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Lipoprotein Lipase Activity of Rat Cardiac Muscle

CHANGES IN THE ENZYME ACTIVITY DURING INCUBATIONS OF ISOLATED CARDIAC-MUSCLE CELLS *IN VITRO*

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1. Isolated cardiac-muscle cells from the hearts of adult rats were shown to retain a high amount of viability during 4h of incubation when viability was assessed by Trypan Blue stain exclusion and intracellular enzyme leakage. 2. The cells also retained their ability to take up O_2 and utilize added substrates over the period of incubation at both 25 and 30°C. 3. When cells from the hearts of fed rats were incubated in a buffered-salts solution at pH 7.4 in the presence of amino acids and heparin, lipoprotein lipase activity in the medium increased progressively. 4. During these incubations the intracellular activity of the enzyme remained constant and the total activity of lipoprotein lipase in the system (cells + medium) increased by 80% over the 4h of incubation at 25°C. 5. In the absence of heparin only low amounts of enzyme activity were detectable in the medium and the total lipoprotein lipase activity in the system remained constant. 6. The measurement of lipoprotein lipase activity in either fresh homogenates of the cells or in homogenates of acetone/diethyl ether-dried powders of the cells had no effect on the overall pattern of activity change during the incubations, although as reported previously the total activity detected with acetone/diethyl ether-dried preparations was approx. 3-fold higher than with fresh cell homogenates. 7. The observations were compared with published data on lipoprotein lipase activity changes in neonatal heart cell cultures maintained *in vitro*.

Lipoprotein lipase (clearing-factor lipase; EC 3.1.1.34), the enzyme that regulates the removal of lipoprotein triacylglycerol fatty acid from the plasma by the extrahepatic tissues (Robinson, 1970), has now been shown to be present in cardiac tissue at more than one site (Chohan & Cryer, 1977, 1978). Thus in addition to being present at the endothelial cell surface, where its functional role in triacylglycerol fatty acid hydrolysis is exercised (Borensztajn & Robinson, 1970; Fielding & Higgins, 1974; Rogers & Robinson, 1974; Borensztajn *et al.*, 1975), the enzyme has also been shown to be present in cardiac-muscle cells from adult rats (Bagby *et al.*, 1977; Chohan & Cryer, 1978, 1979) and in neonatal heart cells maintained in culture (Chajek *et al.*, 1978*a,b*). The possible significance of the lipoprotein lipase activity that resides within cardiac-muscle cells, where it cannot be directly involved in triacylglycerol fatty acid hydrolysis, can be considered initially in the light of two observations. First, the lipoprotein lipase activity found in cardiac cells from adult hearts is substantially localized within endoplasmic-reticulum-derived microsomal vesicles, a location consistent with the possibility that the enzyme may be secreted from the cell (Chohan & Cryer, 1979). Secondly, when mesenchymal cells from the hearts of neonatal animals, maintained in culture, are incubated under appropriate conditions, lipoprotein lipase activity appears extracellularly in the incubation medium (Chajek *et al.*, 1978*a,b*). In the present study the possibility that the cardiac-muscle cell from the adult heart might also act as a source of extracellular lipoprotein lipase has been investigated by incubating isolated cells *in vitro*. The approach follows a path previously shown to be useful in the study of adipose tissue lipoprotein lipase activity in adipocyte-containing incubation systems (Stewart & Schotz, 1971, 1973, 1974; Cryer *et al.*, 1975; Kornhauser & Vaughan, 1975; Spencer *et al.*, 1978).

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Materials and Methods

Materials

Collagenase (type II, 150 units/mg), fatty acid-free albumin and bovine serum albumin (fraction V) were obtained from Sigma (London) Chemical Co., Kingston upon Thames, Surrey KT2 7BH, U.K. Heparin (Pularin) was obtained from Evans Medical, Speke, Liverpool, U.K., and Trypan Blue was from Gibco Bio-cult, Glasgow, Scotland, U.K.

