LOCALIZATION OF CALCIUM IN THE SMOOTH ENDOPLASMIC RETICULUM OF RAT ISOLATED FAT CELLS

C. N. HALES, J. P. LUZIO
Department of Medical Biochemistry, The Welsh National School of Medicine, Heath Park, Cardiff CF4 4XN, Wales

J. A. CHANDLER
Tenovus Institute for Cancer Research, Heath Park, Cardiff, CF4 4XX, Wales

AND L. HERMAN
Department of Pathology, State University of New York, Downstate Medical Center, Brooklyn, New York 11203, U.S.A.

SUMMARY

The cytoplasm of the rat isolated fat cell contains a highly organized, interconnected system of smooth endoplasmic reticulum having close association with the central lipid mass, mitochondria and cytoplasmic lipid droplets. Elements of smooth endoplasmic reticulum approach, but do not fuse with, the plasma membrane.

When fat cells were treated with potassium pyroantimonate during fixation for electron microscopy a precipitate was produced inside the smooth endoplasmic reticulum. Analysis with an AEI-EMMA 4 analytical electron microscope showed that the precipitate contained calcium but not sodium, magnesium or manganese.

It is possible that the smooth endoplasmic reticulum of fat cells may be structurally and functionally analogous to the sarcoplasmic reticulum in skeletal muscle, and that redistribution of calcium from a calcium store inside the smooth endoplasmic reticulum may be a consequence of the action of lipolytic hormones.

INTRODUCTION

Rat isolated white fat cells have been shown to respond to a wide variety of hormones (Rodbell, 1970). It is generally accepted that the effects of lipolytic hormones on such cells are mediated via a rise in intracellular cyclic AMP concentration, and that anti-lipolytic hormones prevent such a rise (Robison, Butcher & Sutherland, 1971). As a result of experiments on the effects on lipolysis of local anaesthetics (Hales, 1970; Hales & Perry, 1970; Siddle & Hales, 1972), and glucocorticoids (Werner, Alm & Low, 1972; Exton et al. 1972), and on EGTA depletion of fat cell calcium (Alm, Efendic & Low, 1970; Efendic, Alm & Low, 1970), it has been suggested that hormonal control of lipolysis may be affected by alteration of the intracellular Ca\(^{2+}\) concentration in addition to alteration of cyclic AMP levels (Rasmussen, 1970; Siddle & Hales, 1972). Insulin activation of rat fat cell pyruvate dehydrogenase may also depend on an alteration of intracellular Ca\(^{2+}\) distribution (Martin, Denton, Pask & Randle, 1972). In a number of other systems Ca\(^{2+}\) has been proposed as a hormone 'second messenger', additional to cyclic AMP (Rasmussen, 1970; Rasmussen, Goodman & Tenenhouse, 1972).
The cytosol concentration of Ca\(^{2+}\) in most mammalian cells appears to be low, with Ca\(^{2+}\) apparently being stored elsewhere in the cell at a much higher concentration (Rasmussen et al. 1972). Hormones may alter cytosol Ca\(^{2+}\) concentration, either by affecting the Ca\(^{2+}\) permeability of the plasma membrane or by regulating its uptake and release from an intracellular store (Rasmussen, 1970; Rasmussen et al. 1972).

The current study was undertaken to investigate the possibility of hormonally induced changes in the ultrastructure of rat isolated fat cells and to look for sites of intracellular ion localization. Since it was initiated, several publications describing the results of ultrastructural studies of rat isolated fat cells have appeared (Pictet et al. 1968; Schotz et al. 1969; Cushman, 1970; Slavin, 1972). It is the purpose of this paper to extend these findings and to report an investigation of the cation content of the smooth endoplasmic reticulum. We present the hypothesis that part of the smooth endoplasmic reticulum of fat cells may be structurally and functionally analogous to the sarcoplasmic reticulum in skeletal muscle.

