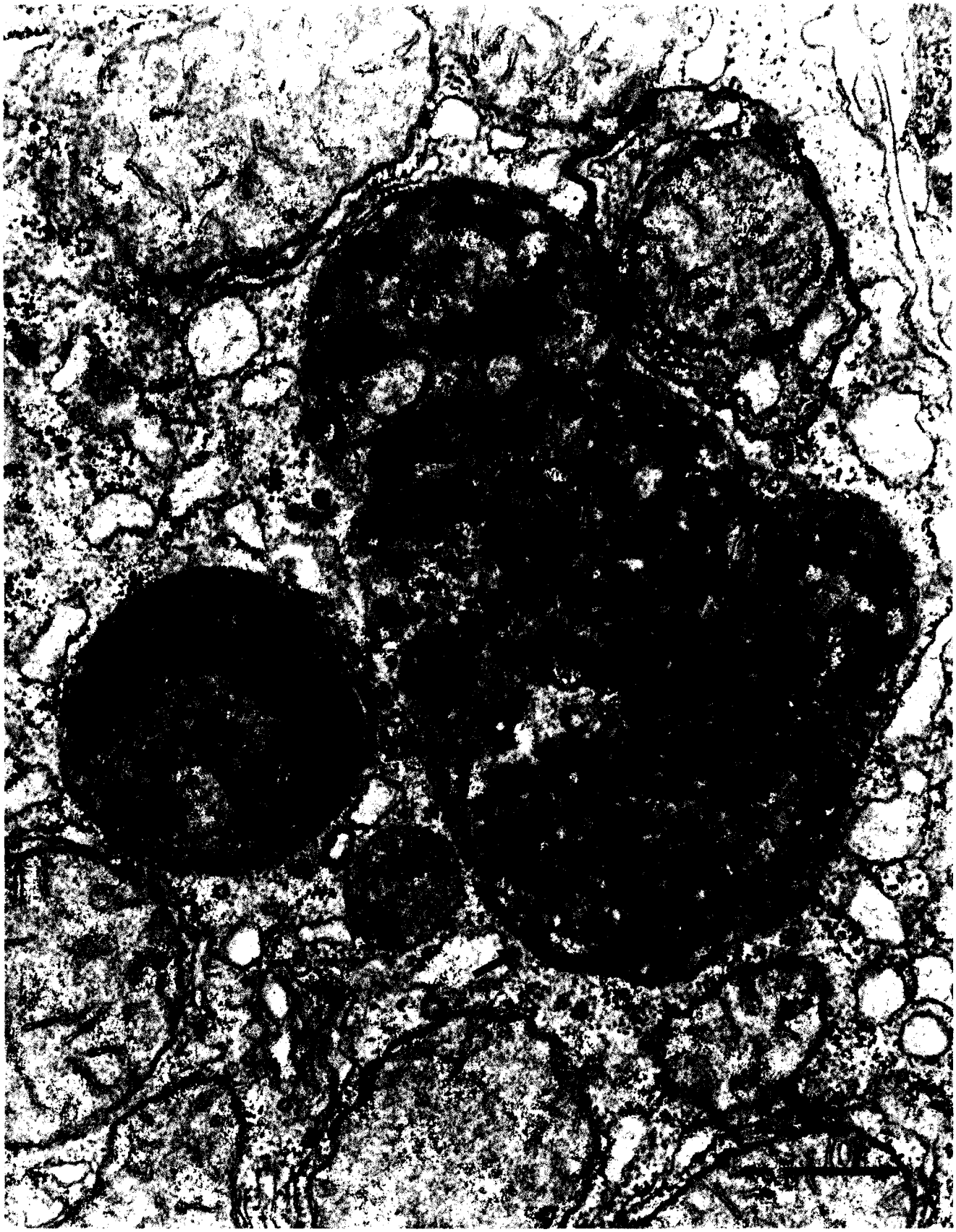

Fig. 1. Portion of a control rat hepatocyte at the age of 12 hours. A huge lysosome-residual body (arrow) containing amorphous material in the form of roughly spherical aggregates, is present. Bar: 0.5 μm.

were small and contained relatively large amounts of undigested glycogen as, described before (Kotoulas et al., 1991).

