Monolayer cell culture of freshly isolated adipocytes using extracellular basement membrane components

Stacy A. Hazen, William A. Rowe,* and Christopher J. Lynch[1]

Departments of Cellular and Molecular Physiology and Medicine,* The Milton S. Hershey Medical Center and College of Medicine, The Pennsylvania State University, Hershey, PA 17033

Abstract Cell biological techniques requiring cells attached to surfaces, such as monolayer cell culture, microspectrofluorometry, and confocal microscopy, have not been readily available for use on adipocytes because they float and tend to lyse when attached to charged non-biological surfaces. A new method for attaching freshly isolated rodent adipocytes to thermanox plastic surfaces using Matrigel (a defined mixture of extracellular matrix components that resembles the basal lamina surrounding adipocytes in vivo) is described. The method takes advantage of an unusual physical characteristic of Matrigel, i.e., that it is a liquid at cold temperatures and a hydrated gel at higher temperatures. To attach the isolated cells, chilled thermanox plastic coverslips were coated with a thin uniform layer of ice-cold Matrigel and inverted into warm floating adipocytes. Adipocytes floated up against the liquid Matrigel and became immediately attached when the Matrigel changed to a gel in response to the warmth of the cells and media. Cell volume measurements of the attached versus freshly isolated cells indicate no significant difference in the centroid cell volume of the attached cells. This indicates that the method does not select for small or large cells. Adipocytes maintained for 6 days in culture did not display any change in their size or differentiated microscopic appearance. The relative concentrations of major proteins in silver-stained SDS-PAGE gels and several differentiation state-dependent proteins, including ATP-citrate lyase, carbonic anhydrase III (CA III), adipocyte lipid binding protein (ALBP), and pyruvate carboxylase, were examined. No significant change was observed in the relative concentrations of these proteins when the Matrigel-cultured adipocytes were compared to freshly isolated cells. In addition, Matrigel-cultured adipocytes retained their ability to respond to insulin, as shown by the positive effects of insulin on ATP-citrate lyase concentrations and the negative effects of insulin on ALBP and CA III concentrations. The results of these experiments indicate that adipocytes on Matrigel may maintain many of their differentiated characteristics. Attachment of adipocytes to Matrigel-coated coverslips also allowed spectrofluorometric measurements to be made using methods normally used for attached cells. Intracellular pH was measured in a spectrofluorometer equipped with a perfusion chamber using the fluorescent pH probe, BCECF. BCECF-loaded adipocytes displayed brief alkalinization in response to NH_4Cl exposure and rebound acidification upon its withdrawal, indicating that the Matrigel does not interfere with dye loading or intracellular pH regulation. This method should be useful for any number of long-term studies on isolated adipocytes or cell biological techniques that require a surface attached cell. —**Hazen, S. A., W. A. Rowe, and C. J. Lynch.** Monolayer cell culture of freshly isolated adipocytes using extracellular basement membrane components. *J. Lipid Res.* 1995. **36:** 868–875.

Supplementary key words adipose tissue • Matrigel-cultured adipocytes • intracellular pH regulation • spectrofluorometry

Freshly isolated adipocytes are a widely used and important tool for studying fat cell physiology and metabolic disorders that affect adipose tissue such as diabetes mellitus and obesity. These cells are relatively easy to isolate because their most prominent feature, a lipid droplet that comprises 80–90% of their intracellular volume, causes them to float in physiological solutions in which many other cells sink. Unfortunately, this same feature and the tendency of physical forces to easily disrupt adipocytes (especially those from obese animals) can confound the application of routine cell biological techniques. For example, studies on gene regulation and protein expression require long-term incubations that necessitate primary culture of the cells. An obvious technical problem is that seeding of monolayer cultures normally requires cells to sink to the bottom of the cell culture dish in media in which adipocytes float. Although primary cultures of adipocytes can be accomplished with the cells floating, this procedure has been reported to lead to a rapid dedifferentiation of the cells (1).

Abbreviations: CA III, carbonic anhydrase III; ALBP, adipocyte lipid binding protein; PBS, phosphate-buffered saline; KRH, Krebs-Ringer HEPES buffer; BSA, bovine serum albumin; DMEM, Dulbecco's modified Eagle's medium; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; BCECF, 2',7'-bis-(2-carboxyethyl)-5-(and 6)-carboxyfluorescein; BCECF-AM, acetoxymethyl ester of BCECF.

[1]To whom correspondence should be addressed.

