

An electron microscopic and biochemical study of the effects of propranolol on the glycogen autophagy in newborn rat hepatocytes

O.B. Kotoulas¹, S.A. Kalamidas¹, P. Miles² and A.C. Hann²

¹Department of Anatomy, Histology and Embryology, Medical School, University of Ioannina, Ioannina, Greece and

²Electron Microscopy Unit, Cardiff School of Biosciences, Cardiff University, Cardiff, Wales, United Kingdom

Summary. The effects of propranolol on the glycogen autophagy in newborn rat hepatocytes were studied by using biochemical determinations, electron microscopy and morphometric analysis. Propranolol lowered the liver cyclic AMP and cyclic AMP-dependent protein kinase activity. It also decreased the formyl-methionyl-leucyl-phenylalanine (FMLP)-inhibitable Ca²⁺-ATPase activity including lysosomal calcium uptake pump. The normal postnatal increase in the volume of autophagic vacuoles and the activity of acid glycogen-hydrolyzing alpha glucosidase were inhibited. Also, the degradation of glycogen inside the autophagic vacuoles was apparently inhibited. The activity of acid mannose 6-phosphatase was increased. These findings indicate that propranolol influences several steps in the sequence of events leading to the breakdown of glycogen in the autophagic vacuoles of newborn rat hepatocytes. This supports our previous studies suggesting that cyclic AMP regulates glycogen autophagy.

Key words: Propranolol, Autophagy, Glycogen, Acid glucosidase, Acid mannose 6-phosphatase

Introduction

Glycogen degradation in the autophagic vacuoles appears to be a selective process under conditions of demand for the production of glucose, such as in newborn animals (Rosenfeld, 1964; Kotoulas and Phillips, 1971; Glaumann et al., 1985; Kotoulas, 1986; Kotoulas et al., 1991; Salehi et al., 1998; Kalamidas and Kotoulas, 2000b). Our previous studies suggested that cyclic AMP regulates the degradation of glycogen in autophagic vacuoles of newborn rat hepatocytes through changes in the activity of lysosomal glycogen-hydrolyzing acid alpha 1,4 glucosidase. The

administration of cyclic AMP, glucagon, adrenalin or caffeine which elevate the tissue levels of the nucleotide, induces certain aspects of autophagy including increase in the total volume of autophagic vacuoles, the activity of acid alpha 1,4 glucosidase and the degradation of glycogen inside autophagic vacuoles. This action may be mediated by the cyclic AMP-dependent protein kinase. It represents a beta-adrenergic function (Kotoulas and Phillips, 1971; Shelburne et al., 1973; Kotoulas, 1986, 1988; Kotoulas et al., 1991; Kalamidas et al., 1994; Holen et al., 1992, 1996; Wang et al., 2001).

Calcium is known to mediate stimulus-response coupling in cells. Cyclic AMP stimulates the activity of membrane-bound calcium uptake pump which compartmentalizes the cation within lysosomes. Accumulation of calcium in the lysosomes promotes autophagy and may induce glycogen degradation inside the autophagic vacuoles (Jeffrey et al., 1970; Caroni and Carafoli, 1981; Lagast et al., 1984; Klempner, 1985; Gordon et al., 1993; Kim and Klionsky, 2000; Kalamidas et al., 2002). Changes in activity of the important lysosomal enzyme acid mannose 6-phosphatase may also be associated with these phenomena (Kalamidas and Kotoulas, 2000a,b; Kalamidas et al., 2002).

In this study, the effects of propranolol, a beta-adrenergic blocking agent which antagonizes cyclic AMP, were studied. Our results indicate that this agent could influence several steps in the sequence of events leading to glycogen autophagy and support our previous studies suggesting that cyclic AMP regulates this process in newborn rat hepatocytes.

Materials and methods

Chemicals

Propranolol hydrochloride (Inderal), Lot PL29/5062 was from Imperial Chemical Industries. The reagents for biochemistry and electron microscopy were obtained as before (Kalamidas and Kotoulas, 2000b). For the

immunogold labelling procedures, rabbit anti-cAMP-dependent protein kinase regulatory subunit IIa (PKA RIIa) affinity-purified antibody recognizing the subunit IIa of this enzyme, was used. Due to the sequence of the peptide selected, no cross-reactivity is seen with other PKA receptor isoforms. Species reactivity is: human, rhesus monkey, rat, mouse. This antibody, Cat. No. AB 1613, Lot No. 18110098, was obtained from Chemicon International Inc. (Temecula, USA). Goat anti-rabbit IgG-15 nm gold conjugate, Cat. No. RPN 422 BS; Lot No. 187688 was from Nycomed-Amersham (Cardiff, UK). All other reagents used in the immunolabelling process were supplied by Sigma (Poole, UK).

Experimental design

All procedures involving the handling, treatment and sacrifice of animals followed the EEC Council Directive 86/609 on the approximation of laws, regulations and administrative provisions of the Member States regarding the protection of animals used for experimental and other scientific purposes (1986) and the Guide for the care and use of laboratory animals (Institute of Laboratory Animal Resources, 1996).

Newborn Wistar rats were obtained from six pregnant females. Five newborn animals were killed at birth. Treated animals were injected intraperitoneally with propranolol at birth and 2 hours after birth (a dose of 16.7 mg/kg each time). Normal animals were used as controls. Injected animals and controls were killed at the age of 6 hours. Parenteral glucose was administered as before. Injected animals and controls were killed at the age of 12 hours (Kalamidas and Kotoulas, 1999, 2000b).