Discussion

Mammalian liver glycogen is accumulated at the end of gestation so vast reserves of the polysaccharide are present at birth. The stress of birth and the hypoglycemia resulting from the loss of maternal blood sugar supply, lead to the secretion of adrenalin and glucagon acting to raise the cyclic AMP tissue levels. The nucleotide activates liver phosphorylase and enhances phosphorolytic degradation of the hyaloplasmic glycogen of hepatocytes to produce glucose. This tides the newborn over a period during which this animal is entirely dependent on its own reserves (Dawkins, 1963; Dawes and Shelley, 1968; Pilkis et al., 1986). Other studies suggest that cyclic AMP also promotes certain mechanisms of autophagy including the induction of hydrolytic pathway of glycogen degradation inside the autophagic vacuoles of hepatocytes. In cases where there

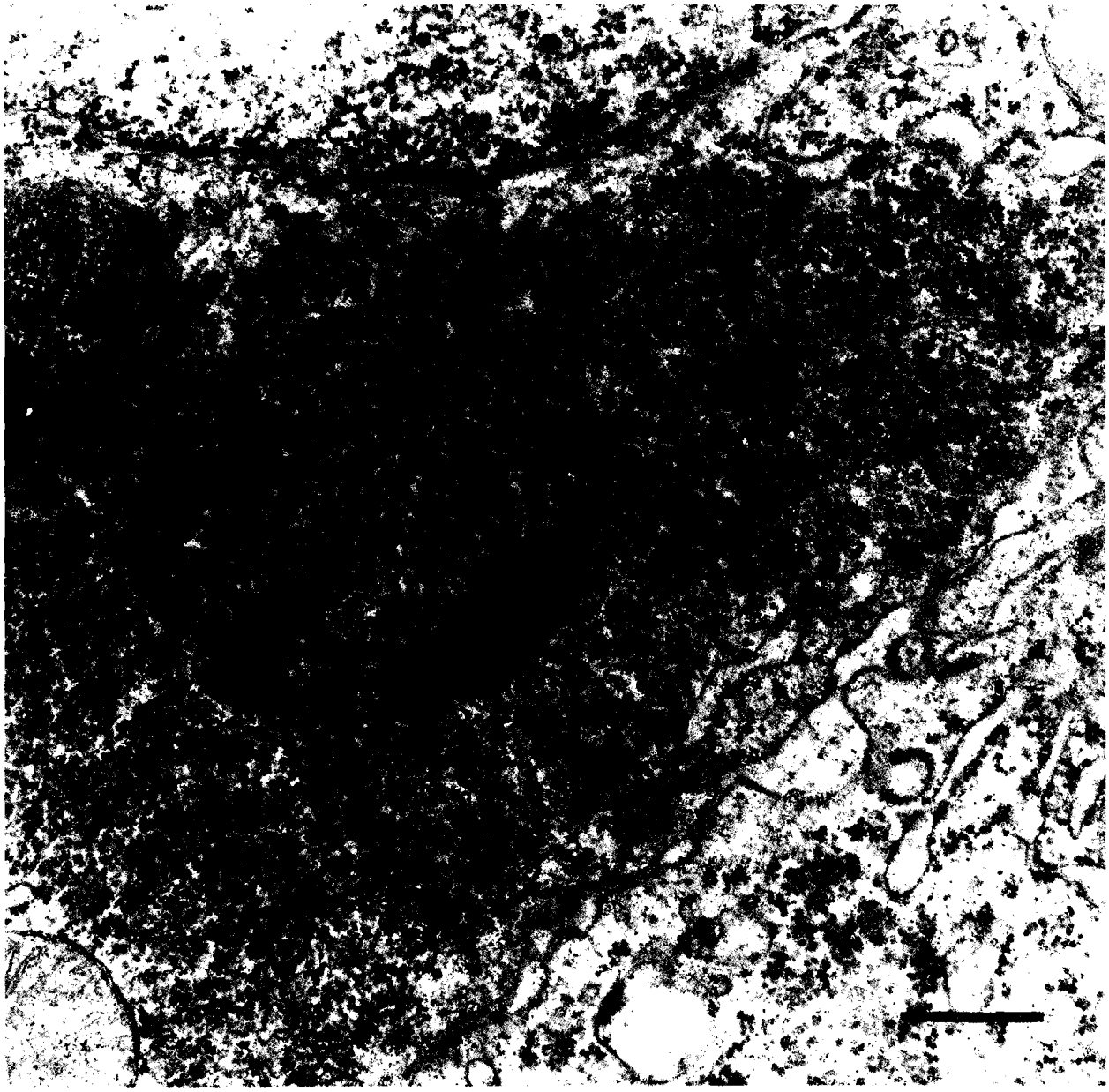

Fig. 2. Portions of two glucose-treated rat hepatocytes at the age of 12 hours. A lysosome (arrow) containing undigested glycogen, is present. Bar: 0.5 μm.

is a demand for the massive liberation of glucose, as in the immediate postnatal period, hydrolytic degradation assists phosphorolytic degradation of glycogen in producing this sugar (Rosenfeld, 1964; Kotoulas et al., 1971; Pfeifer, 1971; Kotoulas, 1986; Kalamidas et al., 1994).

In the newborn rat hepatocytes there is a burst of autophagic activity few hours after birth. Many large glycogen-containing autophagic vacuoles appear at the margins of the hyaloplasmic glycogen deposits (Phillips and Unakar, 1966; Phillips et al., 1967; Kotoulas and Phillips, 1971). The activity of liver lysosomal glycogen-hydrolyzing glucosidase increases at this period. Exogenous cyclic AMP enhances this phenomena, promoting autophagy and glycogen hydrolytic degradation in these cells (Rosa, 1971; Kotoulas, 1981; Kotoulas, 1986; Kalamidas et al., 1994). The nucleotide could exert its action through activation of cyclic AMP-dependent protein kinase. These lysosomal changes depend in part on protein synthesis (Kotoulas, 1988; Holen et al., 1992; Kalamidas and Kotoulas, 1999).