Other shorter term cell biological methods are also problematic when applied to adipocytes, e.g., spectrofluorometric studies for intracellular ion measurements. While some studies have been accomplished with adipocytes stirred in a cuvette (2), magnetic cell stirrers are generally designed for cells that sink and do not rupture easily. In our experience, especially with cells from obese animals, stirrer speeds required to draw the adipocytes down into the spectrofluorometer's light path tend to rupture many cells. To get around this problem, Barnacle glue has been used to attach adipocytes to coverslips that fit into spectrofluorometer cuvettes (3). In our hands, however, the attachment efficiency of this method is poor. In addition, the effects of glue on membrane proteins and membrane functions have not been characterized. Another approach has been to use adipocyte cell lines such as the 3T3-L1 and 3T3-F442A lines that perform well in cell biological studies. However, there are many differences between these cell lines and freshly isolated adipocytes. While these cells have been very useful for studying adipocyte differentiation, they are not thought to be a suitable model for studying obesity (4).

In this communication we describe a method for attaching isolated adipocytes to surfaces. The method partially embeds the cells in an extracellular matrix that may be more physiological and may provide some protection against shearing forces. This method should also facilitate studies of adipocyte cytoskeletal-extracellular matrix interactions and allow the application to freshly isolated adipocytes of cell biological methods that have been technically difficult to perform in the past.

MATERIALS AND METHODS

Adipocyte isolation

Sprague-Dawley rats were purchased from Charles River, housed in the animal facility at the Pennsylvania State University College of Medicine, and fed ad libitium. Intact adipocytes were prepared from epididymal fat pads of anesthetized rats using a method described previously (5). Briefly, the fat pads were removed and rinsed in phosphate-buffered saline (PBS) then in Krebs-Ringer HEPES buffer (KRH) containing 118 mM NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4 \cdot [7H_2O]$, 2.5 mM $CaCl_2$, 1.2 mM KH_2PO_4, 25 mM HEPES, 1 mM EDTA, 2 mM glucose, 200 nM adenosine, and 2% bovine serum albumin Fraction V (BSA). Typically, 2–4% BSA is used during isolation procedures to adsorb fatty acids released from the cells and prevent the lysis of freshly prepared adipocytes. Following collagenase Type 1 (Worthington Biochemical, Freehold, NJ) digestion, the isolated adipocytes were passed through nylon mesh with a 250 micron pore diameter and resuspended in KRH (pH 7.4). The cell suspension was washed twice by centrifugation for 30 sec at 200 *g* in KRH. Each time the infranatant was discarded and the adipocytes were resuspended.

Adipocyte attachment to Matrigel-coated coverslips

Prior to coverslip attachment, the adipocytes were maintained in polypropylene conical tubes containing a physiological buffer (KRH) at 37°C. The thermanox coverslips (13.7 × 22 mm) and pipettes used to apply the Matrigel (Collaborative Research, Bedford, MA) were chilled on ice. Matrigel was thawed at 4°C as a liquid and applied to one surface of the coverslips in a uniform manner. A small drop of Matrigel was placed on the coverslip and then smeared across its length to obtain a thin coating of the protein mixture. Next, each coverslip was inverted and introduced horizontally into the cell layer. The relative density of the fat cells forces them up against the Matrigel-coated surface of the coverslips. The coverslips were held in the cell suspension for 30 sec while the warmth of the cells and buffer caused the Matrigel to gel around the adipocytes, effectively attaching them to the solid surface of the coverslips. The coverslips were then placed cells-up for 30 sec on a paper towel to remove any excess lipid and fluid clinging to the edges of the coverslip and to permit further seating of the cells to the Matrigel before the coverslips were placed into media. Once attached, the cells were used immediately or cultured.

Primary culture

To culture the isolated cells, the coverslips were placed into tissue culture dishes containing Dulbecco's modified Eagle's medium (DMEM) supplemented with 25 mM $NaHCO_3$, 1 mM HEPES, 1.5 mM glutamine, 10 mg/ml of a penicillin/streptomycin mixture and either 10% calf serum or fetal calf serum (as indicated). The cells were maintained in a 5% CO_2 incubator at 37°C, and the medium was changed daily. In some experiments coverslips were fixed daily in 5% paraformaldehyde in PBS for approximately 1 hr and then stored in PBS. After the sixth day of culture, all six paraformaldehyde-fixed coverslips were stained with hematoxylin and eosin.