Biochemical methods

The enzyme activities of glycogen-hydrolyzing acid alpha 1,4 glucosidase, acid mannose 6-phosphatase, cyclic AMP-dependent protein kinase and formyl-methionyl-leucyl-phenylalanine (FMLP)-inhibitable Ca^{2+} -ATPase were assayed in liver homogenates as before (Kalamidas and Kotoulas, 2000b). For the determination of acid mannose 6-phosphatase, tissue homogenates were pretreated for 1 hour in 0.1M sodium acetate buffer at pH 5.2 and 37 °C before incubation with the substrate. Mannose 6-phosphate (20 mM) was used as substrate. For the determination of glucose 6-phosphate-hydrolyzing activity at pH 5.2 (acid glucose 6-phosphatase activity), tissue homogenates were pretreated in the same way before incubation with the substrate (Arion and Nordlie, 1964; Ockerman, 1965; Hugon et al., 1970). Glucose 6-phosphate (20 mM) was used as substrate. Incubation was carried out for 60 min in 0.1 M sodium acetate buffer with 0.05% Triton X-100 at pH 5.2 and 37 °C. The reaction was stopped by 10% ice-cold trichloroacetic acid. Released phosphate was determined according to Fiske and Subbarow (1925). Activity was expressed as micromoles of inorganic phosphorus produced per hour per mg of protein (Zakim

and Vessey, 1973; VandeWerve, 1989; Ajzannay and Mithieux, 1996; Kalamidas and Kotoulas, 2000b). Liver cyclic AMP and glycogen were determined as before (Kalamidas et al., 1994; Kalamidas and Kotoulas, 2000a). The results were statistically evaluated according to Hill (1967).

Electron microscopy

Electron microscopy and morphometric analysis were performed as described before (Kalamidas and Kotoulas, 2000a). Immunogold particles on lysosomal membranes were counted on micrographs enlarged to a final magnification of 40000. Particles were counted per μm of lysosomal membrane profile. Since the sections of tissue were not very thin as compared with the dimensions of particles, no attempt was made to estimate other parameters (Weibel and Gomez, 1962; Weibel, 1969; Kotoulas et al., 1991).

Immunogold labelling procedures

Ultrathin sections 90 nm thick, cut from Epon- or Epon-Araldite-embedded liver tissue, were mounted on pioloform-coated nickel grids and etched for 5 minutes in 2% aqueous periodic acid. To remove excess osmium, the grids were incubated for 10 minutes in saturated aqueous sodium metaperiodate. The sections were blocked for 60 minutes with 5% normal goat serum in 20mM Tris-buffered saline (TBS), pH 7.2, containing 0.2% bovine serum albumin (BSA). The grids containing sections were then incubated overnight at 4 °C in 1:500 dilution of rabbit anti-cAMP-dependent protein kinase regulatory subunit RIIa and washed with six changes of 20 mM TBS+0.5% BSA, pH 8.0, for 10 minutes each. The sections were incubated for 1 hour at room temperature in goat anti-rabbit IgG-15 nm gold conjugate diluted 1:25 in the same washing buffer (TBS+0.5% BSA). After a good rinse with TBS followed by double-distilled water, the grids were counterstained in uranyl acetate and lead citrate. Immunolabelling controls consisted of: 1) sections that were incubated in TBS without rabbit anti-cAMP-dependent protein kinase regulatory subunit and then in goat anti-rabbit IgG-15nm gold conjugate; 2) sections that were incubated in pre-absorbed rabbit anti-cAMP-dependent protein kinase regulatory subunit followed by goat anti-rabbit IgG-conjugated gold; and 3) sections that were incubated in rabbit anti-cAMP-dependent protein kinase regulatory subunit (which was pre-absorbed overnight in goat anti-rabbit IgG) and then in goat anti-rabbit-conjugated gold.

Antibody specificity

Rat liver extracts were separated using SDS-PAGE on 8% gels. The gels were treated according to the methods described by Mednieks et al. (1989) before being transferred to a strip of nitrocellulose paper. The

Propranolol and glycogen autophagy

paper was then blocked overnight in 0.5% BSA in 50 mM TBS, pH 7.6 and incubated for 6 hours in a 1:50 dilution of rabbit anti-cAMP-dependent protein kinase RIIa subunit. Following a thorough washing in TBS, the paper was stained in Coomassie Blue dye. The immunoreactions were compared with standard proteins of known molecular weights (bovine serum albumin, 67KD; ovalbumin, 43KD). The immunoreaction band was positioned between 43KD and 67KD. Its relative mobility was found to be 52KD, based on the molecular weights of the standard proteins.

Results

Biochemical results

The administration of propranolol in newborn rats resulted in decreased liver cyclic AMP concentration, cyclic AMP-dependent protein kinase and FMLP-

inhibitable Ca^{2+} -ATPase activities (Table 1). In the propranolol-treated newborns, the activity of liver glycogen-hydrolyzing acid glucosidase decreased whereas that of acid mannose 6-phosphatase increased. Liver glycogen in propranolol-treated animals showed no significant change as compared to the controls (Table 2).

Glucose 6-phosphate-hydrolyzing activity at pH 5.2 (acid glucose 6-phosphatase activity) was found increased in both the propranolol-treated animals (controls, $7.5 \pm 1.4 \mu\text{moles P}_i/\text{hr/mg protein}$; propranolol-treated, $8.9 \pm 1.6 \mu\text{moles P}_i/\text{hr/mg protein}$, $n=3$, $p<0.05$) and the parenteral glucose-treated animals (controls, $13.3 \pm 3.2 \mu\text{moles P}_i/\text{hr/mg protein}$; glucose-treated, $16.8 \pm 4.6 \mu\text{moles P}_i/\text{hr/mg protein}$, $n=3$, $p<0.05$). This enzyme activity was also determined in the presence or absence of the chelator EGTA (20 mM) in the reaction mixture. The activity was significantly higher ($p<0.05$) in the presence ($9.4 \pm 1.4 \mu\text{moles P}_i/\text{hr/mg protein}$) than

Table 1. Liver cyclic AMP concentration, cyclic AMP-dependent protein kinase and FMLP-inhibitable Ca^{2+} -ATPase activities after propranolol treatment of newborn rats, 6 hours after birth.

TREATMENT	cAMP (pmoles/g wet wt)	cAMP-DEPENDENT PROTEIN KINASE ($\mu\text{moles P}_i/\text{hr/mg protein}$)	FMLP-INHIBITABLE Ca^{2+} -ATPase ($\mu\text{moles P}_i/\text{hr/mg protein}$)
Control	80.8 ± 19.6	56.3 ± 18.4	0.83 ± 0.28
Propranolol	49.3 ± 14.5	23.1 ± 7.9	0.45 ± 0.18
p	<0.05	<0.05	<0.05

Results are means \pm standard deviations. Each value includes 3 observations.