MATERIALS AND METHODS

Male Wistar rats (120-140 g), fed on a stock laboratory diet, were used without previous starvation. Rats were killed by decapitation, and fat cells prepared from the epididymal fat pads by the method of Rodbell (1964). Isolated cells were washed and suspended in Krebs-Ringer bicarbonate buffer (1.3 \(\times\) \(10^{-3}\) M Ca\(^{2+}\), pH 7.4 (Cohen, 1957), containing 4% bovine serum albumin. On some occasions, prior to fixation, cells were incubated in this buffer for 1 h at 37 °C in the presence of various test substances. All glassware with which fat cells came into contact was treated with Siliclad (Clay Adams, Parsippany, N.J., U.S.A.) before use.

The fat cell suspension was centrifuged (300 g, 30 s) and the infranatant removed. For morphological investigation cells were fixed for 30 min at room temperature in 0.1 M cacodylate buffer, pH 7.4 containing 2.5% glutaraldehyde (Sabatini, Bensch & Barnnett, 1963), (Polysciences Inc., Rydall, Pennsylvania), and washed 3 times with cacodylate buffer containing 0.2 M sucrose. The fixed cells were post-osmicated for 30 min in 0.14 M veronal-acetate buffer, pH 7.4 containing 1% OsO\(_4\) (Palade, 1952), followed by suspension in veronal-acetate buffer containing 5% uranyl acetate. The cells were then dehydrated in ascending concentrations of ethanol, washed repeatedly in propylene oxide until no further black material was leached out and embedded in Epon. Thin sections were cut on an LKB Ultrotome III, mounted on uncoated copper grids, stained with a saturated solution of uranyl acetate in 50% ethanol (prepared in the dark), washed with water, stained with tartrate-stabilized lead hydroxide (Millonig, 1961), and again washed with water. Stained sections were examined and photographed with an AEI EM801 electron microscope, using the tilt stage where necessary.

For antimonate localization of cations in fat cells, the method of fixation and staining was modified from that described above. The method of antimonate precipitation used was developed by 2 of the current authors in a study of cation localization in pancreatic islets of Langerhans (Herman, Sato & Hales, 1973), by modification of the technique of Spicer et al. (1968). Although the pyroantimonate precipitation technique was originally devised for sodium localization, sodium is rapidly lost from fat cells in normal medium (Perry & Hales, 1969), the presence of phosphate ions may interfere with the formation of sodium pyroantimonate complexes (Torack & Lavalle, 1970), and pyroantimonate has been shown to precipitate other cations, this lack of specificity making subsequent elemental analysis essential (Herman et al. 1973). Cells were fixed for 30 min at room temperature in 2.5% glutaraldehyde buffered to pH 7.4 with 0.1 M potassium phosphate, and washed 3 times with potassium phosphate buffer containing 10% sucrose. The fixed cells were left overnight in the final buffer wash and then post-osmicated for 30 min in 1% osmium tetroxide-2% potassium pyroantimonate (BDH). The cells were subsequently washed 3 times in 0.14 M veronal-acetate buffer, pH 7.4 and incubated for 2 h in veronal-acetate buffer containing 5% uranyl acetate. The fixed, stained cells were dehydrated, embedded and sectioned as described above. Thin sections were mounted on carbon-coated
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copper grids, examined and photographed in an AEI EM801 electron microscope, and also examined in an AEI-EMMA 4 analytical electron microscope to enable elemental analysis of antimonate deposits. Details of the theory and operation of the analytical electron microscope have been discussed by Marshall & Hall (1966), Cooke & Duncumb (1969), Chandler (1971, 1973), and Weavers (1973).