To examine the effects of insulin, adipocytes were attached to Matrigel-coated coverslips and cultured in DMEM containing 10% fetal calf serum in the presence or absence of 0.5 μg/ml insulin. After 4 days the cells were released from the coverslips using Dispase, Grade II (Boehringer Mannheim, Indianapolis, IN). Dispase effectively degrades Matrigel without damaging the embedded cells. The cultured cells were washed in PBS prior to preparation for SDS-PAGE and immunoblotting procedures.

Cell sizing

Primary cultures of adipocytes were released from the coverslips by Dispase digestion and washed in PBS at 37°C prior to size determination. Centroid cell volumes

and cell volume distributions were determined at 37°C using a customized Coulter counter (Model ZM, Coulter Electronics, Hialeah, FL) with a 120 micron port attached to a computer-driven pulse height analyzer (PCA-II, The Nucleus, Inc, Oak Ridge, TN) as described previously (6). Freshly isolated adipocytes were sized immediately after preparation.

SDS-PAGE and immunoblotting

Freshly isolated and cultured adipocytes were solubilized in sample buffer containing 2.2% SDS taking care not to emulsify the lipid, which interferes with electrophoresis. The lysates were diluted with sample buffer to a protein concentration of approximately 1 mg per ml, boiled for 5 min at 100°C, and then briefly centrifuged to separate the protein in solution from the lipid supernatant. One-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting were performed as previously described (7, 8). After electrophoresis, the gel was either silver-stained or transferred to Nitroplus membrane for immunoblotting as previously described (7, 8). The CA III antisera were a generous gift from Dr. N. Carter (St. George's Medical School, London). The adipocyte lipid binding protein (ALBP) antisera were graciously donated by Dr. D. Bernlohr (Department of Biochemistry, University of Minnesota). Pyruvate carboxylase was detected in Western blots as previously described with avidin-linked alkaline phosphatase (5).

Spectrofluorometry

In order to prevent lysis of the fat cells during spectrofluorometric runs, we used a modification of the cuvette perfusion system described by Kahn et al. (9). The perfused buffers were warmed to ensure that the cells remained at 37°C. A thermostatic cuvette holder was also used to keep the contents of the cuvette at this temperature during experimental runs. The perfusate was introduced to the bottom of the cuvette (approximately 3 ml/min) while the older buffer was aspirated gently from the top of the cuvette. Using this perfusion system, different solutions were easily introduced into the cuvette by alternating the containers from which the perfusate was drawn.

Cells on coverslips used for spectrofluorometric readings were loaded with the intracellular pH indicator, 2',7'-bis-(2-carboxyethyl)-5-(and 6)-carboxyfluorescein (BCECF, from Molecular Probes Eugene, OR). The dye was loaded as the acetoxymethyl ester (BCECF-AM) (10, 11) for 20 min at a concentration of 30 μM in KRH buffer without BSA. The loaded cells were washed in the perfusion chamber of the spectrofluorometer with fresh BSA-free KRH. During experimental runs, BCECF was alternatively excited at 500 nm (the absorption maximum) and 450 nm (the isobestic point) using a dual excitation SPEX spectrofluorometer. These excitation measurements were collected at the emission wavelength 535 nm. The fluorescent intensities were recorded at the two excitation wavelengths every 5 sec and were ratioed (500/450). The ratios were then converted into pH values after calibration of the intracellular pH using the high K^+/nigericin method of Weiner and Hamm (11) and Thomas, Buchsbaum, and Racker (12). A linear calibration curve was generated using high K^+ buffers containing 146 mM KCl, 5 mM glucose, 2 mM glutamine, 10 mM HEPES, 10 mM MES, and 10 mM PIPES, which were introduced to the attached adipocytes in the presence of 10 μM nigericin. The pH values of the high K^+ buffers ranged from 6.4 to 8.0.

RESULTS

Coating coverslips with Matrigel worked best when both the coverslips and pipettes were kept ice-cold rather than at room temperature. Coating the coverslips with a thick layer of Matrigel resulted in dense clumps of cells attaching rather than smooth monolayers of cells. Therefore, it is important that a thin uniform layer of Matrigel is applied when using this method. Also, if the Matrigel is applied when in a slightly gelled state, the cells do not attach as efficiently. The Matrigel should be kept at 4°C until it is used to prevent premature gelling.