Table 2. Liver glycogen concentration, acid glucosidase and acid mannose 6-phosphatase activities after propranolol treatment of newborn rats, 6 hours after birth.

TREATMENT	GLYCOGEN (mg/mg protein)	ACID GLUCOSIDASE ($\mu\text{moles glucose/hr/mg protein}$)	ACID MANNOSE 6-PHOSPHATASE ($\mu\text{moles P}_i/\text{hr/mg protein}$)
Control	0.173 ± 0.082 (at birth, 0.800 ± 0.260)	0.185 ± 0.064 (at birth, 0.105 ± 0.050)	8.9 ± 1.5
Propranolol	0.210 ± 0.095	0.132 ± 0.050	10.9 ± 1.6
P	>0.05	<0.05	<0.05

Results are means \pm standard deviations. Each value includes 5 observations.

Table 3. Comparison of the fractional volumes of autophagic vacuoles and lysosomal glycogen in the hepatocytes from control and propranolol-treated newborn rats, 6 hours after birth.

	VOLUME OF AUTOPHAGIC VACUOLES	VOLUME OF LYSOSOMAL GLYCOGEN	% VOLUME OF AUTOPHAGIC VACUOLES OCCUPIED BY LYSOSOMAL GLYCOGEN
Control	0.90 ± 0.16 (at birth, 0.38 ± 0.08)	0.21 ± 0.08 (at birth, 0.14 ± 0.04)	23 (at birth, 37)
Propranolol	0.56 ± 0.09	0.19 ± 0.06	34
P	< 0.05	> 0.05	

Volumes are expressed as $\mu\text{m}^3/100 \mu\text{m}^3$ of cytoplasm. Values are mean \pm standard errors. Results computed from a total of 30 micrographs and a cytoplasmic area of $1780 \mu\text{m}^2$ at birth, $1850 \mu\text{m}^2$ for control and $1800 \mu\text{m}^2$ for propranolol.

in the absence ($8.2 \pm 1.2 \mu\text{moles P}_i/\text{hr/mg protein}$) of EGTA (Kalamidas et al., 2002).

Electron microscopic results

The term lysosomes refers to lysosomes and related particles including autophagic vacuoles unless otherwise specified (DeDuve and Wattiaux, 1966; Dunn, 1990a,b;

Kornfeld, 1992). At birth, abundant hyaloplasmic glycogen stores were present in the hepatocytes. The fractional volume of hyaloplasmic glycogen was very large (Kotoulas and Phillips, 1971; Maintas et al., 1993). Autophagic vacuoles were rare, small and round. Their fractional volume was small. Lysosomal glycogen occupied a large part of this volume (Table 3). At 6 hours, part of the hyaloplasmic glycogen was mobilized.

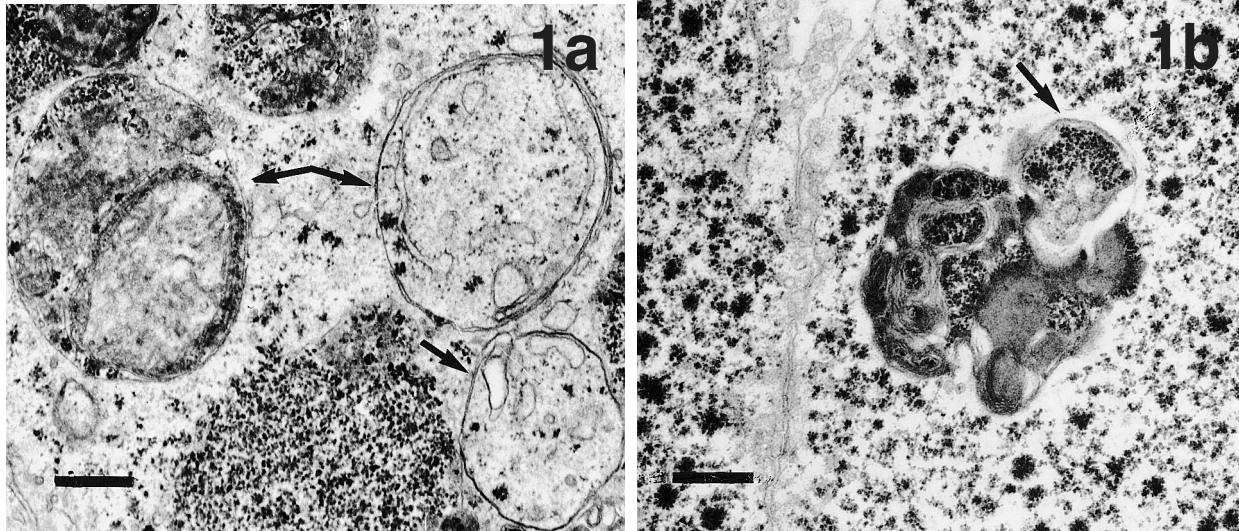


Fig. 1. **a.** Portion of a control rat hepatocyte at the age of 6 hours. Several autophagic vacuoles (arrows) usually containing negligible quantities of undigested glycogen, are seen. Bar: $0.5 \mu\text{m}$. **b.** Portions of two propranolol-treated rat hepatocytes at the age of 6 hours. An autophagic vacuole (arrow) containing a moderate quantity of undigested glycogen, is seen. Bar: $0.5 \mu\text{m}$.