Animals, tissues and cells

Male rats of the M.R.C. hooded strain, from the colony of this Department, were used throughout. The rats weighed 190–210 g in the fed state and were maintained on modified diet 41B (Pilsbury and Co., Birmingham B5 7UG, U.K.). The animals were killed between 08:00h and 09:30h. Immediately after the death of the rats hearts were excised and isolated cardiac cells were prepared from them by the modified procedure of Powell & Twist (1976) as described by Chohan & Cryer (1978). Dry weights and wet weights of cells were determined as described by Farmer *et al.* (1977).

Determination of cell viability during incubations

The viability of freshly prepared cells and of those cells removed from incubation systems at various times was assessed by the Trypan Blue exclusion method of Howard & Pesch (1968). Secondly, the proportion of the original cellular lactate dehydrogenase (EC 1.1.1.27) activity that appeared in the medium during the course of the cell incubations was determined. The cellular and medium activities of lactate dehydrogenase were determined by the method of Bergmeyer & Bernt (1974) as described previously (Chohan & Cryer, 1979). Thirdly, the O_2 uptake of the cells and its response to added substrates and inhibitors was monitored by using either a Clarke-type oxygen electrode (Powell & Twist, 1975) or a manometric technique (Warburg & Krippahl, 1959). For the oxygen-electrode method the system was calibrated with water saturated with O_2 (upper limit) and with the same medium after the addition of sodium dithionite (lower limit). The respiration rate was determined in 1 ml of cell suspension (approx. 50 mg wet wt. of cells/ml of Krebs–Ringer bicarbonate buffer, pH 7.4; see below) by following the rate of O_2 concentration in the medium for period of at least 5 min. For the continuous measurement of O_2 uptake over longer periods, a manometric technique was employed Warburg & Krippahl, 1959). These incubations were carried out under an atmosphere of O_2/CO_2 (19 : 1) in manometric vessels and by techniques previously shown to be suitable for isolated-cell studies (Krebs *et al.*, 1974; Dani *et al.*, 1977). The final concentration of cells used in this method was also approx. 50 mg wet wt. of cells/ml.

Incubation conditions for the study of cardiac-muscle cell lipoprotein lipase activity

In all the incubation experiments of this type, cells were suspended at concentrations of between 45 and 60 mg wet wt./ml of medium. The basal medium used was the Krebs–Ringer bicarbonate buffer used in the isolation of the cardiac-muscle cells (Powell & Twist, 1976). The buffer, at pH 7.4, had the following composition (mmol/litre): NaCl, 120.5; $NaHCO_3$, 13.1; KCl, 2.6; KH_2PO_4, 1.18; $MgSO_4$, 1.18; glucose, 11.1. To this basal medium was added an amino acid mixture such that the final concentration of all the amino acids in the medium was equal to twice their concentration in rat plasma (Miller & Biegelman, 1967; East *et al.*, 1973). The final approximate concentrations of individual amino acids in the medium were (mmol/litre): L-alanine, 0.8; L-arginine hydrochloride, 0.35; L-aspartic acid, 0.15; L-cysteine hydrochloride, 0.12; L-glycine, 0.6; L-glutamic acid, 0.1; L-histidine, 0.15; L-isoleucine, 0.26; L-lysine, 0.7; L-methionine, 0.09; L-phenylalanine, 0.15; L-proline, 0.65; L-serine, 1.1; L-threonine, 0.5; L-tryptophan, 0.14; L-tyrosine, 0.18; L-valine, 0.38; L-asparagine, 0.05; L-leucine, 0.3; L-glutamine, 0.75. Such an amino acid mixture was employed previously in fat-cell incubation systems (Cryer *et al.*, 1975). The cells were incubated in either the presence or absence of 1 unit of heparin/ml.