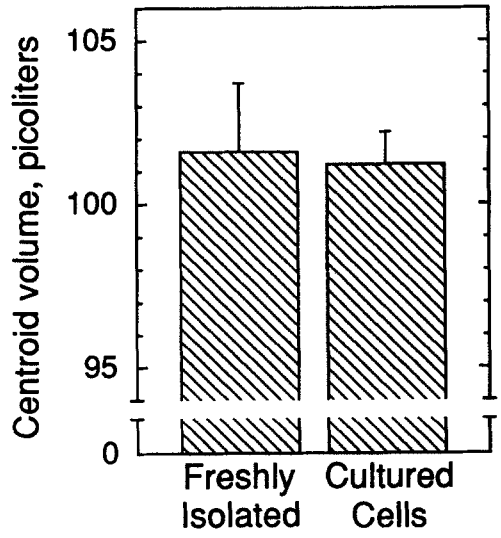

Fig. 1. Effect of Matrigel attachment method on adipocyte centroid volume. Adipocytes were isolated from 315–350 g Sprague-Dawley rats. Average cell volumes and volume distributions (not shown) were determined as described in Methods. Freshly isolated adipocytes were sized directly while cultured cells were first released by Dispase digestion of Matrigel and then washed in warm PBS. The bar shows the mean and standard error of the centroid volume. The results are representative of two such experiments, each measurement represents averaged data from 5,000–10,000 cells.

Characterization of Matrigel-attached adipocytes in primary culture

Primary culture of adipocytes was accomplished by introducing the coverslips into culture dishes and then adding medium to the dishes. To determine whether or not the Matrigel method of attachment selectively attaches larger cells or smaller cells, cell volumes were determined (**Fig. 1**). Comparison of single cell volume distributions (data not shown) and the computer-determined centroid (center of computer-generated volume distribution histograms) of the single cell volume distribution (Fig. 1) revealed no significant differences between freshly isolated adipocytes and attached adipocytes. These findings indicated that the Matrigel method does not appear to favor the attachment of a particular cell size.

Although isolated adipocytes are often used immediately, we have been able to culture cells attached with Matrigel for extended periods of up to 6 days. We examined adipocytes that were attached to Matrigel-coated coverslips under light microscopy after fixation in paraformaldehyde and staining with hematoxylin and eosin. We did not observe any significant change in appearance between cells fixed in 5% paraformaldehyde on the first day of culture from those fixed on subsequent days (**Fig. 2**). Cultured adipocytes retained their lipid droplet and rounded phenotype.

To further characterize the cultured adipocytes, SDS-PAGE and immunoblotting were performed. On silver-stained SDS-PAGE gels we reproducibly observed a similar protein banding pattern between freshly isolated adipocytes and cultured adipocytes (**Fig. 3**). Western blotting indicated that cells cultured for a period of four days did not appear to lose the ability to express tissue-specific differentiation-dependent proteins such as α-ATP citrate lyase (**Fig. 4**), CA III (**Fig. 5**), ALBP (**Fig. 6**) and pyruvate carboxylase (**Fig. 7**).

Cells cultured using this method also retained their insulin sensitivity (Figs. 4–6). Thus, α-ATP citrate lyase concentrations increased when insulin (0.5 μg/ml media) was added to the culture medium (Fig. 4). In contrast, insulin addition led to a decrease in the relative concentrations of CA III (Fig. 5) and ALBP (Fig. 6), but had no effects after 4 days on pyruvate carboxylase concentrations (Fig. 7).

