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INTRACELLULAR AVAILABLE CALCIUM DETERMINATION IN

ADIPOCYTES USING INDO-1

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SHARON SLUSAR GRABSKI

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Gerald L. Davis

Major professor

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INTRACELLULAR AVAILABLE CALCIUM DETERMINATION IN ADIPOCYTES USING INDO-1

By

SHARON SLUSAR GRABSKI

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ABSTRACT

INTRACELLULAR AVAILABLE CALCIUM DETERMINATION IN ADIPOCYTES USING INDO-1

By

Sharon Slusar Grabski

This research developed a method utilizing Indo-1 AM, collagen, and the ACAS 470 for the evaluation of intracellular available calcium in single tissue derived adipocytes. This method permits measuring nanomolar calcium changes in 0.1 minute intervals. The glucose transport assay studies indicated that 2.5 mM Indo-1 AM and collagen did not alter insulin stimulated glucose transport curves. A possible left shift in glucose transport curves at higher Indo-1 AM concentrations was indicated. The effect of collagen on glucose transport was studied by devising a method utilizing collagen coated microcarrier beads. The basal calcium level of adipocytes incubated with or without glucose in their medium was measured using the ACAS 470. No significant difference was found. Insulin stimulation of these basal adipocytes support the theory that calcium is involved in the insulin-glucose transport process.

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CHAPTER I

PURPOSE AND DESIGN

INTRODUCTION

Knowledge of what factors influence extra- and intracellular available calcium levels, as well as what mechanisms calcium modulates, is vital to our understanding of subcellular processes. Studies suggest a correlation between intracellular available calcium levels and insulin action in several types of cells including the adipocyte [1,2]. Insulin action is a complex cascade of mechanisms involving receptors, second messengers, transport proteins, enzymes, calcium, various mediators and effectors, most of whose interactions are unclear. It is known that insulin is the primary regulator of plasma glucose transport into adipocytes and that glucose transport is the rate limiting step in plasma glucose uptake by adipocytes. It has been proposed that insulin induces the translocation of glucose transport proteins from an intracellular pool to the cell membrane [3,4]. The contribution of this translocation towards the total glucose transport mechanism has been debated. Other theorized mechanisms include 1) an insulin induced modification of plasma membranes [5] and 2) an intracellular regulatory metabolite alteration of glucose transport kinetics [6].

The glucose transporter protein has been identified and studied in a variety of cells including HepG2 erythrocytes and 3T3-L1 adipocytes. The HepG2/erythrocyte glucose transporter is not activated by insulin although the cells contain insulin receptors. These cells have a non-insulin dependent mechanism for activating their glucose transporters. 3T3-L1 adipocytes have glucose transporters that are activated by insulin binding to

receptors thus they have an insulin dependent mechanism for glucose transport. When the HepG2/erythrocyte-type glucose transporters are expressed in 3T3-L1 adipocytes, they translocate to plasma membranes in response to insulin as do the 3T3-L1 glucose transporters [7]. Therefore translocation is regulated not by tissue specific transporters but by other tissue specific factors such as messengers.

Recent evidence strongly suggests correlation between insulin regulated glucose transport and intracellular available calcium. Quin 2-AM when incubated with adipocytes chelates intracellular calcium forming a biologically inactive Quin 2:Ca^{2+} thus decreasing available cytosolic calcium. In rat adipocytes, high concentrations of Quin 2 inhibit glucose transport by 55% for insulin and 85% for insulin mimetic agents such as Vanadate, Concanavalin A, H_2O_2 and TPA (12-O-tetradecanoyl phorbol 13 acetate) in the stimulated portion of a dose response curve [8]. This suggests that the glucose transport mechanisms activated by these chemically diverse compounds all depend on intracellular calcium, possibly in a common calcium dependent mechanism. It was noted that the basal (unstimulated) transport level of these compounds (Vanadate, Concanavalin A, H_2O_2 and TPA) was unaffected by the Quin 2, suggesting the presence of another transport mechanism which is calcium independent at basal levels. However, other data suggests a calcium dependent role at the basal level. Researchers observed an increase both in basal level glucose transport and calcium efflux in adipocytes when using H_2O_2 and vanadate when compared to cells not treated with any agent [9]. Calcium flux antagonists, such as verapamil, lowered basal glucose transport [10]. At present, the precise role of calcium in these processes is the subject of debate.

To resolve the calcium role debate, a determination of mechanisms involved in the insulin binding-calcium interaction is needed, as well as a determination of where calcium

acts in the insulin-glucose transport cascade. Calcium may act only in the receptor-plasma membrane area producing no measurable change or there may be a mechanism in which there are measurable cytosolic calcium changes. One study used Fura-2, a fluorescent calcium indicator that has an excitation spectrum change when bound to calcium. The Fura-2 labeled adipocyte suspensions had increased calcium concentrations in response to insulin [1]. Theories concerning the function of this calcium concentration change include that of activation and translocation of calcium. One activation postulation is that a cellular calcium sensitive protein kinase C controls phosphorylation of proteins such as the glucose transporter protein [11]. Studies suggest that this glucose transport protein is activated by insulin through an unknown mechanism which might involve protein kinase C [12]. Another postulation based on work with Swiss 3T3 cells and fibroblasts is that hormones stimulate both diacyglycerol and inositol 1,4,5 triphosphate. Both then act as second messengers, diacyglycerol stimulates protein C kinase and Inositol mobilizes calcium to form a bifurcating signalling system [13,14,15,16]. To investigate calcium-insulin interactions further it is necessary to observe and evaluate dynamic calcium distributions in isolated single cells and determine what effect insulin, insulin agonists, and antagonists have on their calcium distribution.