Measurement of lipoprotein lipase activity

Total lipoprotein lipase activities in both cells and media were determined as the rate of non-esterified fatty acid released from an apolipoprotein C_{II}-activated triacylglycerol emulsion (Intralipid; Vitrum, Stockholm, Sweden) at 30°C as described by Cryer & Jones (1978). Enzyme activity was determined directly in the media samples (separated by centrifugation). Cells were prepared for the assay either as fresh aqueous homogenates or as homogenates of acetone/diethyl ether-dried powders (Chohan & Cryer, 1979). The enzyme was characterized in all the preparations by the degree of inhibition observed when 0.6 M-NaCl was present in the assay (>85% in all cases) and by the obligatory requirement for plasma in the assay. All the assays contained heparin at a final concentration of 1 unit/ml. Activities have been expressed as μmol of non-esterified fatty acids released/h per g fresh wt. of cells or as units/10^6 viable cells. The activities are given as means ± S.D. and Student's *t* test (Fisher & Yates, 1957) was used to assess the significance of the differences between means.

Results

Viability of isolated heart cells during incubation

When isolated cardiac-muscle cells prepared from the hearts of fed rats were incubated at 25°C in Krebs–Ringer bicarbonate buffer containing glucose (see the Materials and Methods section), the viability of the cells remained high throughout. Thus 97% of the lactate dehydrogenase activity detectable in the system at zero time was intracellular and after 4h of incubation this activity had only decreased by 9%. Similarly, the proportion of cells that excluded Trypan Blue decreased by only 7% from 84 to 77% during the 4h of incubation. These data represent mean values obtained in six separate experiments in which viability was assessed hourly. The values in all cases had a standard deviation of less than 5%. Such studies were carried out as a routine in all the other incubations and the observed viabilities were within the limits shown in all cases in both the presence and absence of heparin.

After incubation for 4h at 25°C most of the cells still retained the rod-like morphology characteristic of cardiac-muscle cells.

As a metabolic criterion of their viability the O_2 uptake of the cells was determined at intervals and also continuously throughout the incubation period.

When cells (at between 30 and 60mg wt./ml) were incubated in the Krebs–Ringer bicarbonate buffer at 25°C either in the presence or in the absence of heparin the rate of O_2 uptake determined with the Clarke-type oxygen electrode system remained the same for each sampling time for up to 5h (Table 1). The measured rate of O_2 uptake observed was also linear when each sample was examined. When 10mM-lactate was added to the cell suspension after their basal O_2 uptake rate had been determined, there was a stimulation observed in the rate of O_2 uptake by the cells. Although the stimulation of O_2 uptake in the presence of lactate was significant ($P < 0.02$) at 0, 3, 4 and 5h, it was not at 1 and 2h when the group of experiments was considered. However, for individual suspension the stimulation was noticeable for each incubation at all the times of study. The effect of added lactate was immediate (within 15–30s of addition) as was the effect of 3mM-KCN, which inhibited O_2 uptake by the cells completely (results not shown).

When the O_2 uptake of cells was monitored by the Warburg technique it was found necessary to raise the temperature of incubation to 30°C to achieve adequate rates of equilibration between the gaseous and aqueous phases of the system. Under these conditions, as Fig. 1 shows, the rate of O_2 uptake of isolated heart-muscle cells in the basic Krebs–Ringer medium was linear up to 2.5h, after which an apparent acceleration in respiration was observed. This acceleration in O_2 uptake rate was not noticeable when 10mM-lactate was present in the medium. 10mM-Lactate caused a stimulation in O_2 uptake by the cells, which was significantly different ($P < 0.02$) from the basal rate at all times after 1h of incubation.

Stability of soluble cardiac lipoprotein lipase activity

To assess the potential loss of lipoprotein lipase activity that might occur during isolated cell incubations (when enzyme might be expected to appear in the incubation medium) preliminary studies on the stability of soluble enzyme in the proposed cell incubation medium were carried out. Soluble enzyme for this purpose was prepared from homogenates (30% w/v) made in the Krebs–Ringer bicarbonate buffer defined above. The homogenates were centrifuged at $100000g_{av.}$ for 1h and the supernatant was used as a source of soluble enzyme. Fig. 2 shows that when the soluble enzyme was incubated in the Krebs–Ringer buffer at 25°C in the presence of 1 unit of heparin/ml the decline in enzyme activity over 6h of incubation was only 10%. In the absence of heparin,