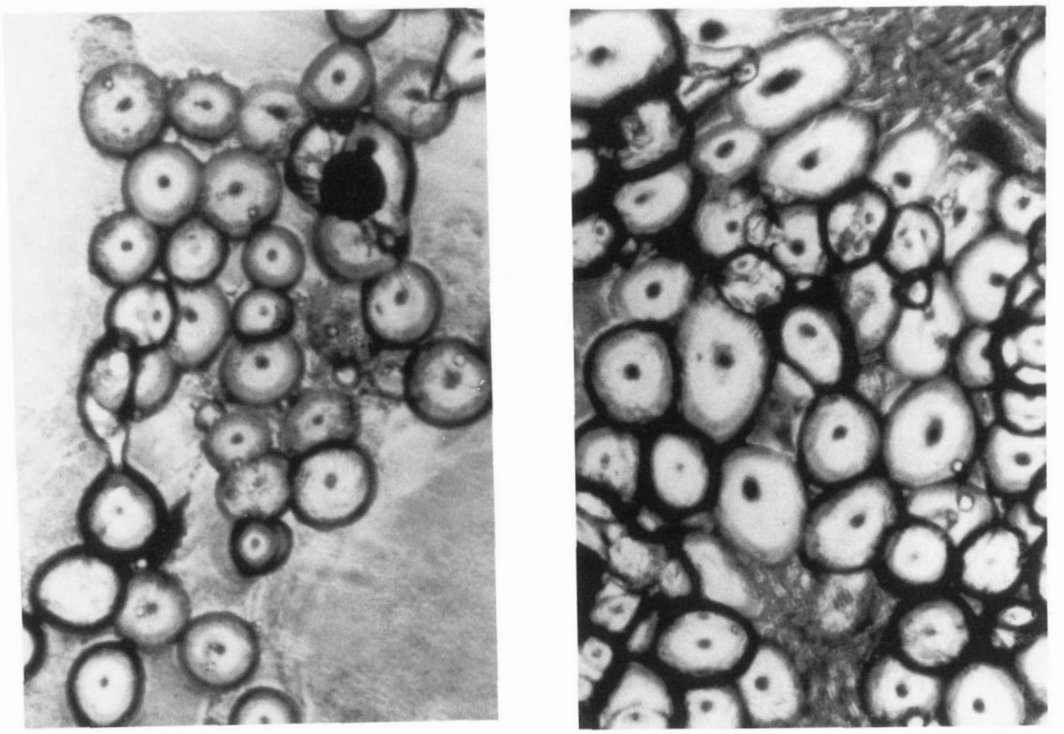

Fig. 2. Attachment and primary culture of adipocytes attached to Matrigel-coated coverslips. Isolated rat adipocytes were attached to Matrigel-coated coverslips and cultured for 1 (left panel) or 6 (right panel) days. The cells were fed DMEM containing 10% calf serum and incubated at 37°C in a humidified atmosphere of 95% air/5% CO_2. After culture, these cells were fixed in 5% paraformaldehyde in PBS. After fixation, the cells were stained with hematoxylin and eosin. Photomicrographs were prepared at 40× using a phase contrast microscope. The dark object in the middle of the cells is a refraction artifact due to the lipid droplet rather than the cell nucleus.

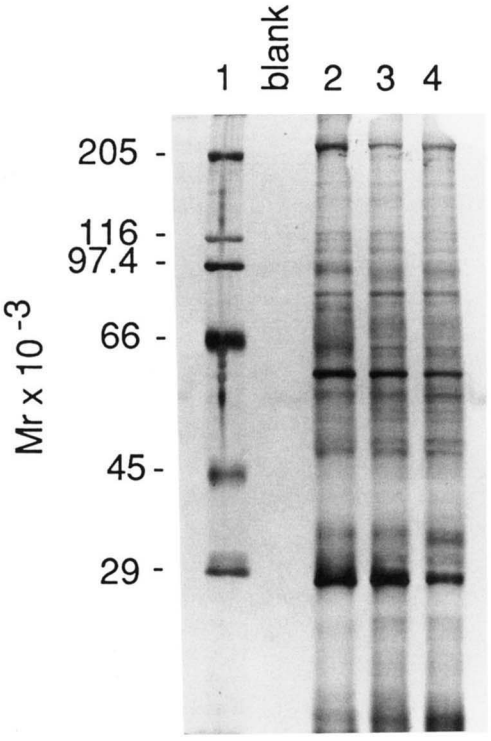

Fig. 3. Silver-stained proteins from freshly isolated and cultured adipocytes using the Matrigel attachment method. Proteins (20 μg) from freshly isolated adipocytes (lane 2) or adipocytes cultured for 4 days (lanes 3 and 4) were solubilized in sample buffer and separated on a 7.5% polyacrylamide SDS-PAGE gel followed by silver staining. The cultured cells were fed DMEM containing 10% fetal calf serum in the absence (lane 3) or presence (lane 4) of insulin (0.5 μg/ml media). The cultured cells were released with Dispase and washed with PBS prior to solubilization.

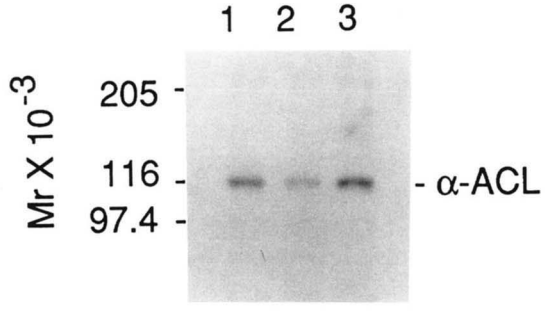

Fig. 4. Insulin regulation of α-ATP citrate lyase in Matrigel-cultured rat adipocytes. Proteins (50 μg) from freshly isolated adipocytes (lane 1) or adipocytes cultured for 4 days (lanes 2 and 3) were solubilized in sample buffer and separated on a 7.5% polyacrylamide SDS-PAGE gel then transferred to Nitroplus membrane. The membrane was immunoblotted using an α-ATP citrate lyase polyclonal antibody (a gift from Dr. C. Rubin). The cultured cells were fed DMEM containing 10% fetal calf serum in the absence (lane 2) or presence (lane 3) of insulin (0.5 μg/ml media). Attached cells were released with Dispase and washed with PBS prior to solubilization.

