The Subcellular Location, Maturation and Response to Increased Plasma Glucagon of Ruthenium Red-Insensitive Calcium-Ion Transport in Rat Liver

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1. The subcellular distribution and maturation of Ruthenium Red-insensitive Ca^{2+} transport activity were determined in livers of rats ranging in age from 3 days pre-term to 10 weeks of adult life and compared with those of glucose 6-phosphatase, 5'-nucleotidase and Ruthenium Red-sensitive Ca^{2+} transport. Initial rates of Ruthenium Red-insensitive Ca^{2+} transport were highest in those fractions enriched in glucose 6-phosphatase, i.e. the microsomal fraction; this fraction was devoid of Ruthenium Red-sensitive Ca^{2+} transport activity. Although the heaviest fraction (nuclear) contained significant amounts of 5'-nucleotidase activity it was devoid of Ruthenium Red-insensitive Ca^{2+} transport activity. 2. Foetal rat liver contains minimal amounts of Ruthenium Red-insensitive Ca^{2+} transport activity, glucose 6-phosphatase and 5'-nucleotidase activities. These begin to be expressed concomitantly soon after birth; Ruthenium Red-insensitive Ca^{2+} transport is maximal by 3 to 4 days and remains so for up to at least 10 weeks of adult life. Glucose 6-phosphatase also reaches a peak at 3–4 days, but then rapidly decreases to approach adult values. Maximal activity of 5'-nucleotidase in the microsomal and nuclear fractions is seen about 4–6 days after birth; this enzyme activity remains increased for up to about 10 days and then falls, but not as rapidly as glucose 6-phosphatase. It is tentatively suggested that the bulk of the Ruthenium Red-insensitive Ca^{2+} transport is attributable to the system derived from the endoplasmic reticulum. 3. Administration of glucagon to adult rats enhances by 2–3-fold the initial rate of Ruthenium Red-insensitive Ca^{2+} transport in the intermediate but not the microsomal fraction. The hormone-induced effect is fully suppressed by co-administration of puromycin, is dose-dependent with half-maximal response at approx. $1\text{ }\mu\text{g}$ of glucagon/100 g body wt. and time-dependent exhibiting a half-maximal response about 1 h after administration of the hormone. 4. Ruthenium Red-insensitive Ca^{2+} transport in the post-mitochondrial fraction of foetal liver also responds to the administration *in situ* of glucagon. The response, which also is prevented by co-administration of puromycin, is maximal in those fetuses nearing term. The suggestion is made that these effects of the hormone on Ruthenium Red-insensitive Ca^{2+} transport are an integral part of the physiological network in the liver cell.

It is becoming increasingly evident that many of the effects of Ca^{2+} on physiological and biochemical events in the cell are difficult to evaluate rationally in the absence of knowledge about the regulation of cell Ca^{2+} . Considerable evidence exists that mitochondria in many tissues and species play a predominant role in the regulation of cell Ca^{2+} (reviewed by Bygrave, 1977, 1978a,c). Recently increasing attention has been given to the possibility that the Ca^{2+} -transport system located in the endoplasmic reticulum of non-muscle tissue may also be important in the regulation of cell Ca^{2+} (see below and Bruns *et al.*, 1976).

The reports of Moore *et al.* (1974, 1975) were

Abbreviations used: Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid; butyl-PBD, 5-(4-biphenyl)-2-(4-*t*-butylphenyl)-1-oxa-3,4-diazole.

among the first to describe the energy-dependent sequestration of Ca^{2+} by a microsomal fraction from rat liver. Important features of this transport system included a high affinity for Ca^{2+} , an absolute requirement for energy of ATP hydrolysis, but an apparent relatively slow rate of Ca^{2+} transport. One report contained data showing that, although the rate of Ca^{2+} transport differed between male and female rats, no major change in transport could be observed when the age of the rat varied between 3 weeks and 10 weeks *post partum* (Moore *et al.*, 1975).