Parenteral glucose, which abolishes postnatal hypoglycemia, the natural stimulus for glucagon secretion, or propranolol, which antagonizes cyclic AMP, inhibits autophagy and glycogen hydrolytic degradation inside the autophagic vacuoles. The autophagic vacuoles are few and small. The glycogen included in these organelles remains largely undigested. The activity of liver lysosomal glycogen-hydrolyzing glucosidase is low. The cyclic AMP-dependent protein kinase activity is also low (Dawkins, 1963; Kotoulas et al., 1971, 1991; Kalamidas et al., 1994; Kalamidas and Kotoulas, 1999). Insulin, which opposes the action of glucagon, produces similar results (Kotoulas, 1981; Pilkis et al., 1986; Skoglund et al., 1987; Maintas et al., 1993).

There is a paucity of information regarding the sequence of events distal to cyclic AMP and cyclic AMP-dependent protein kinase, leading to the increase in glycogen-hydrolyzing acid glucosidase activity and hydrolytic degradation of glycogen inside the autophagic vacuoles. Calcium is widely recognized to influence stimulus-response coupling in cells. Cyclic AMP is known to modify the probability of opening calcium channels and the activity of the membrane-bound calcium pump (Lagast et al., 1984; Itoh et al., 1988; Rohrkasten et al., 1989; Baron et al., 1990; Kotoulas et al., 1991). Preliminary experiments showed that phorbol myristate acetate (PMA), which may promote the input of calcium to lysosomes, increases the activity of acid glucosidase (controls, 0.226±0.060 μmoles/ hr/ mg protein, PMA-treated animals, 0.298±0.073 μmoles/hr/ mg protein, $p<0.05$). Formyl- methionyl-leucyl-phenylalanine (FMLP)-inhibitable Ca^{2+}-ATPase activity including lysosomal calcium uptake pump activity, was found to be increased in the PMA-treated animals (Caroni and Carafoli, 1981; Lagast et al., 1984; Klempner, 1985).