Examinations of the precipitates were made with EMMA for calcium, manganese, sodium and magnesium. Positive readings were found only for calcium and the conditions of operation chosen were: 100 kV, probe diameter 0.1 μm, probe current 0.01-0.03 μA. Counting time was 100 s at each point of analysis. The crystal spectrometers were used to detect calcium since the energy dispersive analyser was unable to resolve the peaks of calcium and antimony (Ca Kα = 3.691 keV, Sb Kα = 3.624 keV). The pentaerythritol (PET) crystals were chosen with one spectrometer being set permanently on peak position and the other on background. A given number of precipitates at a given number of different cell foci were measured and the mean values presented to demonstrate relative calcium distribution (see Table 1 with reference to Fig. 1, p. 4). The precipitates varied in size at each place but for quantitative comparison the same probe area was used at each place. The shape of the probe was varied from circular to elliptical without changing the probe current, simply by adjustment of the microscope stigmator. In addition, analyses were made on sections that had not been fixed with the antimony salt solution.

RESULTS

Incubation of the isolated fat cells with various hormones (adrenalin, 10 μg/ml; ACTH, 10 μg/ml; insulin, 50 μU/ml), and other substances (dibutyryl cyclic AMP, 5 mM; theophylline, 1 mM; propranolol, 10 μg/ml), prior to fixation produced no apparent overall changes in the morphology of the cells. Photographs of fat cell sections shown in the current paper were chosen for clarity of the image, irrespective of the pre-fixation treatment of the cells.

The overall morphology of the rat isolated white fat cell was observed to be in accordance with previously published work (Rodbell, 1967; Schotz et al. 1969; Cushman, 1970; Slavin, 1972). The extensive membrane system with the appearance of smooth-surfaced endoplasmic reticulum (ER) and its interesting relationships to other subcellular organelles prompted further electron-microscopic investigation.

Smooth endoplasmic reticulum and the central lipid droplet

The central lipid droplet was observed to be surrounded by a smooth membrane system previously described as saccules of smooth endoplasmic reticulum (Pictet et al. 1968), as a fenestrated double membrane (Schotz et al. 1969), or as a fenestrated envelope (Cushman, 1970). This smooth membrane system appeared to be part of a highly organized, interconnected system of smooth ER found throughout the cytoplasm and having close association with mitochondria and cytoplasmic lipid droplets as well as with the central lipid mass (Figs. 2, 4). The smooth ER surrounding the central lipid mass could often be followed round the entire portion of the cell present in the section being examined, but was apparently not immediately applied to the lipid.

The region of the interface between the cytoplasm and the central lipid mass is clearly one of great functional significance since it is probably the site at which lipid deposition and mobilization take place. Careful ultrastructural study of this region revealed 2 different structures, an electron-opaque line (Figs. 2-4), or thin filaments entering the lipid droplet as previously described by Schotz et al. (1969). The relationship between these alternative structures was not resolved.
Smooth endoplasmic reticulum and mitochondria

A feature of the smooth ER system was its close association with mitochondria. Smooth ER was frequently observed not only to pass close to mitochondria, but also to encircle them (Figs. 2, 4).

Smooth endoplasmic reticulum and the plasma membrane

In many sections smooth ER was seen to approach the plasma membrane. A close relationship of the 2 membranes was observed, both at sites of invagination of the plasma membrane (Fig. 4), and when no such invagination occurred (Figs. 2, 3). Whenever smooth ER appeared to fuse with the plasma membrane the section was further examined using the tilt stage. With this technique it was found that, in every case examined, the smooth ER and plasma membranes were discrete and did not fuse (Fig. 3).

Fig. 1. Schematic representation of regions (A–I) of fat cell analysed with EMMA.

Intracellular cation localization

After fixation of fat cells in glutaraldehyde and postfixation in osmium tetroxide/pyroantimonate a dense precipitate was observed in thin sections (Figs. 5–7). This precipitate appeared to be mainly localized adjacent to the central lipid droplet (Fig. 5). Mitochondria and nuclei were usually free of precipitate. In a few sections precipitate was associated with the plasma membrane (Fig. 6). Examination of the precipitate at higher magnification revealed that some of the precipitation particles were deposited in the smooth ER system (Fig. 7). In some sections lines of dense precipitate ran into the cytoplasm away from the central lipid droplet (Fig. 6), the pattern being very similar to that observed for smooth ER (Fig. 2). Fine precipitate, randomly scattered and not associated with any organelle, was observed in the
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ground substance of the cytoplasm (Figs. 6, 7). The significance of this precipitation is uncertain since it varied considerably from one preparation to another. No correlation of this deposition was noted with any ultrastructural feature.