The study reported here was undertaken to extend knowledge about the potential physiological significance of ATP-dependent microsomal Ca^{2+} transport in rat liver. In undertaking the study, full use was made of a recently described technique for measuring the initial rates of the transport system

(Bygrave, 1978b), of the finding that Ruthenium Red is the most suitable agent yet described for distinguishing mitochondrial from non-mitochondrial Ca^{2+} transport by liver *in vitro* (Ash & Bygrave, 1977), of knowledge that maturation of mitochondrial Ca^{2+} transport in rat liver occurs rapidly at the time of birth of the animal (Bygrave & Ash, 1977) and finally of knowledge that glucagon administration to rats *in vivo* induces cycloheximide- and puromycin-sensitive changes to the mitochondrial Ca^{2+} transport system as revealed by measurements *in vitro* (Hughes & Barritt, 1977; V. Prpic, T. L. Spencer & F. L. Bygrave, unpublished work).

Data in the present report show that the bulk of the Ruthenium Red-insensitive Ca^{2+} transport throughout liver development correlates more with the microsomal marker enzyme glucose 6-phosphatase than with the plasma-membrane marker enzyme 5'-nucleotidase. They show too that the initial rates of Ruthenium Red-insensitive Ca^{2+} transport are very low in perinatal liver, but increase sharply at about 3 days *post-partum*. Finally it is shown that this transport responds to increased concentrations of plasma glucagon in both foetal and adult animals. The response to glucagon is prevented by co-administration of the inhibitor of protein synthesis, puromycin.

Experimental

Animals

The animals used were rats of the Dark Agouti strain. A breeding colony was established by housing usually two or three adult female rats with one adult male for an unrestricted period of time. Cages were checked each day for the arrival of new litters; neonatal age was determined by regarding the day of birth as day zero.

The age of foetal young was determined by examining female rats for the presence of a vaginal plug, which generally remained visible for a period of up to 24 h after copulation. Calculation of foetal age was based on a gestation period of 22 days, and the day after an overnight mating was taken as day -22.

Preparation of membrane fractions

Neonatal rats were killed by decapitation; adult rats were first stunned and then killed by cervical dislocation. With young rats, whole litters or pooled litters of the same age were killed at the one time.

The livers were rapidly removed and immersed in ice-cold isolation medium (250 mM-sucrose, 5 mM-Hepes, 0.5 mM-EGTA, pH 7.4). After several washes to remove excess blood the livers were minced with scissors and the isolation medium was adjusted to

give a 10% (w/v) homogenate. Neonatal and adult livers were homogenized in a Thomas B and Thomas C glass homogenizer respectively by using one or two strokes of a motor-driven Teflon pestle. Foetal livers were homogenized by hand in a Thomas B homogenizer. The homogenate was filtered through a double layer of cheesecloth to remove unbroken cells and connective tissue. The filtered homogenate was then fractionated by the following protocol.

(i) The homogenate was centrifuged at 750g for 10 min in an RC2-B Sorvall centrifuge to remove nuclei and cell debris; this fraction is henceforth referred to as the 'nuclear fraction'.

(ii) The 'mitochondrial fraction' was prepared by centrifuging the post-nuclear supernatant fraction at 12100g for 10 min.

(iii) Postmitochondrial supernatant fraction was centrifuged at 24800g for 20 min. This fraction has been designated as an 'intermediate fraction'; it served to remove remaining lighter ruptured mitochondria that may otherwise have contaminated the final light-membrane (microsomal) fraction.

(iv) The supernatant fraction from step (iii) was then centrifuged at 105000g for 60 min in a Beckman model L-2 ultracentrifuge, to yield the 'microsomal fraction'.

The pellet obtained from each fractionation step was resuspended in an appropriate volume of 250 mM-sucrose/5 mM-Hepes, pH 6.8.

Protein determination

Protein content was measured by a modified biuret method (Szarkowska & Klingenberg, 1963). Crystalline bovine serum albumin was used as a standard.

Calcium transport

Uptake of radioactive Ca^{2+} was measured by membrane filtration. Transport assays were performed at 37°C (Moore *et al.*, 1975; Bygrave, 1978b), in the following incubation medium: 100 mM-KCl/5 mM-imidazole/histidine buffer (pH 6.8)/5 mM- MgCl_2 /3.3 mM-ammonium oxalate, containing 0.5–0.8 mg of protein/ml. After a preincubation time of 2 min, 5 mM-ATP, pH 6.8, was added and the assay was initiated by the addition of 20 μM - $^{45}\text{Ca}^{2+}$. The final reaction volume was 1.43 ml. $^{45}\text{Ca}^{2+}$ transport was measured in the presence and absence of 1.4 μM -Ruthenium Red (see the Results section).