Previous studies by other investigators suggested that phosphorylation-dephosphorylation mechanisms regulate autophagic activity (Seglen and Gordon, 1984; Seglen et al., 1996; Blommaart et al., 1997; Stromhaug, 1997; Kassner et al., 1999). Moreover, modifications of the dephosphorylation competence of lysosomes may control lysosomal function. Changes in acid mannose 6-phosphatase are important in this respect since dephosphorylation of acid hydrolases may be controlled by the compartmentalization of this phosphatase activity to distinct populations of lysosomes. Hence, lysosomes could exist in a dephosphorylation-incompetent and dephosphorylation-competent state, depending on the level of their mannose 6-phosphatase activity (Griffiths et al., 1988, 1990; Einstein and Gabel, 1991; Bresciani et al., 1992; Kundra and Kornfeld, 1998). Acid hydrolases may be delivered to the dephosphorylation-incompetent subpopulation of lysosomes by the cation-dependent mannose 6-phosphate receptor. Extracellular stimuli such as serum-free medium promote the dephosphorylation-incompetent state (Einstein and Gabel, 1991; Bresciani and VonFigura, 1996).

In newborn rat liver, acid mannose 6-phosphatase activity was low at birth but gradually increased during the first 12 hours of life. The protein synthesis inhibitor, actinomycin, prevented this change. This increase in enzyme activity may represent a developmental phenomenon. Several enzymes involved in various metabolic processes show a similar increase during this postnatal period (Walker, 1968). A transient fall in the level of mannose 6-phosphatase activity observed at the age of 6 hours roughly coincided with the burst of autophagic activity occuring at this age. The administration of cyclic AMP which induced autophagy in the newborn hepatocytes, resulted in decreased activity of this enzyme. The administration of agents that inhibited autophagy in these animals, such as parenteral glucose and propranolol, produced opposite results.

The data presented in this paper are not sufficient to claim the association of mannose 6-phosphatase activity with the autophagic activity. However, they raise the possibility that cyclic AMP-induced autophagic mechanisms in the newborn rat hepatocytes are associated with an acid mannose 6-phosphatase activity-deficient state of lysosomes. The autophagic vacuoles formed as a result of cyclic AMP administration could belong to a class of organelles which are inefficient in the dephosphorylation of mannose 6-phosphate and accumulate phosphorylated acid hydrolases (Einstein and Gabel, 1991; Bresciani and VonFigura, 1996). The phosphomannosyl recognition marker may promote the segregation of hydrolases into the autophagic vacuoles under our experimental conditions (Fischer et al., 1980). Since the dephosphorylation competence of lysosomes could change within few hours in response to various stimuli (Einstein and Gabel, 1989, 1991; Ohsawa et al., 1998), the possibility is raised that the cyclic AMP-induced decrease in acid mannose 6-phosphatase activity may be due, in part, to a transition of an active to an inactive form of this enzyme.

Other lysosomal enzymes such as the different forms of acid phosphatase may contribute to mannose 6-phosphate-dephosphorylating activity (Griffiths et al., 1990; Bresciani et al., 1992; Hayman and Cox, 1994; Bresciani and VonFigura, 1996). In this study, cyclic AMP- and glucose-treated animals showed no statistically significant changes in the total and tartrate-resistant p-nitro-phenyl phosphate-hydrolyzing acid phosphatase activities. Also, glucose-treated animals showed no significant change in the total ß-glycerophosphate-hydrolyzing acid phosphatase activity (Kotoulas et al., 1971).

The development of autophagic vacuoles into huge and elongated organelles during the last part of the 12-hour postnatal period in newborn rat hepatocytes, suggests the formation of an extended tubular compartment as described in other cells. This compartment represents a functional lysosome which may be the site where the biosynthetic, endocytic and autophagic pathways meet (Griffiths et al., 1990; Einstein and Gabel, 1991; Rabinowitz et al., 1992; Kalamidas et al., 1994; Meikle et al., 1999). The enhancement of development of this compartment by cyclic AMP and its inhibition by glucose suggest that the formation of this organelle may represent a step in the sequence of events related to cyclic AMP-induced autophagy in these cells.

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