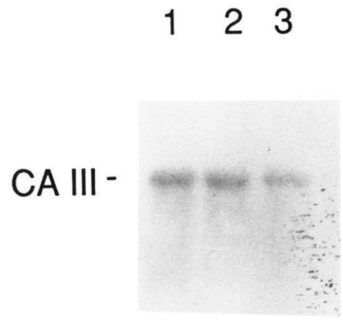

Fig. 5. Insulin regulation of CA III in Matrigel-cultured rat adipocytes. Proteins (50 μg) from freshly isolated adipocytes (lane 1) or adipocytes cultured for 4 days (lanes 2 and 3) were solubilized in sample buffer and separated on a 7.5% polyacrylamide SDS-PAGE gel then transferred to Nitroplus membrane. The membrane was immunoblotted using CA III antisera (a gift from Dr. N. Carter). The cultured cells were fed DMEM containing 10% fetal calf serum in the absence (lane 2) or presence (lane 3) of insulin (0.5 μg/ml media). Attached cells were released with Dispase and washed with PBS prior to solubilization.

Using Matrigel-attached adipocytes for spectrofluorometric experiments

For these experiments the cells were put into spectrofluorometer cuvettes, and the 500 nm/450 nm fluorescence ratios were determined every 5 sec. Adipocytes that were loaded with BCECF-AM were perfused from the bottom of the cuvette to the top with modified KRH (contains 133 mM NaCl and 10 mM HEPES) for 6 min. Brief alkalization of the cytoplasm occurred upon exposure of the attached adipocytes to KRH containing 20 mM NH_4Cl for 6 min after which the NH_4Cl-free KRH buffer was re-introduced to the cells (**Fig. 8**). To determine the intracellular pH, cells were permeablized to H^+ at the end of the experiment using the high K^+/nigericin method (11, 12), and the 500/450 fluorescence ratios were determined at different pH levels. A typical intracellular pH versus 500/450 fluorescence ratio calibration curve is shown in **Fig. 9,** demonstrating linearity in the Matrigel-attached

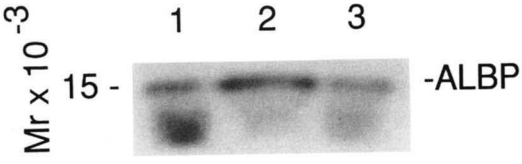

Fig. 6. Insulin regulation of adipocyte lipid binding protein (ALBP) in Matrigel-cultured rat adipocytes. Proteins (50 μg) from freshly isolated adipocytes (lane 1) or adipocytes cultured for 4 days (lanes 2 and 3) were solubilized in sample buffer and separated on a 10% polyacrylamide SDS-PAGE gel then transferred to Nitroplus membrane. The membrane was immunoblotted using ALBP antisera (a gift from Dr. Bernlohr). The cultured cells were fed DMEM containing 10% fetal calf serum in the absence (lane 2) or the presence (lane 3) of insulin (0.5 μg/ml media). Attached cells were released with Dispase and washed in PBS prior to solubilization.

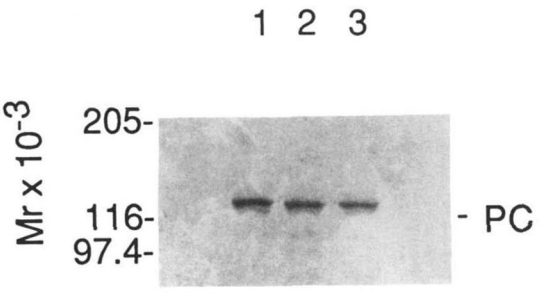

Fig. 7. Pyruvate carboxylase (PC) in Matrigel-cultured rat adipocytes. Proteins (50 μg) from freshly isolated adipocytes (lane 1) or adipocytes cultured for 4 days (lanes 2 and 3) were solubilized in sample buffer and separated on a 7.5% polyacrylamide SDS-PAGE gel then transferred to Nitroplus membrane. The blot was incubated with biotin, then probed with avidin-linked alkaline phosphatase to detect biotin requiring enzymes. The cultured cells were fed DMEM containing 10% fetal calf serum in the absence (lane 2) or the presence (lane 3) of insulin (0.5 μg/ml media). Attached cells were released with Dispase and washed in PBS prior to solubilization.

cells. It was important to exclude BSA from the high K^+/nigericin buffers, as BSA has been previously found to scavenge nigericin (13).