At specified time intervals, a 100 μl sample of the reaction medium was removed and filtered through a membrane filter (0.45 μm porosity), prewashed with 250 mM-sucrose/5 mM-Hepes. The membrane filters were washed again to remove non-sequestered $^{45}\text{Ca}^{2+}$, and then dissolved in 10 ml of methyl-Cellosolve scintillant (6 g of butyl-PBD in 400 ml of

2-methoxyethanol and 600 ml of toluene). Radioactivity retained by the filters was measured by liquid-scintillation spectrometry. The specific radioactivity of the radioactive isotope in each incubation was determined by counting an unfiltered 100 μ l sample.

Ruthenium Red-insensitive transport refers to that Ca^{2+} transported in the presence of 1.4 μM -Ruthenium Red. Ruthenium Red-sensitive transport refers to that Ca^{2+} transported in the absence of Ruthenium Red minus that transported in the presence of Ruthenium Red.

Enzyme assays

Glucose 6-phosphatase. Glucose 6-phosphatase was determined at 37°C in an incubation medium containing 50 mM-potassium maleate, pH 6.5, and 10–40 μg of protein. The reaction was initiated after a 5 min preincubation by the addition of 10 mM-glucose 6-phosphate, pH 6.5. The final reaction volume was 0.5 ml. Background activity was determined by control incubations, which did not receive the substrate addition. Assays were terminated after a 30 min incubation with 1 ml of 20% trichloroacetic acid/4% sodium dodecyl sulphate/4% ascorbic acid.

The release of phosphate was measured colorimetrically by the method of Baginski *et al.* (1966). The specific activity was expressed as μmol of phosphate released/30 min per mg of protein.

5'-Nucleotidase. The assay for 5'-nucleotidase was adapted from the method of Emmelot & Bos (1967).

The reaction medium contained 47 mM-Tris/HCl/4.7 mM-MgCl₂ (pH 7.8), 0.06% Triton X-100 and 10–40 μg of protein. The reaction was initiated after a 5 min preincubation at 37°C by the addition of 5 mM-AMP, pH 7.8. The final reaction volume was 0.5 ml.

Phosphate release was measured as above.

Hormone administration

For foetal experiments, pregnant rats of the Dark Agouti strain were used. Laparotomies were performed under Nembutal anaesthesia. A proportion of the foetuses received subcutaneous injections through the uterine wall, of 10 μg of glucagon in 1 mM-NaOH made up to a final volume of 10 μl with 0.9% NaCl. The remaining foetuses were injected with 10 μl of 0.9% NaCl, to serve as controls. Adopting this experimental approach, the control and treated foetuses were exactly the same age and were subjected to the same maternal influences. Then 5 h later, the mother was killed by cervical dislocation, and the foetuses were removed. The crown-to-rump body lengths were measured as an indication of relative maturity. The livers were then rapidly excised and postmitochondrial supernatant fractions

from individual liver homogenates were prepared by the fractionation procedure outlined above. Ruthenium Red-insensitive Ca^{2+} transport was measured in the postmitochondrial supernatant fractions.

In experiments involving newborn animals, Dark Agouti strain rats were used. The crown-to-rump body length was measured and correlated with the known age of the neonatal animals. Each was injected intraperitoneally with 2.5 μg of glucagon/g body wt., made up to a delivery volume of 10 μl with 0.9% NaCl. Controls were injected with 10 μl of 0.9% NaCl. Then 5 h later, the newborn were killed by decapitation, and their livers removed. Glucagon-treated livers were pooled separately from control livers. Postmitochondrial supernatant fractions were prepared by the fractionation procedure outlined above. Ca^{2+} transport in the presence of 1.4 μM -Ruthenium Red was assayed in the postmitochondrial supernatant fractions.

In experiments involving adult animals, male rats of the Wistar strain were injected intraperitoneally with a glucagon dosage ranging from 0.1 to 30 μg /100 g body wt. Controls received no injection. After 1 h, the rats were killed by cervical dislocation and the livers were rapidly excised. In time-course experiments, however, livers were taken after periods of 30 min, 2 h or 4 h. The fractionation procedure detailed above was modified in the following manner: (a) the 'nuclear fraction' was prepared by centrifuging the liver homogenate at 900 g for 5 min in an RC2-B Sorvall centrifuge; (b) the post-nuclear supernatant fraction was then centrifuged at 4500 g for 5 min; the remaining fractions were assayed in both the intermediate and microsomal fractions.