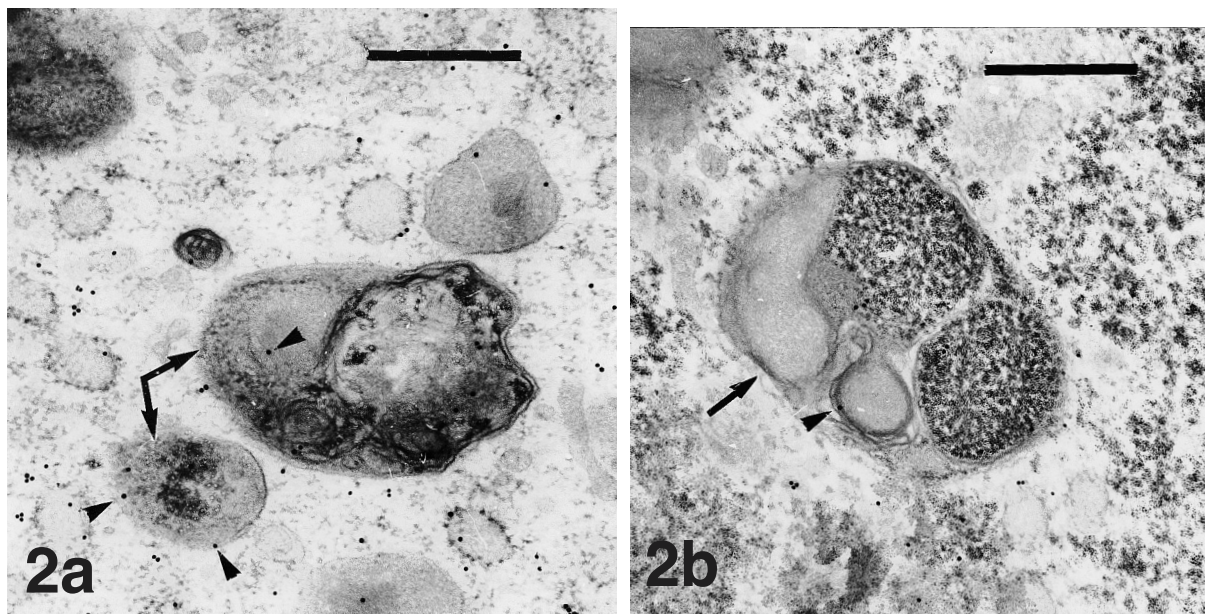


Fig. 2. **a.** Immunogold labelling for PKA RIIa subunits of a control rat hepatocyte at the age of 6 hours. Two lysosomes (arrows) with immunogold particles (arrowheads) are seen. One of the particles is associated with the delimiting membrane of lysosome: another is in close vicinity to this membrane. Bar: $0.5 \mu\text{m}$. **b.** Immunogold labelling for PKA RIIa subunits of a propranolol-treated rat hepatocyte at the age of 6 hours. A lysosome (arrow) with one immunogold particle (arrowhead) is seen. No particle is seen on the delimiting membrane of lysosome. Bar: $0.5 \mu\text{m}$.

Propranolol and glycogen autophagy

The fractional volume of hyaloplasmic glycogen was reduced (Kotoulas and Phillips, 1971; Maintas et al., 1993). Numerous large, oval or irregular, autophagic vacuoles often exceeding 1 μm in diameter, appeared. Their fractional volume was increased. They were usually located at the margins of hyaloplasmic glycogen areas and contained small or moderate quantities of undigested glycogen. Lysosomal glycogen occupied a small part of their volume (Kotoulas and Phillips, 1971; Table 3 and Fig. 1a).

The administration of propranolol produced no significant change in the hyaloplasmic glycogen at the age of 6 hours. The normal postnatal lysosomal changes were largely inhibited. Autophagic vacuoles were fewer and smaller than in the controls and often appeared less than 1 μm in diameter. Their fractional volume was decreased. They were round, oval or irregular. A larger fraction of the volume of autophagic vacuoles was occupied by undigested glycogen, as compared to the controls (Table 3, Fig. 1b). Preliminary experiments with immunogold labelling for the RIIa subunits of cyclic AMP-dependent protein kinase (PKA), showed gold particles over various cell constituents such as lysosomes, peroxisomes, endoplasmic reticulum and hyaloplasm. Gold particles in contiguity with the delimiting membrane of lysosomes, were counted. The staining intensity of RIIa subunits was reduced in the delimiting membrane of lysosomes of the propranolol-treated animals (controls, 1.20 ± 0.23 particles per μm of membrane profile, propranolol-treated, 0.83 ± 0.18 particles per μm of membrane profile, $n=15$, $p<0.05$). Immunogold particles in the vicinity to the delimiting membrane, were not counted. However, such particles may also belong to oblique sections of this membrane (Fig. 2a,b).

Discussion

Mammalian liver glycogen is accumulated at the end of gestation. Vast reserves of the polysaccharide are present in the hyaloplasm of hepatocytes at birth. They are progressively mobilized during the first 12 hours after birth. This is due to glucagon secretion and tissue cyclic AMP elevation (Kotoulas and Phillips, 1971; Maintas et al., 1993). Cyclic AMP also promotes the sequestrational step of hepatocytic autophagy and increases the lysosomal glycogen-hydrolyzing (but not the maltose-hydrolyzing) activity of acid glucosidase (Kotoulas, 1986; Kotoulas et al., 1991; Kalamidas et al., 1994; Holen et al., 1996). Autophagic vacuoles are few and small at birth and a large part of their volume (37%) is occupied by glycogen. They increase in number and size during this period of postnatal glycogen mobilization. Lysosomal glycogen-hydrolyzing activity of acid glucosidase is low at birth but increases thereafter (Kotoulas and Phillips, 1971; Kotoulas et al., 1971; Kotoulas, 1981; Kalamidas et al., 1994).

Autophagic vacuoles can selectively degrade glycogen in cases where there is a demand for the

massive liberation of glucose, as in this postnatal period (Rosenfeld, 1964; Kotoulas and Phillips, 1971; Pfeifer, 1971; Kalamidas and Kotoulas 2000b). Formation of autophagic vacuoles is spatially and biochemically related to the breakdown of glycogen in newborn hepatocytes. These organelles are not distributed randomly throughout the cell but are predominantly found at the junction of glycogen areas with glycogen-free areas (Kotoulas and Phillips, 1971; Kalamidas and Kotoulas, 2000b).

At the age of 6 hours, the autophagic vacuoles became large and numerous. Despite the fact that their volume was markedly increased as compared to the birth, the volume of lysosomal glycogen in the cell was not proportionally increased. This was apparently due to the concomitant lysosomal glycogen degradation. Only a small part (23%) of the volume of autophagic vacuoles was occupied by undegraded glycogen. Lysosomal glycogen-hydrolyzing activity of acid glucosidase was increased as compared to the birth.