Fig. 1 represents schematically the regions of the fat cell analysed with EMMA. Calcium was the only cation found to give positive readings using EMMA; magnesium, manganese and sodium were undetectable. A limitation of the analysis was that precipitates varied in size and were always smaller than the probe diameter (0.1 \( \mu \)m). Nevertheless the results indicate a specific distribution of calcium localization as shown in Table 1. The values represent calcium X-ray counts averaged over a number of areas of the same type in a counting period of 100 s. No attempt was made to assess mass fractions of calcium in the tissue sections because of the presence of heavy metal stains. Within each section X-ray counts are directly proportional to local calcium mass values, and assuming constant section mass thickness, represent relative calcium concentration values.

Table 1. Calcium X-ray counts for pyroantimonate-treated rat fat cells

<table>
<thead>
<tr>
<th>Area of fat cell analysed with EMMA</th>
<th>X-ray counts in 100 s, mean ± s.e.m.</th>
<th>n</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Dense precipitate at the interface of cytoplasm with central lipid droplet</td>
<td>286 ± 49</td>
<td>18</td>
<td>0.01</td>
</tr>
<tr>
<td>B. Dense precipitate within smooth ER adjacent to the central lipid droplet</td>
<td>246 ± 39</td>
<td>7</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>C. Precipitate associated with central lipid droplet</td>
<td>210 ± 36</td>
<td>9</td>
<td>0.01</td>
</tr>
<tr>
<td>D. Cytoplasmic matrix</td>
<td>123 ± 38</td>
<td>7</td>
<td>0.05</td>
</tr>
<tr>
<td>E. Plasma membrane</td>
<td>80 ± 19</td>
<td>8</td>
<td>0.05</td>
</tr>
<tr>
<td>F. Mitochondria</td>
<td>165 ± 76</td>
<td>4</td>
<td>0.10</td>
</tr>
<tr>
<td>G. Interface of cytoplasm and central lipid droplet lacking precipitate</td>
<td>137 ± 32</td>
<td>7</td>
<td>0.01</td>
</tr>
<tr>
<td>H. Central lipid droplet lacking precipitate</td>
<td>18 ± 22</td>
<td>6</td>
<td>0.01</td>
</tr>
<tr>
<td>I. Precipitate external to plasma membrane</td>
<td>326 ± 97</td>
<td>5</td>
<td>0.01</td>
</tr>
</tbody>
</table>

X-ray counts normalized to 0.01 \( \mu \)A beam current. n, number of observations at each cell area. P, significance of the difference from the X-ray counts in area H estimated by Student’s t test.

DISCUSSION

Cushman (1970) commented on the extensive smooth-surfaced endoplasmic reticulum of the rat isolated fat cell. The current study shows the ultrastructural inter-relationship of this system with the central lipid droplet, mitochondria and the plasma membrane. It is difficult to believe that the striking morphology of this highly developed system does not have important implications for the function of the tissue.

A similarly highly organized system of smooth membranes is observed in skeletal muscle (the sarcoplasmic reticulum), and in terms of regulation adipose tissue may be considered to pose similar anatomical problems to skeletal muscle. In both tissues it is believed that the effect of substances acting at the plasma membrane is transmitted to a centrally situated mass with a clearly defined and regular geometry in relation to the
plasma membrane. In skeletal muscle, this mass is the contractile protein activated by events at the neuromuscular junction. Activation of contraction has been shown to be mediated via release of Ca²⁺ from the sarcoplasmic reticulum (Ebashi, Endo & Ohtsuki, 1969). Localization of calcium has been demonstrated in the sarcoplasmic reticulum of skeletal muscle using oxalate precipitation (Constantin, Frazini-Armstrong & Podolsky, 1965), and also by the technique of pyroantimonate precipitation in conjunction with X-ray micro-analysis (Yarom & Chandler, 1972).