Table 1. *O_2 uptake of isolated cardiac-muscle cells after incubation at 25°C*

Immediately after preparation cardiac-muscle cells from the hearts of fed rats were incubated in the basal medium plus heparin as described in the Materials and Methods section. The cells from eight hearts were pooled and incubated at 25°C in 13ml of medium. The final cell concentration for the three independent experiments shown was between 30 and 60mg wet wt. of cells/ml. At the time intervals shown 1ml portions of the incubation cell suspensions were removed and the rate of O_2 uptake was measured using a Clarke-type oxygen electrode in both the absence and presence of 10mM-sodium lactate (see the Materials and Methods section). The O_2 uptake measurements were also done at 25°C. The values given are means ± S.D. For cells taken at 0, 1 and 5h of incubation the inclusion of 3mM-KCN completely inhibited O_2 uptake by the cells in both the absence and presence of lactate.

	O_2 uptake (μl of O_2/min per g fresh wt. of cells)					
Incubation (h) ...	0	1	2	3	4	5
Added substrate						
None	7.5 ± 4	5.4 ± 3	5.2 ± 3	7.3 ± 2	9.3 ± 1	7.6 ± 3
10mM-Lactate	13.5 ± 2	8.8 ± 2	8.0 ± 3	11.7 ± 1	14.0 ± 1	16.8 ± 3

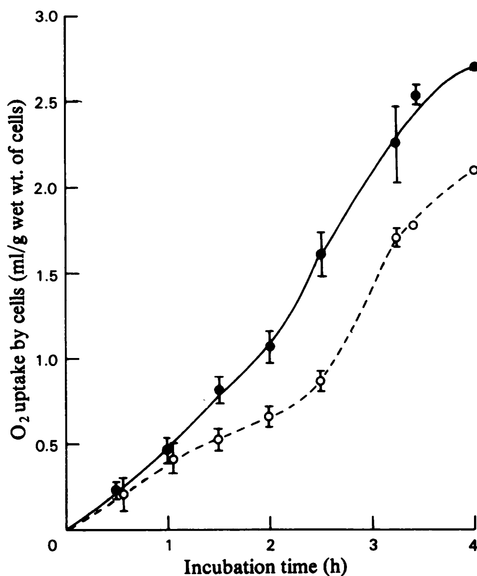

Fig. 1. *O_2 uptake of isolated cardiac-muscle cells monitored continuously during incubation at* 30°C
Cells were suspended in 3 ml of medium (see the Materials and Methods section) at a concentration of 56 mg wet wt. of cells/ml. The suspensions were incubated in a Warburg constant-volume manometric apparatus at 30°C with shaking as described in the Materials and Methods section. The uptake of O_2 was monitored and the respiration of the cells was followed in the presence (●) and absence (○) of 10 mM-sodium lactate. The lactate was added from the side arm of the incubation flask after a 10 min equilibration period in the shaking apparatus at 30°C. The values shown are means ± S.E.M. for three separate incubations.

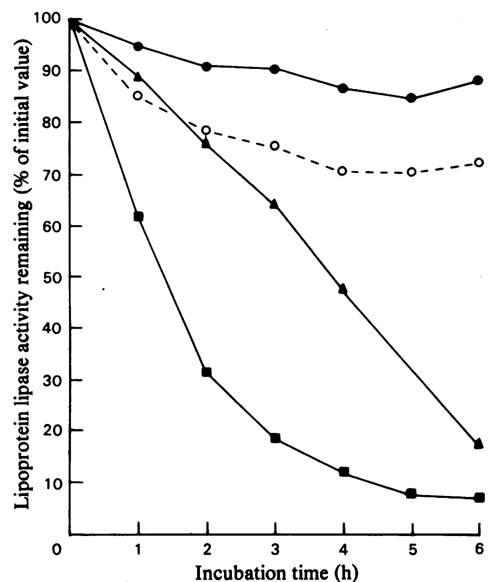

Fig. 2. *Effect of incubation temperature on the stability of soluble lipoprotein lipase activity*
Soluble enzyme activity was prepared from heart tissue homogenates by taking the supernatant after centrifugation at 100000 $g_{av.}$ for 1 h. The homogenate (30%, v/v) was prepared in Krebs/Ringer bicarbonate containing 11 mM-glucose at pH 7.4. The Figure shows the pattern of lipoprotein lipase activity decline during incubations at 25°C plus heparin (1 unit/ml) (●), at 25°C without heparin (○), at 30°C plus heparin (▲) and at 37°C plus heparin (■). At 30 and 37°C the omission of heparin had no effect on the pattern of activity decay.