Glycogen breakdown in lysosomes could be inhibited by the inhibition of formation of autophagic vacuoles or/and by the inhibition of degradation of polysaccharide inside these organelles. Autophagic vacuoles in the propranolol-treated animals, at the age of 6 hours, did not reach their normal number and size. Despite the fact that the volume of these organelles was reduced, the volume of lysosomal glycogen in the cell was not reduced. This was apparently due to the inhibition of lysosomal glycogen degradation. A large part (34%) of the volume of autophagic vacuoles was occupied by undegraded glycogen. Moreover, lysosomal glycogen-hydrolyzing activity of acid glucosidase was decreased. Autophagic vacuoles in the treated animals were to some extent, morphologically and biochemically similar to those at birth (Kotoulas and Phillips, 1971; Kotoulas, 1981; Kalamidas et al., 1994; Kalamidas and Kotoulas, 2000b). These results with propranolol, a beta adrenergic antagonist which lowers tissue cyclic AMP, support our previous studies suggesting that the nucleotide regulates glycogen autophagy (Kotoulas, 1986; Kalamidas et al., 1994).

The decreased levels of liver cyclic AMP and cyclic AMP-dependent protein kinase activity, produced by the administration of propranolol, were apparently due to the well known effects of this agent on the adenylate cyclase and cyclic AMP accumulation in the liver (Hoffman and Lefkowitz, 1996). Cyclic AMP-dependent protein kinase is the major intracellular receptor for cyclic AMP. Following propranolol administration, cyclic AMP stimulus is removed and the dissociation of catalytic and regulatory subunits of this kinase and their intracellular translocation, are inhibited.

The association of regulatory subunit RII with the membranes of lysosomes and related structures, has been previously reported (DeCamilli et al., 1986; Jungmann et al., 1986; Griffiths et al., 1990). The functional significance of RIIa subunit reduction on the delimiting membrane of lysosomes, is obscure. The distribution of

RII subunit may reflect, to a large extent, the distribution of the type II holoenzyme. However, translocation mechanisms for the RII and other subunits in a nonstoichiometric relationship incompatible with the transfer of holoenzyme, have also been proposed. Dissociation of the RIIa subunit from membrane-anchoring proteins may occur. Cyclic AMP dependent protein kinase II (PKA II) is targeted to certain subcellular compartments through binding to A kinase anchoring proteins (AKAPs). Disruption of the RII binding to the AKAP, may impair PKA II-dependent effects on the lysosomal membrane. Changes in the cyclic AMP-induced regulation of RIIa gene and inhibition of the synthesis of RIIa protein due to the decreased cyclic AMP tissue levels or changes resulting from the altered degradation of PKA subunits due to the persistently modified cyclic AMP levels, may also take place. The propranolol-induced change in RIIa subunits may reflect a modification in the signal transducing mechanism influencing the lysosomal membrane functions including calcium pump (Jungmann et al., 1986; Griffiths et al., 1990; Spaulding, 1993; Skalhegg and Tasken, 1997; Dell'Acqua and Scott, 1997; Kalamidas et al., 2002).

Cyclic AMP-dependent protein kinase stimulates the activity of lysosomal calcium uptake pump and promotes the accumulation of calcium into the lysosomes (Lagast et al., 1984; Klempner, 1985). Hence, the decreased activity of FMLP-inhibitable Ca^{2+} -ATPase including lysosomal calcium pump, observed in our propranolol-treated animals, could be explained on the basis of the cyclic AMP-antagonizing action of propranolol. This agent may also exert a direct inhibitory effect on this calcium pump (Griffiths et al., 1990; Mackrill, 1999; Jan et al., 2000; Dash et al., 2001; Almotrefi et al., 2001).

Accumulation of calcium in the lysosomes influences lysosomal functions including glycogen autophagy (Jeffrey et al., 1970; Kim and Klionsky, 2000; Kalamidas et al., 2002; Inbal et al., 2002). Since cyclic AMP promotes the normal postnatal development of autophagic vacuoles, activity of glycogen-hydrolyzing acid alpha 1,4 glucosidase and degradation of lysosomal glycogen (Kotoulas, 1986; Kalamidas et al., 1994; Wang et al., 2001), the antagonizing action of propranolol on cyclic AMP, cyclic AMP-dependent protein kinase and lysosomal calcium pump, could explain the inhibitory effect of the drug on these lysosomal phenomena. Parenteral glucose which prevents the postnatal glucagon secretion and cyclic AMP tissue elevation or insulin which opposes the action of glucagon, produces similar results (Kotoulas, 1981; Maintas et al., 1993; Kalamidas and Kotoulas, 2000b; Cheng et al., 2002).

Propranolol-treated animals showed increased activity of the important lysosomal enzyme acid mannose 6-phosphatase. This could result from decreased intralysosomal accumulation of calcium and a modified extent of the formation of an endogenous chelate. Parenteral glucose-treated animals also exhibited increased acid mannose 6-phosphatase activity

(Arion and Nordlie, 1964; VandeWerve, 1989; Mithieux et al., 1990; Kalamidas and Kotoulas, 2000b; Kalamidas et al., 2002).

Lysosomes exist in a dephosphorylation-competent and dephosphorylation-incompetent state, depending on the level of their acid mannose 6-phosphatase activity. Cyclic AMP-induced autophagic vacuoles may belong to a class of lysosomal organelles which contain decreased levels of this activity. There are different functional systems for targeting of acid hydrolases to cellular organelles (Kornfeld, 1986, 1992; Griffiths et al., 1988; Gonzalez-Noriega et al., 1989, 1993; Gonzalez-Noriega and Michalak, 2001). Following the administration of propranolol, acid hydrolases may predominantly be delivered to classes of organelles containing increased levels of acid mannose 6-phosphatase activity (Hugon et al., 1970; Fischer et al., 1980; Sly, 1985; Einstein and Gabel, 1991; Ma et al 1992; Bresciani and VonFigura, 1996; Kalamidas and Kotoulas, 2000a,b).