Treatment with puromycin

With the foetal experiments described above, individual foetuses received a combined injection of 10 μg of glucagon plus 175 μg of puromycin made up to 10 μl with 0.9% NaCl. For the adult experiments outlined above, a combined injection of 30 μg of glucagon/100 g body wt. plus 4.2 mg of puromycin/100 g body wt. was delivered intraperitoneally. Puromycin-treated animals were killed and fractions were prepared as described above.

Materials

$^{45}\text{Ca}^{2+}$ was obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Ruthenium Red was purchased from Sigma Chemical Co., St Louis, MO, U.S.A., and recrystallized by the method of Fletcher *et al.* (1961).

Nucleotides were from Boehringer, Mannheim, West Germany.

Membrane filters (0.45 μm porosity) were obtained from the Gelman Instrument Co., Ann Arbor,

MI, U.S.A. D-Glucose 6-phosphate (disodium salt), glucagon (crystalline) and puromycin dihydrochloride (crystalline) were from Sigma.

Nembutal (pentobarbitone sodium) was purchased from Abbott Laboratories Pty., Sydney, Australia. All other materials were of analytical-reagent grade.

Results

Assay of Ruthenium Red-insensitive Ca^{2+} transport

Fig. 1(a), which is representative of a number of experiments, illustrates the time course of Ca^{2+} transport by the microsomal fraction isolated from rat liver at three different stages of development. As described by Bygrave (1978b), and seen especially in the adult system, the rate of transport is most rapid at the earliest times and usually within the first minute of incubation. By contrast with the relatively high initial rate observed in the adult fraction, those in perinatal liver are significantly lower (see below).

Fig. 1(b) confirms the finding (Ash & Bygrave, 1977; Bygrave, 1978b) that Ca^{2+} transport by the microsomal fraction is insensitive to concentrations of Ruthenium Red that completely block transport of the ion into liver mitochondria (Reed & Bygrave, 1974), and fully endorses its usefulness in distinguishing mitochondrial from non-mitochondrial Ca^{2+} transport, i.e. that potentially attributable to the microsomal fraction and/or plasma membrane.

Subcellular distribution of Ruthenium Red-insensitive Ca^{2+} transport in adult liver

To assess which subcellular organelles were responsible for the observed Ruthenium Red-

insensitive Ca^{2+} transport activity, the liver homogenate was fractionated as described in the Experimental section. Five different fractions were obtained, designated nuclear, mitochondrial, intermediate, microsomal and soluble, and in each the following were measured: Ca^{2+} transport both in the presence and in the absence of Ruthenium Red, glucose 6-phosphatase and 5'-nucleotidase. The assumption was made that Ruthenium Red-sensitive Ca^{2+} transport would be enriched in the mitochondrial fraction, that glucose 6-phosphatase activity would reflect the presence of microsomal membranes and that 5'-nucleotidase activity would reflect the presence of plasma membranes (Fleischer & Kervina, 1974).

Representative data (Fig. 2) are taken from such an experiment carried out with adult rat liver. It is seen that the initial rate of Ruthenium Red-insensitive Ca^{2+} transport is greatest in the microsomal fraction and gradually diminishes in the heavier fractions. Ruthenium Red-sensitive Ca^{2+} transport is absent from the microsomal fraction and almost completely absent from the intermediate fraction. Clearly, it is predictably enriched in the mitochondrial fraction. The relatively high activity in the nuclear fraction indicates the presence of large amounts of mitochondrial Ca^{2+} -transport activity in the heavier liver cell fractions, an aspect of Ca^{2+} transport dealt with elsewhere (Bygrave *et al.*, 1978).

The distribution of glucose 6-phosphatase activity, but not that of 5'-nucleotidase, follows exactly that of Ruthenium Red-insensitive Ca^{2+} transport, permitting the tentative suggestion that this activity is localized in those cell fractions enriched in glucose 6-phosphatase, i.e. the microsomal fraction.