however, the decline over the same period was 30%. These losses in enzyme activity were smaller than the extensive losses observed when the enzyme was incubated at either 30°C (82% at 6 h) or 37°C (95% at 6 h). At 30 and 37°C the overall loss of enzyme activity was unaffected by the presence of heparin and/or the inclusion of Intralipid (5% v/v; washed by flotation at a density of 1.006 g/ml) in the medium.

Incubation of isolated cardiac-muscle cells at 25°C

When isolated cardiac-muscle cells prepared from the hearts of fed rats were incubated at 25°C in the Krebs–Ringer bicarbonate buffer, pH 7.4, containing amino acids (see the Materials and Methods section) the activity of lipoprotein lipase determined in fresh homogenates of the cells was unaltered over 4 h of incubation (Fig. 3*b*). Under these conditions low amounts of enzyme activity appeared in the medium, but these did not lead to any overall change in the total lipoprotein lipase activity of the incubation system as a whole (cells + medium). The pattern of enzyme activity change was, however, significantly altered when similar cell incubations were carried out in the medium containing 1 unit of heparin/ml. Fig. 3(*a*) shows the data from a series of experiments where cells from fed animals were incubated at 25°C in the presence of heparin. In the presence of heparin there was a progressive increase in the activity of lipoprotein lipase in the incubation medium. The final activity in the medium after 4 h of cell incubation was 5-fold higher than the activity seen in the absence of heparin (Fig. 3*a* versus 3*b*). The activity of the enzyme in cells incubated in the presence of heparin showed a small progressive, but statistically insignificant, increase when compared with the activity of cells incubated without heparin. This increase, however, taken together with the

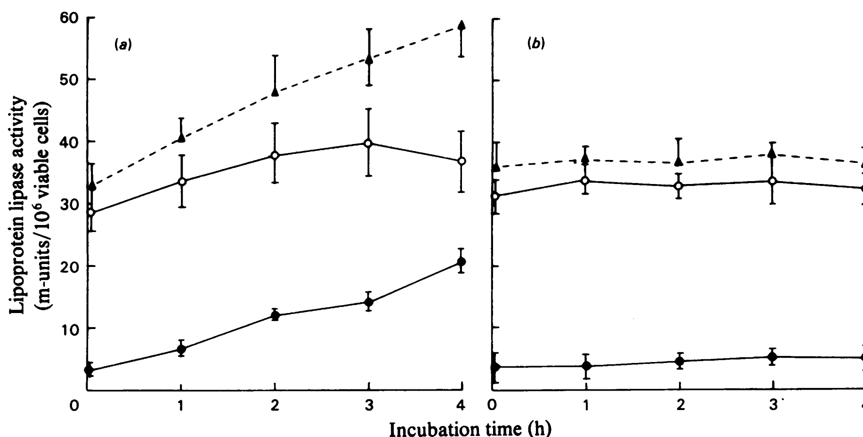

Fig. 3. *Changes in lipoprotein lipase activity when isolated cardiac-muscle cells were incubated either in the presence (a) or absence (b) of heparin at 25°C*

For each experiment the isolated cardiac-muscle cells prepared from the hearts of eight fed rats were incubated in 13 ml of Krebs–Ringer bicarbonate buffer containing 10 mM-glucose and amino acids at pH 7.4 (see the Materials and Methods section). The cell suspensions were incubated at 25°C either in the absence (*b*) or presence (*a*) of 1 unit of heparin/ml. For the measurement of lipoprotein lipase activity duplicate 1 ml samples of the cell suspension were removed at the times indicated. Duplicate assays at each time point were combined to give a mean. The points indicate means ± S.D. for nine independent experiments in the case of plus heparin (*a*) and six independent experiments in the case of incubations done in the absence of heparin (*b*). The activities determined separately in the medium (●) and cells (○) have been combined to show the net total enzyme activity in the system (▲) (medium + cells). At each of the time points small samples of the suspensions were taken for viability and dry-weight determinations together with medium lactate dehydrogenase assays. The lipoprotein lipase activity is given as nmol of non-esterfied fatty acid released/h per 10^6 viable cells (m-units/10^6 viable cells) (see the text).