Mannose 6-phosphate-hydrolyzing activity is very similar in many respects to glucose 6-phosphate-hydrolyzing activity. The latter activity is also present in the lysosomes and autophagic vacuoles. These activities may be due to one, common enzyme catalyzing reversible transphosphorylation between mannose (6-phosphate) and glucose (6-phosphate) (Arion and Nordlie, 1964; Ockerman, 1965; Ericsson, 1969; Hugon et al., 1970; Sakai et al., 1989). They are modulated by calcium and the amplitude of calcium effect on each activity may depend on the concentrations of this cation, mannose 6-phosphate or glucose 6-phosphate (VandeWerve, 1989; Ajzannay et al., 1993; Ajzannay and Mithieux, 1996; Kalamidas et al., 2002). Changes in these activities could modify the intralysosomal composition in nonphosphorylated and phosphorylated sugars, influence the transport of sugars through the lysosomal membrane and participate in a transducing mechanism in the regulation of lysosomal function including lysosomal glycogen degradation (Wells et al., 1981; Warren, 1989; Jonas et al., 1990; St-Denis et al., 1995; Salehi et al., 1998; Michalak et al., 1999).

Acknowledgements. We thank Mr. Guy Pitt, Mr. Christos Economou, Miss Katerina Vourdas and Mr. Apostolos Pihias for expert technical assistance.

References

- Ajzannay A. and Mithieux G. (1996). Glucose 6-phosphate and mannose 6-phosphate are equally and more actively hydrolyzed by glucose 6-phosphatase during hysteretic transition within intact microsomal membrane than after detergent treatment. *Arch. Biochem. Biophys.* 326, 238-242.
- Ajzannay A., Minassian C., Riou J. P. and Mithieux G. (1993). Glucose-6-phosphate phosphohydrolase of detergent-treated liver microsomal membranes exhibits a specific kinetic behaviour towards glucose 6-phosphate. *Eur. J. Biochem.* 212, 335-338.
- Almotrefi A.A., Basco C., Moorji A. and Dzimirri N. (2001). Evidence for the binding of beta-adrenoreceptor blockers to microsomal Na^+/K^+