In adipose tissue, hydrolysis of triglyceride is activated by a number of hormones, most of which are currently believed to have their primary action at the plasma membrane (Rodbell, 1972). Subsequent to binding to the plasma membrane, the hormones stimulate intracellular production of cyclic AMP. This in turn may activate lipolysis by mechanisms analogous to those mediating the activation of glycogenolysis (Hales, Chalmers, Perry & Wade, 1968; Wade, Chalmers & Hales, 1970; Huttunen, Steinberg & Mayer, 1970; Huttunen & Steinberg, 1971). However, results indicating the importance of ions in the regulation of lipolysis have also suggested that a rise in intracellular cyclic AMP concentration is not in itself enough to activate lipolysis (Hales et al. 1968; Hales & Perry, 1970; Siddle & Hales, 1972). As discussed in the Introduction, there is considerable evidence to suggest that alteration of cytosol calcium concentration may be required for control of lipolysis, and the possibility that an intracellular store of calcium may be mobilized during hormonal stimulation of lipolysis has been suggested (Rasmussen, 1970; Efendic et al. 1970). In experiments with rat isolated fat cells it has not been possible to show that extracellular calcium is essential for catecholamine-stimulation of lipolysis. Removal of calcium from the extra-cellular medium has been shown to diminish adrenalin-stimulated lipolysis only at low hormone concentrations (Mosinger & Vaughan, 1967). More recently Efendic et al. (1970) found that preincubation of human adipose tissue pieces in calcium-free medium in the presence of the calcium chelator EGTA increased the inhibition of noradrenalin-stimulated lipolysis produced simply by addition of EGTA to the incubation medium. Moreover, although theophylline- and dibutyryl cyclic AMP-stimulated lipolysis were not inhibited by the presence of EGTA in the incubation medium, preincubation with EGTA resulted in inhibition. These authors suggested that preincubation of adipose tissue pieces with EGTA depleted endogenous stores of calcium which could be mobilized in the stimulation of lipolysis. In searching for a structural basis for such an intracellular calcium pool we have used the pyroantimonate precipitation technique previously employed to localize calcium in the pancreatic β cell (Herman et al. 1973), and we have shown calcium localization in the smooth ER of the rat isolated fat cell.

It may appear surprising that using the pyroantimonate X-ray microanalysis technique, calcium was not found to be associated with other intracellular structures, for instance mitochondria, which in most tissues have the ability to absorb and store calcium. In addition, calcium localization within the smooth ER system was localized mainly to that ER close to the central lipid droplet. Several explanations might be offered for these apparent anomalies. The technique is likely to be dependent both on whether calcium is complexed or free within cellular structures, and on the methods
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used for fixation, staining and dehydration. Similarly, it is possible that other cations present in the cell do not precipitate with pyroantimonate. It has been suggested that the presence of phosphate ions (as used in the current investigation), might interfere with the formation of sodium pyroantimonate complexes (Torack & Lavalle, 1970; Tisher, Weavers & Cirksena, 1972), and also that electron microscopy fixatives may affect cellular accumulation of cations (Krames & Page, 1968; Yarom, Ben-Ishay & Zinder, 1972). Perry & Hales (1969) have reported that the half time of efflux of intracellular ⁸⁶Na⁺ from rat isolated fat cells into normal medium was 2.8 min. Despite these problems it seems clear that at least part of the smooth ER system of the rat fat cell contains calcium as a predominant cation.

Microsomal fractions prepared from muscle, liver, kidney and brain have been shown to be capable of ATP-dependent calcium uptake (Yoshida, Kadota & Fujisawa, 1966; Ohtsuki, 1969; Diamond & Goldberg, 1971). If fat cell smooth ER is similarly capable of calcium transport and storage, it is possible that, as in muscle (Ebashi et al. 1969), the close proximity of elements of the smooth ER system to the plasma membrane may allow activation of smooth ER calcium uptake or release as a response to events at the cell surface. Such an activation may be effected by cyclic AMP. In muscle theophylline can stimulate Ca²⁺ release by the sarcoplasmic reticulum (Weber & Herz, 1968).