activity increase in the medium, produced a net lipoprotein lipase activity increase in the system, which represented an 80% increase over the original activity during the 4 h of incubation, compared with a complete lack of overall increase in the system incubated in the absence of heparin. The total activities at 0 and 4 h respectively were 33 ± 4 and 57 ± 5 (9 experiments). The patterns of activity change noted were not altered with the enzyme activity was expressed either as units per g wet wt. of cells or per 10^6 total cells. Also when cell incubations were done at higher medium heparin concentrations (up to 5 units/ml) no detectable difference in the rate of enzyme appearance in the medium to that seen in the presence of 1 unit/ml was observed (results not shown).

In some of the experiments described above where cells were incubated with and without heparin at 25°C portions of the cells, taken at the intervals shown, were used for the preparation of acetone/diethyl ether-dried powders (Chohan & Cryer, 1978). In four experiments in the 'plus-heparin case' and two experiments in the 'minus-heparin case', direct comparisons of lipoprotein lipase activity determinations were made in both acetone/diethyl ether-dried powders of cells and aqueous homogenates of freshly removed cells. Although as reported previously (Chohan & Cryer, 1978, 1979) acetone/diethyl ether-dried powder preparations of heart cells demonstrated an approx. 3-fold higher lipoprotein lipase activity than fresh cell homogenates, incubation of the cells under the conditions described did not alter the magnitude of this difference at any of the incubation times.

Discussion

The cell-characterization studies reported here indicate that the cardiac-muscle cells isolated from adult rats had a high amount of viability when first prepared, which they retained during relatively long incubations. This maintenance of high viability over long periods appears to be a general characteristic of isolated cardiac-muscle cells from a variety of adult mammalian sources including, for example, rat (Berry *et al.*, 1970; Carlson *et al.*, 1978), rabbit (Dani *et al.*, 1977) and dog (Liu & Spitzer, 1978). This property, recognized by a number of workers (see above), indicates their potential usefulness in many metabolic studies, including those pursued here involving the study of lipoprotein lipase and the control of its activity in the heart. In addition to retaining their ability to exclude vital stain and main-

tain the intracellular location of cytoplasmic enzymes during incubation the cells also took up O_2 at rates compatible with previous studies (Berry *et al.*, 1970; Dani *et al.*, 1977) and with rates thought to maintain intracellular metabolite and energy balance via tight respiratory coupling (Powell & Twist, 1976; Dani *et al.*, 1977). In addition the rate of respiration in the cells prepared here was sensitive to added substrate, in common with other heart muscle-cell preparations, showing a high degree of metabolic intactness (see the references above).