Propranolol and glycogen autophagy

- ATPase in guinea pig heart preparations. *Can. J. Physiol. Pharmacol.* 79, 8-12.
- Arion W.J. and Nordlie R.C. (1964). Liver microsomal glucose 6-phosphatase, inorganic pyrophosphatase and pyrophosphate-glucose phosphotransferase. *J. Biol. Chem.* 239, 2752-2757.
- Bresciani R. and VonFigura K. (1996). Dephosphorylation of the mannose 6-phosphate recognition marker is localized in late compartments of the endocytic routes. Identification of purple acid phosphatase (uteroferrin) as the candidate phosphatase. *Eur. J. Biochem.* 238, 669-674.
- Caroni P. and Carafoli E. (1981). Regulation of Ca²⁺-pumping ATPase of heart sarcolemma by a phosphorylation-dephosphorylation process. *J. Biol. Chem.* 256, 9371-9373.
- Cheng A., Dube N., Gu F. and Tremblay M.I. (2002). Coordinated action of protein tyrosine phosphatases in insulin signal transduction. *Eur. J. Biochem.* 269, 1050-1059.
- Dash R., Kadambi V.J., Schmidt A.G., Tepe N.M., Biniakiewicz D., Gerst M.J., Canning A.M., Abraham W.T., Hoit B.D., Liggett S.B., Lorenz J.N. and Kranias E.G. (2001). Interactions between phospholamban and beta-adrenergic drive may lead to cardiomyopathy and early mortality. *Circulation* 103, 889-896.
- DeCamilli P., Moretti M., Donini S.D., Walter U. and Lohmann S. M. (1986). Heterogeneous distribution of cAMP receptor protein RII in the nervous system: Evidence for its intracellular accumulation on microtubules, microtubule-organizing centers, and in the area of the Golgi complex. *J. Cell Biol.* 103, 189-203.
- DeDuve C. and Wattiaux R. (1966). Functions of lysosomes. *Annu. Rev. Physiol.* 28, 435-492.
- Dell'Acqua M.L. and Scott J.D. (1997). Protein kinase A anchoring. *J. Biol. Chem.* 272, 12881-12884.
- Dunn W.A. Jr. (1990a). Studies on the mechanisms of autophagy: Formation of the autophagic vacuole. *J. Cell Biol.* 110, 1923-1933.
- Dunn W.A. Jr. (1990b). Studies on the mechanisms of autophagy: Maturation of the autophagic vacuole. *J. Cell Biol.* 110, 1935-1945.
- EEC. (1986). Council Directive 86/609 on the approximation of laws. Regulations and administrative provisions of the Member States regarding the protection of animals used for experimental and other scientific purposes. *Official Journal of the European Communities*, L 358, 1-29.
- Einstein R. and Gabel C.A. (1991). Cell- and ligand-specific dephosphorylation of acid hydrolases: Evidence that the mannose 6-phosphatase is controlled by compartmentalization. *J. Cell Biol.* 112, 81-94.
- Ericsson J.L.E. (1969). Studies on induced cellular autophagy. II. Characterization of the membranes bordering autophagosomes in parenchymal liver cells. *Exp. Cell Res.* 56, 393-405.
- Fischer H.D., Gonzalez-Noriega A., Sly W.S. and Morrè D.J. (1980). Phosphomannosyl-enzyme receptors in rat liver. *J. Biol. Chem.* 255, 9608-9615.
- Fiske C.H. and Subbarow Y. (1925). The colorimetric determination of phosphorus. *J. Biol. Chem.* 66, 375-400.
- Glaumann H., Ahlberg J., Berkenstam A. and Henell F. (1985). Protein degradation in the lysosomes. Comparison between morphometric and biochemical assessments. *J. Ultrastruct. Res.* 91, 253.
- Gonzalez-Noriega A. and Michalak C. (2001). Mannose 6-phosphate-independent endocytosis of beta glucuronidase. II. Purification of a cation-dependent receptor from bovine liver. *Biochim. Biophys. Acta* 1538, 152-161.
- Gonzalez-Noriega A., Coutino R., Saavedra V.M. and Barrera R. (1989). Adsorptive endocytosis of lysosomal enzymes by human fibroblasts: presence of two different functional systems that deliver an acid hydrolase to lysosomes. *Arch. Biochem. Biophys.* 268, 649-658.
- Gonzalez-Noriega A., Ferrera P., Coutino R. and Michalak C. (1993). Isolation of an inhibitor for the independent mannose 6-phosphate receptor endocytosis of bovine beta-glucuronidase by human fibroblasts. *Arch. Biochem. Biophys.* 306, 331-336.
- Gordon P.B., Holen I., Fosse M., Rotnes J.S. and Seglen P.O. (1993). Dependence of hepatocytic autophagy on intracellularly sequestered calcium. *J. Biol. Chem.* 268, 26107-26112.
- Griffiths G., Hoflack B., Simons K., Mellman I. and Kornfeld S. (1988). The mannose 6-phosphate receptor and the biogenesis of lysosomes. *Cell* 52, 329-341.
- Griffiths G., Hollinshead R., Hemmings B.A. and Nigg E.A. (1990). Ultrastructural localization of the regulatory (RII) subunit of cyclic AMP-dependent protein kinase to subcellular compartments active in endocytosis and recycling of membranes receptors. *J. Cell Sci.* 96, 691-703.
- Hill A.B. (1967). *Principles of medical statistics*. Oxford University Press. New York.
- Hoffman B.B. and Lefkowitz R.J. (1996). Catecholamines, sympathomimetic drugs, and adrenergic receptor antagonists. In: Goodman and Gilman's *The pharmacological basis of therapeutics*. Hardman J.G. and Limbird L.E. (eds). McGraw-Hill Co. Inc. N.York. pp 199-248.
- Holen I., Gordon P.B., Stromhaug P.E. and Seglen P.O. (1996). Role of cAMP in the regulation of hepatocytic autophagy. *Eur. J. Biochem.* 236, 163-170.
- Holen I., Gordon P.B. and Seglen P.O. (1992). Protein kinase-dependent effects of okadaic acid on hepatocytic autophagy and cytoskeletal integrity. *Biochem. J.* 284, 633-636.
- Hugon J.S., Borgers M. and Maestracci D. (1970). Glucose 6-phosphatase and thiamine pyrophosphatase activities in the jejunal epithelium of the mouse. *J. Histochem. Cytochem.* 18, 361-364.
- Inbal B., Bialik S., Sabanay I., Shani G. and Kimchi A. (2002). DAP kinase and DRP-1 mediate membrane blebbing and the formation of autophagic vesicles during programmed cell death. *J. Cell Biol.* 157, 455-468.
- Institute of Laboratory Animal Resources. (1996). *Guide for the care and use of laboratory animals*. National Academy Press. Washington, D.C.
- Jan C.R., Tseng C.J., Chou K.J. and Chiang H.T. (2000). Novel effects of propranolol. Release of internal Ca²⁺ followed by activation of capacitative Ca²⁺ entry in Madin Darby canine kidney cells. *Cell. Signal.* 12, 265-269.
- Jeffrey P.L., Brown D.H. and Illingworth-Brown B. (1970). Studies of lysosomal alpha glucosidase. I. Purification and properties of the rat liver enzyme. *Biochemistry* 9, 1403-1415.
- Jonas A.J., Conrad P. and Jobe H. (1990). Neutral-sugar transport by rat liver lysosomes. *Biochem. J.* 272, 323-326.
- Jungmann R.A., Constantinou A.I., Squinto S.P., Kwast-Welfeld J. and Schweppe J.S. (1986). Regulation of lactate dehydrogenase gene expression by cAMP-dependent protein kinase subunits. In: *Metabolic regulation: application of recombinant DNA techniques*. *Ann. NY Acad. Sci.* 478, 147-161.
- Kalamidas S.A. and Kotoulas O.B. (1999). The degradation of glycogen in the lysosomes of newborn rat hepatocytes: glycogen-, maltose- and isomaltose-hydrolyzing acid alpha glucosidase activities in liver. *Histol. Histopathol.* 14, 23-30.
- Kalamidas S.A. and Kotoulas O.B. (2000a). Studies on the breakdown of glycogen in the lysosomes. The effects of hydrocortisone. *Histol.*