Fig. 1. Ca^{2+} transport by the microsomal fraction isolated from rats of various ages

The basic incubation medium contained, in a final volume of 1.5 ml: 100 mM-KCl, 5 mM-imidazole/histidine buffer, pH 6.8, 3.3 mM-ammonium oxalate, 5 mM-ATP, 5 mM-MgCl₂ and 0.5–0.8 mg of microsomal protein. Where indicated Ruthenium Red was present at 1 μM . The reaction was initiated by the addition of 20 μM - Ca^{2+} containing 0.3 μCi of $^{45}\text{Ca}^{2+}$. Reaction temperature was 37°C. For further details see the Experimental section. (a) Rats were 10 weeks old (○), 2 days old (△) or 2 days pre-term (●); (b) Ruthenium Red was absent (○) or present (●).

Developmental profile of Ruthenium Red-insensitive Ca^{2+} transport in liver

Experiments similar to those described in Fig. 2 were carried out repeatedly, but at various stages of liver development. Fig. 3(a) shows that in all of the microsomal fractions Ruthenium Red-insensitive Ca^{2+} transport activity is low in foetal liver, but quickly rises soon after birth to reach maximum rates by 3–4 days. Glucose 6-phosphatase activity also increases sharply soon after birth to reach a maximum at 3–4 days, but by contrast with Ruthenium Red-insensitive Ca^{2+} transport it falls sharply after 4 days and thereafter gradually decreases to approach adult values, consistent with the observations of Burch *et al.* (1963). The activity of 5'-nucleotidase similarly increases soon after birth, but maximum rates are not seen until 4–6 days. The activity of this enzyme falls subsequently but not to the same extent as glucose 6-phosphatase. By contrast with these three enzyme activities, that of Ruthenium Red-sensitive Ca^{2+} transport in this fraction is near-absent throughout development (cf. Fig. 2).

Fig. 3(b) shows the same four activity profiles in the intermediate fraction. The pattern for glucose 6-phosphatase is not very different from that of this enzyme in the microsomal fraction. On the other hand, 5'-nucleotidase activity is considerably lower at most stages of development; the sharp rise seen early in development in the microsomal fraction is absent. Despite this, Ruthenium Red-insensitive Ca^{2+} transport activity in this fraction is as high as that of the microsomal fraction especially from 3 to 10 days after birth. After this time the activity decreases to approach adult values. Again throughout development, Ruthenium Red-sensitive Ca^{2+} transport remains minimal and invariable.

Fig. 3(c) shows that of the four activities in the mitochondrial fraction only that of Ruthenium Red-sensitive Ca^{2+} transport displays any significant variation with development. At and soon after birth this activity increases sharply to attain near-adult rates by several days after birth. This finding is in full agreement with previous observations made in this laboratory (Bygrave & Ash, 1977).

Fig. 3(d) shows changes in the above four activities in the nuclear fraction. Both Ruthenium Red-insensitive Ca^{2+} transport and glucose 6-phosphatase activities are near-minimal throughout development. Especially significant is the finding that 5'-nucleotidase activity reaches maximal activity at 7–12 days after birth yet Ruthenium Red-insensitive Ca^{2+} transport remains practically absent. As predicted from earlier work (Bygrave & Ash, 1977) Ruthenium Red-sensitive Ca^{2+} transport reaches maximal rates soon after birth. It is important to point out that measurements of Ruthenium Red-sensitive Ca^{2+}

transport as made in this study are not optimal for that system. Despite this, however, the developmental profile for this activity in this work deviates only slightly from that obtained when optimum conditions are used (see Bygrave & Ash, 1977).

Fig. 2. Subcellular distribution of Ruthenium Red-insensitive and -sensitive Ca^{2+} transport, glucose 6-phosphatase and 5'-nucleotidase in various fractions of liver from adult rats

Subcellular fractions were isolated as described in the Experimental section and Ca^{2+} transport and the enzyme assays carried out also as described therein. The experiment is one of several that gave similar results. The fractions are nuclear (N), mitochondrial (Mt), intermediate (I), microsomal (Mc) and soluble (S). The horizontal bar represents the activity in the homogenate. (a) Ruthenium Red-insensitive Ca^{2+} transport, (b) Ruthenium Red-sensitive Ca^{2+} transport, (c) glucose 6-phosphatase, (d) 5'-nucleotidase.