With the availability of such cells our objective was to develop media and conditions under which the mechanisms of lipoprotein lipase activity change in the heart could be studied. Although media for such incubation studies have been described for intact adipose tissue (Salaman & Robinson, 1966; Wing & Robinson, 1968; Robinson & Wing, 1971; Cryer *et al.*, 1973; Ashby *et al.*, 1978) and isolated adipocytes (Stewart & Schotz, 1971, 1973, 1974; Cryer *et al.*, 1975; Spencer *et al.*, 1978), not only do these media have an ionic composition inappropriate to heart cells (Powell & Twist, 1976; Farmer *et al.*, 1977), but many also contain serum or other components with either variable compositions or ill-defined properties with regard to the system *in vitro* under consideration. It was our intention therefore to develop a medium that was of a minimum complexity compatible with a successful outcome. Thus the basic medium chosen was the Krebs–Ringer buffer containing glucose at pH 7.4 used in the isolation of the cells. In this medium at 25°C the cells retained viability and lipoprotein lipase in the soluble state showed an acceptable degree of stability. To this basal medium the only other addition was an amino acid mixture of known composition and physiologically relevant concentration (see the Materials and Methods section). In this medium the cells maintained their intracellular lipoprotein lipase activity, but it was only on the addition of heparin to the medium that significant changes in lipoprotein lipase activity in the system occurred. The extraction of lipoprotein lipase into the incubation medium when adipose tissue (Salaman & Robinson, 1966; Cryer *et al.*, 1973) and adipocytes (Cryer *et al.*, 1975; Kornhauser & Vaughan, 1975) are incubated has been noted previously and the magnitude of the measured release was reported to be most marked at 25°C. This is probably because the enzyme is relatively stable at this temperature (Fig. 2; Cryer *et al.*, 1973). Incubation of cultured heart cells from neonatal animals with heparin has also been shown to cause a release of cellular lipoprotein lipase into the medium (Chajek *et al.* 1978*a*). In the case of cultured cells, however, although the total activity of enzyme in the system increased, the cellular activity declined significantly. The heart cells from mature rat hearts studied here, therefore, appear to represent a potentially useful tool in the further study of the modulation of lipoprotein lipase activity and will complement studies with the perfused organ or cultured neonatal cells. It is noteworthy that the heparin-induced release of lipoprotein lipase from cultured neonatal heart cells exhibited many of the characteristics of that observed with the perfused heart (Chajek *et al.*, 1975, 1978*a*). Thus in such cells the highest rate of release occurred shortly after the introduction of heparin and proceeded much more slowly thereafter. In the adult rat cardiac-muscle cells the changes are progressive and appear to represent the operation of a system at steady state and where the enzyme source is intracellular (Chohan & Cryer, 1979).

In this context it has been suggested that heparin binds to cardiac cells in culture and that this bound glycosaminoglycan interferes with the attachment of newly secreted lipoprotein lipase molecules and thus enhances their appearance in the medium (Chajek *et al.*, 1978*a*). A slightly different interpretation of events may, however, be possible. Thus heparin is known to bind lipoprotein lipase with a high affinity (Olivecrona & Bengtsson, 1978) and prior cellular binding of the heparin might merely enhance the ability of the cells to retain the enzyme at their surfaces. Alternatively the heparin may act to release the enzyme from the cell surface by competing for the binding site of a membrane-associated polyanion capable of interacting with lipoprotein lipase in a manner similar to that postulated to occur at the endothelium (Olivecrona *et al.*, 1977).

In the present study the mass of enzyme protein in the system was not assessed and this remains to be considered before possible enzyme activation can be discerned.

In conclusion the system described also has the advantage of using fully differentiated cells, at a stage when their capacity to proliferate has become limited (Klinge & Stocker, 1968; Zak, 1974). This is in contrast with the cell culture systems that have used both myogenic and mesenchymal cells from neonatal rat hearts in studies of lipoprotein lipase activity control (Chajek *et al.*, 1977, 1978*a*,*b*). Such cells from immature animals are not fully differentiated, and recent evidence suggests that not all myogenic cells, for example, will give rise to fully differentiated muscle cells either *in vitro* (Chajek *et al.*, 1978*a*) or *in vivo* (Arguello *et al.*, 1978). Similarly, heart-derived mesenchymal cells have features common to fibroblasts, smooth-muscle cells and endothelial cells *in vitro* (Chajek *et al.*, 1978*a*) and are the major cell types present in the non-contractile endocardial cushions of the heart (Arguello *et al.*, 1978). Thus although in adult heart less than 40% of the cardiac mass consists of fully differentiated muscle cells (Morkin & Ashford, 1968; Grove *et al.*, 1969), our previous demonstration of the predomin-

ant localization of heart-cell-associated lipoprotein lipase with muscle cells and its absence from non-muscle cells (Chohan & Cryer, 1978) together with the demonstrated ability of the cells to give rise to extracellular enzyme without depletion of intracellular activity indicate the potential role of the cardiac-muscle cell in the provision of functional extracellular (endothelial-bound) enzyme in the adult heart. Prospectively, aspects of the operation of the lipoprotein lipase system of the heart and its control may now, by the use of cardiac-muscle cell incubations, become experimentally accessible.

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