Fig. 8 shows that exposure to NH_4Cl resulted in an increase in the fluorescence ratio (500/450) that corresponds

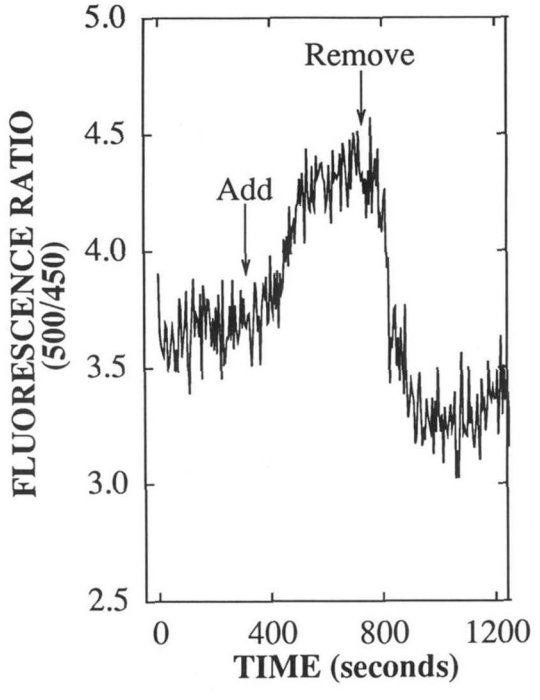

Fig. 8. Effects of ammonium chloride on BCECF fluorescence ratios. Adipocytes were attached to Matrigel-coated coverslips and loaded with 30 μM BCECF-AM. The cells were treated with a KRH buffer containing 20 mM NH_4Cl. After addition of NH_4Cl as indicated by the first arrow on the graph (Add), the cells underwent a brief intracellular alkalinization then acidification when the weak base was removed as indicated by the second arrow on the graph (Remove). The excitation ratio was recorded before, during, and after NH_4Cl exposure and plotted against time. The rise in the fluorescence excitation ratio (500ex/450ex) in the presence of NH_4Cl corresponds to an increase in pH_i.

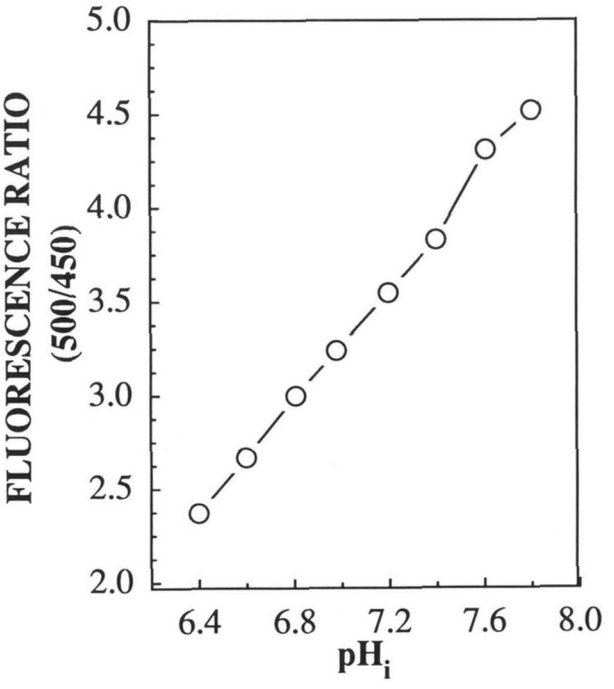

Fig. 9. BCECF calibration curve of intracellular pH. An intracellular pH calibration curve was made using freshly isolated rat adipocytes. The adipocytes were attached to thermanox coverslips by the Matrigel method we developed. Intracellular pH was adjusted to different levels using the nigericin/high K^+ buffer method (11, 12).

to an increase in pH_i. This increase in pH_i is due to an influx of NH_3 that binds free protons to produce ammonium ions (NH_4^+), thus decreasing the concentration of H^+ inside the cell. The fluorescence ratio, and consequently the pH_i, dropped below the initial value after the removal of NH_4Cl bathing the adipocytes. It is hypothesized that this "acidification" after NH_4Cl removal is due to shifting equilibrium of NH_4^+/NH_3 toward NH_3 (with concomitant proton release) as the NH_3 leaves the cell along its concentration gradient (14). These observations agree with studies of NH_4Cl exposure performed on a variety of other cell types (14, 15). Thus, it would seem that the attachment to Matrigel does not interfere with transmembrane ion movements that are necessary for intracellular pH regulation in adipocytes.