Fig. 3. Developmental profile of Ruthenium Red-insensitive and -sensitive Ca^{2+} -transport, glucose 6-phosphatase and 5'-nucleotidase activities in different fractions of rat liver assessed over a range of ages of the rat

Rat livers at the ages indicated were fractionated and the assays on each fraction carried out as described in the Experimental section. ●, Ruthenium Red-insensitive Ca^{2+} transport; ○, Ruthenium Red-sensitive Ca^{2+} transport; ▲, glucose 6-phosphatase, △, 5'-nucleotidase. The assays were carried out with the microsomal (a), intermediate (b), mitochondrial (c) and nuclear (d) fractions.

Ruthenium Red-insensitive Ca^{2+} transport in microsomal fractions isolated after the administration of glucagon to adult rats in vivo

Experiments of Yamazaki (1975), Hughes & Barritt (1977) and those of this laboratory (V. Prpic, T. L. Spencer & F. L. Bygrave, unpublished work), have shown that administration of glucagon to rats *in vivo* induces an alteration in the Ca^{2+} -transport properties of the mitochondria subse-

quently isolated from liver. It was decided therefore to determine whether changes were induced also in Ruthenium Red-insensitive Ca^{2+} transport carried out by the microsomal and intermediate fractions.

The experiment described in Fig. 4, one of three giving essentially identical results, shows that increased plasma glucagon concentrations induce an alteration in Ruthenium Red-insensitive Ca^{2+} transport in the intermediate fraction, but not in the microsomal fraction prepared from the liver. The change is such that the initial rate is enhanced 2–3-fold; the steady-state rate (see Bygrave, 1978b) was enhanced also, but generally not to the same extent.

Data in Fig. 5, obtained with the intermediate fraction, shows that the effects of glucagon administration are completely suppressed when puromycin is co-administered with the hormone (Fig. 5a), and that the degree of enhancement of the initial rate of transport is both dependent on the concentration of injected glucagon (Fig. 5b) and on the time after which the hormone is injected (Fig. 5c).

Ruthenium Red-insensitive Ca^{2+} transport activity in the postmitochondrial fraction after the administration of glucagon in vivo to perinatal rats

The experiments were undertaken in light of the findings of Greengard & Dewey (1967, 1968) that the activity of glucose 6-phosphatase in foetal liver can be expressed prematurely by the administration *in vivo* of glucagon and in the light of knowledge that plasma glucagon concentrations change markedly around the time of birth (Girard *et al.*, 1974).

The age of the animal in the present study was determined as accurately as possible from measurements of the crown-to-rump body length (cf. Greengard & Dewey, 1967). Because of the low yields of microsomal and intermediate fractions that are obtained from a single foetus, it was decided to use only a post-mitochondrial fraction from each animal (see the Experimental section).

Fig. 6 shows the initial rates of Ruthenium Red-insensitive Ca^{2+} transport in the post-mitochondrial fraction of foetal liver (Figs. 6a and 6b) and that from the liver of the newborn rat (Fig. 6c). The increase in the initial rate of Ca^{2+} transport in the control animal from the 41 mm-long foetus to the 48 mm-long newborn animal is exactly as predicted from previous experiments (cf. Fig. 3). However, most evident is that glucagon induces the most pronounced effect when administered at the late foetal stage (Fig. 6b).

The activity of glucose 6-phosphatase was measured also in the postmitochondrial fraction in both the control and glucagon-treated animals. Fig. 7 compares the degree of stimulation of glucose 6-phosphatase by glucagon with that of Ruthenium Red-insensitive Ca^{2+} transport. It is seen that although maximum stimulation of each activity occurs

Fig. 4. Ruthenium Red-insensitive Ca^{2+} transport by the microsomal and intermediate fractions of rat liver mitochondria after the administration *in vivo* of glucagon to adult rats

Male animals were injected with $30 \mu\text{g}$ of glucagon/100 g body wt. (●) 60 min before exposing the liver and initiating preparation of the microsomal (a) and intermediate (b) fractions. The control animal (○) received an injection of 0.9% NaCl. Ca^{2+} transport was measured exactly as described in Fig. 1.