- Histopathol. 15, 29-35.
- Kalamidas S.A. and Kotoulas O.B. (2000b). Glycogen autophagy in newborn rat hepatocytes. *Histol. Histopathol.* 15, 1011-1018.
- Kalamidas S.A., Kotoulas O.B., Kotoulas A.O. and Maintas D.B. (1994). The breakdown of glycogen in the lysosomes of newborn rat hepatocytes: The effects of glucose, cyclic 3' 5' -AMP and caffeine. *Histol. Histopathol.* 9, 691-698.
- Kalamidas S.A., Kotoulas O.B. and Hann A.C. (2002). Studies on glycogen autophagy. The effects of phorbol myristate acetate, ionophore A23187 or phentolamine. *Microsc. Res. Tech.* 57, 507-511.
- Kim J. and Klionsky D.J. (2000). Autophagy, cytoplasm-to-vacuole targeting pathway, and pexophagy in yeast and mammalian cells. *Annu. Rev. Biochem.* 69, 303-342.
- Klempner N.S. (1985). An adenosine triphosphate-dependent calcium uptake pump in human neutrophil lysosomes. *J. Clin. Invest.* 76, 303-310.
- Kornfeld S. (1986). Trafficking of lysosomal enzymes in normal and diseased states. *J. Clin. Invest.* 77, 1-6.
- Kornfeld S. (1992). Structure and function of the mannose 6-phosphate/insulinlike growth factor II receptors. *Annu. Rev. Biochem.* 61, 307-330.
- Kotoulas A.O., Kotoulas O.B. and Kalamidas S. (1991). An electron microscopic and biochemical study of the effects of cyclic 3'5'-AMP, ergotamine or propranolol on the lysosomes of newborn rat hepatocytes. *Histol. Histopathol.* 6, 421-426.
- Kotoulas O.B. (1981). An electron microscopic and biochemical study of the effects of insulin on newborn rat hepatocytes. *Pathol. Res. Pract.* 172, 138-147.
- Kotoulas O.B. (1986). The effects of cyclic 3',5'-AMP on the lysosomes of newborn rat hepatocytes. *J. Ultrastruct. Res.* 97, 210-215.
- Kotoulas O.B. (1988). Effects of actinomycin D on the lysosomes of newborn rat hepatocytes. *Anat. Rec.* 220, 103-107.
- Kotoulas O.B. and Phillips M.J. (1971). Fine structural aspects of the mobilization of hepatic glycogen. I. Acceleration of glycogen breakdown. *Am. J. Pathol.* 63, 1-7.
- Kotoulas O.B., Ho J., Adachi F., Weigensberg B.I. and Phillips M.J. (1971). Fine structural aspects of the mobilization of hepatic glycogen. II. Inhibition of glycogen breakdown. *Am. J. Pathol.* 63, 23-34.
- Lagast H., Pozzan T., Waldvogel F.A. and Lew P.D. (1984). Phorbol myristate acetate stimulates ATP-dependent calcium transport by the plasma membrane of neutrophils. *J. Clin. Invest.* 73, 878-883.
- Ma Z., Grubb J.H. and Sly W.S. (1992). Divalent cation-dependent stimulation of ligand binding to the 46-kDa mannose 6-phosphate receptor correlates with divalent cation-dependent tetramerization. *J. Biol. Chem.* 267, 19017-19022.
- Mackrill J.J. (1999). Protein-protein interactions in intracellular Ca²⁺-release channel function. *Biochem. J.* 337, 345-361.
- Maintas D.B., Kotoulas O.B. and Kotoulas A.O. (1993). A quantitative description of the insulin-induced ultrastructural changes in newborn rat hepatocytes. *Histol. Histopathol.* 8, 235-242.
- Mednieks M.I., Jungmann R.A., Fischer C. and Hand A.R. (1989). Immunogold localization of the type II regulatory subunit of cyclic AMP-dependent protein kinase. Monoclonal antibody characterization and RII distribution in rat parotid cells. *J. Histochem. Cytochem.* 37, 339-346.
- Michalak M., Corbett E.F., Mesaeli N., Nakamura K. and Opas M. (1999). Calreticulin: one protein, one gene, many functions. *Biochem. J.* 344, 281-292.
- Mithieux G., Vega F.V. and Riou J.P. (1990). The liver glucose-6-phosphatase of intact microsomes is inhibited and displays sigmoid kinetics in the presence of α -ketoglutarate-magnesium and oxaloacetate-magnesium chelates. *J. Biol. Chem.* 265, 20364-20368.
- Ockerman P.A. (1965). Glucose 6-phosphatase in human jejunal mucosa. Properties demonstrating the specific character of the enzyme activity. *Biochim. Biophys. Acta* 105, 22-33.
- Pfeifer U. (1971). Tageszeitliche rhythmik der zellularen autophagie. *Naturwissenschaften* 58, 152.
- Rosenfeld E.L. (1964). Control of glycogen metabolism. Ciba Foundation Symposium. Whelan W.J. and Cameron M.P. (eds). J. & A. Churchill Ltd. London.
- Sakai M., Luo S.Q., Tanaka T. and Ogawa K. (1989). Distribution of electric charges and concanavalin A binding sites on autophagic vacuoles and lysosomes in mouse hepatocytes. *Exp. Cell Res.* 185, 143-153.
- Salehi A., Mosen H. and Lundquist I. (1998). Insulin release transduction mechanism through acid glucan 1,4- α -glucosidase activation is Ca²⁺ regulated. *Am. J. Physiol.* 274, E459-468.
- Shelburne J.D., Arstila A.U. and Trump B.F. (1973). Studies on cellular autophagocytosis: cyclic AMP- and dibutyryl cyclic AMP-stimulated autophagy in rat liver. *Am. J. Pathol.* 72, 521-534.
- Skalhegg B.S. and Tasken K. (1997). Specificity in the cAMP/PKA signaling pathway. Differential expression, regulation, and subcellular localization of subunits of PKA. *Front. Biosci.* 2, d331-342.
- Sly W.S. (1985). Receptor-mediated sorting and transport of acid hydrolases to lysosomes. In: Protein transport and secretion. Gething M.-J. (ed). Cold Spring Harbor Lab. New York. pp 121-125.
- Spaulding S.W. (1993). The ways in which hormones change cyclic adenosine 3',5'-monophosphate-dependent protein kinase subunits and how such changes affect cell behaviour. *Endocr. Rev.* 14, 632-650.
- St-Denis J.-F., Berteloot A., Vidal H., Annabi B. and VandeWerve G. (1995). Glucose transport and glucose 6-phosphate hydrolysis in intact rat liver microsomes. *J. Biol. Chem.* 270, 21092-21097.
- VandeWerve G. (1989). Liver glucose 6-phosphatase activity is modulated by physiological intracellular Ca²⁺ concentrations. *J. Biol. Chem.* 264, 6033-6036.
- Wang Z., Wilson W.A., Fujino M.A. and Roach P.J. (2001). Antagonistic controls of autophagy and glycogen accumulation by Snf1p, the yeast homolog of AMP-activated protein kinase and the Cyclin-dependent kinase Pho85p. *Mol. Cell Biol.* 21, 5742-5752.
- Warren L. (1989). Stimulated secretion of lysosomal enzymes by cells in culture. *J. Biol. Chem.* 264, 8835-8842.
- Weibel E.R. (1969). Stereological principles for morphometry in electron microscopic cytology. *Int. Rev. Cytol.* 26, 235-302.
- Weibel E.R. and Gomez D.M. (1962). A principle for counting tissue structures on random sections. *J. Appl. Physiol.* 17, 343-348.
- Wells W.W., Collins C.A. and Kurtz J.W. (1981). Metabolic regulation of lysosomal activity. In: Lysosomes and lysosomal storage diseases. Callahan J.W. and Lowden J.A. (eds). Raven Press. New York. pp 17-30.
- Zakim D. and Vessey D.A. (1973). Techniques for the characterization of UDP-glucuronyltransferase, glucose-6-phosphatase, and other tightly-bound microsomal enzymes. *Met. Biochem. Anal.* 21, 1-37.