Thus, there may be a structural basis, not only for a mobilizable calcium store within the fat cell, but also for a mechanism of stimulating mobilization as a result of events at the cell surface. The existence of such a mechanism would be of obvious importance if alteration of cytosol Ca²⁺ concentration is indeed necessary for the mediation of hormone action on fat cells. The observed close association of smooth ER with mitochondria agrees with earlier observations of a close topographical relationship between mitochondrial membranes and other cytoplasmic membranes both in adipose tissue and other tissues (Sheldon et al. 1962; Bernhard & Rouiller, 1956; Sheldon, 1956). This relationship may also be important in control of cytosol Ca²⁺ concentration, since in muscle there is considerable evidence that mitochondria play an important role in its control (Rasmussen et al. 1972; Carafoli & Azzi, 1972).

The initial part of this work was carried out in the Department of Biochemistry, University of Cambridge. We are grateful to Professor Sir Frank Young for the facilities and encouragement he provided at that time. Professor D. H. Northcote and Dr F. B. P. Wooding provided much helpful guidance and discussion. We are also grateful to Margaret Hodgkins, Diana Jones, Gillian Rowe and Gwyneth Parry for their skilled technical assistance and to Len Jewitt and Ralph Marshall for the photography. The work was supported by grants from the British Diabetic Association, Science Research Council, the Wellcome Research Foundation, and by the Tenovus organization. J.P.L. held a studentship for training in research methods from the Medical Research Council and L.H. was supported by USPHS Research Grant CA-06081, USPHS Special Fellowship AM 39072 and SUNY Research Foundation Grant-in-Aid 71062. Part of this work is to be submitted for a Ph.D. degree in the University of Wales by J.A.C.
REFERENCES


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Fig. 2. Electron micrograph of a portion of isolated rat fat cell cytoplasm showing the smooth endoplasmic reticulum system (er). I, central lipid droplet; m, mitochondrion; pm, plasma membrane. The elements of the smooth endoplasmic reticulum system close to the central lipid droplet are apparently continuous (arrows) with those elements running throughout the cytoplasm, close to the mitochondria and approaching the plasma membrane (double arrow). × 45,000.

Fig. 3A-C. Portion of isolated rat fat cell cytoplasm showing an element of smooth endoplasmic reticulum (er) closely approaching the plasma membrane (pm). l, central lipid droplet. The 3 micrographs represent different tilt stage angles: A, untilted; B, 20° right; and C, 20° left. The use of the tilt stage shows that this element of smooth endoplasmic reticulum does not fuse with the plasma membrane. × 90,000.
Fig. 4. Elements of smooth endoplasmic reticulum (er) surround a mitochondrion (m) in a portion of isolated rat fat cell cytoplasm. l, central lipid droplet; pm, plasma membrane. × 100 000.
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Fig. 5. Electron micrographs of isolated rat fat cells after glutaraldehyde-osmium-pyroantimonate fixation. /, central lipid droplet; m, mitochondrion; pm, plasma membrane. There is a linear array of irregularly placed, dense precipitate adjacent to the central lipid droplet of each cell. × 30,000.

Fig. 6. Portion of isolated rat fat cell cytoplasm after glutaraldehyde-osmium-pyroantimonate fixation. /, central lipid droplet; pm, plasma membrane. There is dense precipitate along the lipid droplet which appears to extend into the cytoplasm (arrows), as well as precipitate in association with the plasma membrane. × 120,000.

Fig. 7. Portion of isolated rat fat cell cytoplasm after glutaraldehyde-osmium-pyroantimonate fixation. /, central lipid droplet; pm, plasma membrane. The dense precipitate is bound by membrane (arrows) of smooth endoplasmic reticulum. × 120,000.