Fig. 5. Properties of glucagon-induced, Ruthenium Red-insensitive Ca^{2+} transport in the intermediate fraction of adult rats. In (a) rats were injected with glucagon ($30 \mu\text{g}/100 \text{ g}$ body wt.) in the absence (●) or in the presence (○) of 4.2 mg of puromycin/100 g body wt.; ▲, 0.9% NaCl-injected rat. In (b) Ruthenium Red-insensitive Ca^{2+} transport was measured after the administration *in vivo* of the indicated concentrations of glucagon. In each of the experiments, the time of injection before killing the rat was 60 min. In (c) $30 \mu\text{g}$ of glucagon/100 g body wt. was administered and the animal was killed at the times indicated.

Fig. 6. Time courses for Ruthenium Red-insensitive Ca^{2+} transport by the post-mitochondrial fraction of rat fetuses of different ages after the administration *in utero* of glucagon

Foetuses of pregnant rats were exposed briefly by caesarean section and glucagon ($10 \mu\text{g}$ into foetuses or $250 \mu\text{g}/100 \text{ g}$ body wt. into newborn) was given (●) to individual animals. Control animals (○) were given 0.9% NaCl. After 5 h the animals were killed and the postmitochondrial fractions prepared and Ca^{2+} transport was measured in the presence of Ruthenium Red. Further details are contained in the Experimental section. The crown-to-rump body lengths were (a) 27 mm, (b) 41 mm and (c) 48 mm (newborn).

Fig. 7. Comparison of the stimulation by glucagon of Ruthenium Red-insensitive Ca^{2+} transport (●) and glucose 6-phosphatase (○) in post-mitochondrial fractions of liver from foetal and newborn rats

Fig. 8. Inhibition by puromycin of glucagon-induced Ruthenium Red-insensitive Ca^{2+} transport by the post-mitochondrial fraction of foetal liver

The experiments described in Fig. 6 were carried out with foetuses of crown-to-rump body length of 41 mm. Where indicated, puromycin (175 µg) (▲) was injected with 10 µg of glucagon (●). The control animal (○) was given 0.9% NaCl.

immediately before birth, i.e. when the crown-to-rump body length is approx. 41 mm, the degree of stimulation of Ruthenium Red-insensitive Ca^{2+} transport greatly exceeds that of glucose 6-phosphatase.

Finally Fig. 8 shows that Ruthenium Red-insensitive Ca^{2+} transport in the postmitochondrial fraction no longer is responsive to the hormone when puromycin is co-administered with the glucagon.

Discussion

The data in the present paper provide a body of new information about several aspects of the control of Ca^{2+} in the liver cell, particularly in regard to the role of the non-mitochondrial Ca^{2+} -sequestering systems. That these systems were in the main not of mitochondrial origin was assessed by their insensitivity to Ruthenium Red. This compound is a potent inhibitor of mitochondrial Ca^{2+} transport (Moore, 1971; Vasington *et al.*, 1972; Reed & Bygrave, 1974) and ample evidence now exists that the substance does not inhibit microsomal Ca^{2+} transport (Ash & Bygrave, 1977; Bygrave, 1978b), nor Ca^{2+} transport across the plasma membrane of intact cells (Blackmore *et al.*, 1978) or Ca^{2+} binding to plasma-membrane preparations (McDonald *et al.*, 1976).

Experiments involving cell fractionation provided evidence that the bulk of the Ruthenium Red-insensitive Ca^{2+} transport in liver is associated with membrane fractions, especially those of the light fractions, enriched in glucose 6-phosphatase (Figs. 2 and 3). Although this finding points to the endoplasmic reticulum as being the source of the majority of the Ruthenium Red-insensitive Ca^{2+} -transport activity, the data do not exclude the possibility that contaminating plasma membranes in these fractions might also be responsible for some of the Ca^{2+} -transport activity. However, by contrast with glucose 6-phosphatase activity, 5'-nucleotidase was enriched in both the light (microsomal) and heavy (nuclear) fractions, consistent with knowledge that on rupture this membrane forms large (non-vesicular) membrane sheets, which sediment at low *g* values, and vesicles that sediment at high *g* values (Fleischer & Kervina, 1974). Consistent with the tentative conclusion expressed above that the bulk of the Ruthenium Red-insensitive Ca^{2+} transport in the microsomal fraction is attributable to the endoplasmic reticulum and not to the plasma membrane, are data showing that plasma-membrane vesicles derived from this fraction and devoid of much of the glucose 6-phosphatase activity, do not transport Ca^{2+} at anywhere near the rates of that by the microsomal fraction (T. L. Spencer & F. L. Bygrave, unpublished work). Also plasma membranes obtained from adipocytes exhibit low Ca^{2+} -transporting abilities (McDonald *et al.*, 1976). Further evidence for this conclusion can be seen in the maturation profile (Fig. 3), whereby about 3 days after birth there is a massive increase in both glucose 6-phosphatase and Ruthenium Red-insensitive Ca^{2+} transport under conditions where an increase in 5'-nucleotidase activity is only slight (Fig. 3b).