DISCUSSION

Freshly isolated fat cells tend to lyse upon attachment to glass or other charged surfaces (e.g., cell culture plastics) and float in cell culture medium. Perhaps this is why most primary cultures of fat tissue have been aimed largely at adipocyte precursor preparations (16–19). However, Garvey, Olefsky, and Marshall (20) and Marshall (21) have established a means of culturing isolated adipocytes by floating the fat cells in airtight, conical poly-

propylene tubes. Under these culturing conditions, the cells change after a day or two. This change is marked by the loss of lipid content and the conversion of the floating rounded mature adipocyte to a sinking fibroblast-like cell. Hajduck and colleagues (1) characterized this process as "de-differentiation."

The ability to successfully culture cells on matrix molecules such as laminin, fibronectin, and collagen may help alleviate these problems. We have devised a method for attaching adipocytes to thermanox coverslips using Matrigel. Matrigel is a commercially available mixture of extracellular matrix proteins. These proteins are prepared from extracts of Engelbreth-Holm-Swarm (EHS) mouse sarcoma basement membranes. This laminin-rich mixture is a liquid form at 4°C. Once warmed above 22°C, Matrigel gels irreversibly; however, Dispase will digest the gel, releasing the attached cells unharmed.

This method does not appear to preferentially attach adipocytes of a particular size as cell volume distributions and centroid cell volumes were not significantly different between cultured cells and freshly isolated adipocytes (Fig. 1). Even after 6 days of culture using the Matrigel method, we did not observe any loss of lipid or conversion of adipocytes to a more fibroblastic morphology (Fig. 2) often seen in floating cultures (1). Cultured adipocytes maintained their complement of major proteins (Fig. 3) and several tissue-specific proteins that we tested including α-ATP citrate lyase (Fig. 4), CA III (Fig. 5), ALBP (Fig. 6) and pyruvate carboxylase (Fig. 7). In addition, the Matrigel-cultured adipocytes retained their ability to respond to insulin as demonstrated by the effect of insulin on α-ATP citrate lyase (Fig. 4), CA III (Fig. 5), and ALBP (Fig. 6). Adipocyte pyruvate carboxylase concentrations have been reported to be altered in different metabolic states such as genetic obesity and diabetes mellitus; however, it is unclear whether these changes are a direct result of insulin action on the adipocytes or on the levels of other hormones that might, in turn, be able to regulate pyruvate carboxylase expression (22). Our findings suggest that the latter may be the case, i.e., as insulin supplementation of the media did not alter the levels of PC after 4 days in culture (Fig. 8). Taken together, these findings suggest that the adipocytes do not de-differentiate when cultured on Matrigel.

We originally devised the Matrigel method for use in spectrofluorometry experiments. Adipocytes were attached to Matrigel-coated thermanox coverslips and used for spectrofluorometry. Changes in pH_i were monitored through the use of the fluorescent pH indicator BCECF loaded as the acetoxymethyl ester BCECF-AM. The fluorescent intensity of this dye and its excitation ratios are linear within the physiological pH range of 6.4 to 8.0 (Fig. 9). By use of the Matrigel-coated coverslip technique described, we have been able to obtain monolayers of cells to use for our recordings. The attached cells are readily loaded with BCECF-AM, remain attached throughout the course of our pH challenges, and did not exhibit the wide fluctuation in fluorescence observed with stirred adipocytes. So far, we have been able to reproduce the types of responses observed in other cells showing that pH_i rises upon exposure to NH_4Cl and falls below initial pH_i values when this weak base is withdrawn (Fig. 8) and have been able to calibrate the fluorescence ratios to intracellular pH (Fig. 9).

Although some researchers have attempted to use Barnacle glue to attach adipocytes to coverslips (3), we have found that Matrigel is more effective and surely more closely resembles the basement membrane components that surround adipocytes in vivo (23). Our procedure is beneficial for examining the morphological and biochemical characteristics of freshly isolated cells and will enable investigators to perform experiments that must be accomplished over the course of several days.

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