It seems significant that Ruthenium Red-insensitive Ca^{2+} transport does not decrease concomitant with either that of glucose 6-phosphatase or 5'-

nucleotidase (Fig. 3). We would regard this as evidence that the liver cell recognizes the transport process to be of sufficient physiological importance that it ensures this does not occur. It is of interest that the maturation profile for Ruthenium Red-insensitive Ca^{2+} transport in liver as seen here, bears a close resemblance to that of Ca^{2+} transport by the sarcoplasmic reticulum of developing chicken pectoralis muscle (Martonosi *et al.*, 1977). Ca^{2+} transport was minimal in chick embryos, but increased greatly immediately after hatching to reach near-maximal values by about 15 days of adult life.

The finding that Ruthenium Red-insensitive Ca^{2+} transport in liver is maximally developed by 3–4 days after birth may be compared with the maturation profile of Ruthenium Red-sensitive Ca^{2+} transport, which shows a period of rapid development over birth such that it is maximal by 1–2 days *post partum* (Bygrave & Ash, 1977). Thus the liver cell by about 3 days *post partum* has fully acquired the ability to regulate intracellular Ca^{2+} via the Ca^{2+} -transport systems located in the mitochondria and endoplasmic reticulum.

Even further evidence for the physiological importance to the liver cell of microsomal Ca^{2+} transport emanates from experiments in which microsomal Ca^{2+} -transport activity is enhanced several-fold by increased plasma glucagon concentrations (Fig. 4). Especially significant in these experiments were the quite low concentrations of the hormone that were needed to be administered *in vivo* to induce the changes in Ca^{2+} transport, the dose-dependency, the time-dependency after administration and the sensitivity of the effect to puromycin. Equally significant was the finding that Ruthenium Red-insensitive Ca^{2+} transport in only the intermediate fraction and not the microsomal fraction was susceptible to increased concentrations of the hormone. The molecular basis of this hormone-induced effect may involve specific protein phosphorylation systems (Greengard, 1978).

The effects of glucagon reported herein also may be compared with those seen on mitochondrial Ca^{2+} transport. Studies of Hughes & Barritt (1977) and in this laboratory (V. Prpic, T. L. Spencer & F. L. Bygrave, unpublished work), have shown that the concentrations of glucagon that induce the above changes in Ruthenium Red-insensitive Ca^{2+} transport also induce mitochondria to retain Ca^{2+} for prolonged periods (see also Dorman *et al.*, 1975). Thus the action of glucagon on both the Ruthenium Red-sensitive and -insensitive Ca^{2+} -transport systems would be to induce the liver cell to decrease the cytoplasmic Ca^{2+} concentration. The effects this would have on liver cell metabolism have not yet been fully assessed.

The present study has provided strong evidence

that Ruthenium Red-insensitive Ca^{2+} transport is a target for hormone action in liver just as it appears to be in the heart (reviewed by Sulakhe & St. Louis, 1976) and as such extends knowledge about the way in which the concentration of the ion in the cytoplasm may be controlled *in situ*.

The relation of the effects of glucagon on Ruthenium Red-insensitive Ca^{2+} transport in adult liver tissue (Figs. 4 and 5) to that in foetal liver (Fig. 6) is uncertain at this time; a common property clearly evident is the sensitivity to puromycin. Despite this uncertainty, however, it seems significant that the concentrations of glucagon and insulin change very markedly around the time of birth (Girard *et al.*, 1974). It remains an intriguing question as to whether changes in the concentrations of these hormones at this time of liver cell differentiation are the stimuli for inducing the expression of the mitochondrial and microsomal Ca^{2+} -transporting systems. Already it has been suggested that the gene expression leading to the maturation of Ca^{2+} transport by sarcoplasmic reticulum in cultured chicken muscle may be regulated by intracellular Ca^{2+} concentrations (Martonosi *et al.*, 1977).

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