Recent developments in both instrumentation and calcium binding fluorescent dyes now make it possible to measure intracellular available calcium, its distribution within the cell and millisecond fluxes in its concentration. Grynkiewicz, Poenie, and Tsien have synthesized a series of fluorescent dyes which combine a tetracarboxylate chelating site with stilbene chromophores resulting in enhanced quantum efficiency and photochemical stability when compared to previous dyes such as Quin 2. This group of dyes, which

include Fura-2 and Indo-1, are very sensitive to and specific for available cytosolic calcium [17,18,19]. Fura-2, when bound to calcium, exhibits a shift in its excitation (absorbance) spectra. Indo-1, when bound to calcium, exhibits a shift in its emission spectra. They provide a bright fluorescence (high quantum yield) at low dye concentrations. This bright fluorescence compared to Quin 2 permits shorter observation times and smaller tissue volumes [17]. It is claimed they cause little or no intracellular disruption of normal function through artificial changes in cellular calcium as observed with Quin 2. To observe cellular calcium using fluorescent compounds Meridian Instruments has developed the ACAS-470 laser fluorescent microscope to study anchored viable single cells. It excites a cell with its laser and then simultaneously measure two different emission wavelengths. Both forms of intracellular Indo-1, calcium free and calcium bound, have the same excitation wavelength but different emission wavelengths. The ACAS is capable of measuring both emission wavelengths at the same time, thus eliminating potential errors that result when wavelengths must be readjusted during experiments. The combination of these new technologies for use in single attached adipocytes will provide tools for understanding the distribution and role of intracellular available calcium.

PROJECT AND OBJECTIVES

The objective of this thesis was the development of a method utilizing Indo-1 for the evaluation of intracellular available calcium in single attached adipocytes. This technique will be used to study the postulated relationships among plasma glucose transport into cells, insulin, and intracellular available calcium. Adipocytes, derived from rat epidyimal fat pads, were chosen because of their well documented ability to participate

in plasma glucose transport in response to insulin and insulin minetic stimulation. The objective has been accomplished through the following steps:

1. Development of techniques and optimal conditions for the study of intracellular available calcium in tissue derived adipocytes using adherence compounds, Indo-1, and the ACAS-470.
2. Determination of the intracellular available calcium distribution in unstimulated adipocytes.
3. Evaluation of changes in intracellular adipocyte calcium concentrations in response to stimuli.

MATERIALS AND INSTRUMENTATION

Indo-1 is a fluorescent indicator developed by Tsien for the evaluation of intracellular calcium concentrations [17,18,19]. The cell-permeant derivative, Indo-1 acetoxymethyl ester (Indo-1 AM) is loaded into a cell where it is cleaved by cellular esterases to Indo-1 free acid. Indo-1 free acid binds very specifically to calcium, giving rise to two forms of Indo-1 in the cell, calcium bound Indo-1 and free Indo-1. Both forms absorb radiation at 353-363 nm, but are distinguishable by their emission wavelengths. Calcium bound Indo-1 emits radiation at 405 nm while free Indo-1 emits radiation at 480 nm. By using the ratio of the two emissions magnitudes calcium measurements are independent of the actual dye concentration in the cell and do not require internal standardization. Indo-1 has a higher quantum efficiency than other fluorescent dyes such as Quin 2. Therefore another advantage is that a brighter, more easily detectable fluorescence occurs using Indo-1 at a lower dye concentration than with the other dyes.

This decreases the chances of Indo-1 perturbing cell processes since less dye is introduced into the system.

The ACAS 470 (Anchored Cell Analysis and Sorting) workstation is designed for the study of viable anchorage dependent single cells [20,21]. It employs a computerized fluorescent microscope with a five watt argon laser source necessary for the ultraviolet wavelengths required for Indo-1 use. A computer controls the stage for rapid, very accurate stage positioning when doing sequential cell analysis and precise targeting of the laser beam on cells. The laser excites Indo-1 at the proper wavelength (353-363 nm) and the ACAS simultaneously detects the emission wavelengths of both free Indo-1 (480 nm) and calcium bound Indo-1 (405 nm). The fluorescence detector data is processed by a microcomputer which displays it as fluorescent images and stores the data for further analysis.

The methods, results, and discussion section of this thesis are divided into two parts. The first part (Chapter Two) addresses objective I, the development an Indo-1 AM/adherence technique for adipocytes and the study of the technique's effect on glucose transport. The second part (Chapter Three) addresses objectives II and III, which demonstrates these techniques on the ACAS with a series of experiments measuring free calcium in adipocytes under different conditions. An appendix will describe operation of the ACAS-470, safety and calcium curve formula.

CHAPTER II

ADHERENCE AND INDO-1 TECHNIQUE STUDIES

INTRODUCTION

This objective of this Chapter is the development of techniques and optimal conditions for the study of intracellular available calcium in tissue derived adipocytes using Indo-1 and the ACAS 470. In order to perform any research using adipocytes, Indo-1 AM and the ACAS-470 the feasibility and optimal conditions of two techniques had to be determined. These were:

1. What compounds could be used for adipocyte adherence (anchoring) to a slide; and
2. That Indo-1 AM was capable of entering adipocytes.

Explanations on how these conditions were determined is explained in the first part of this chapter. After it was concluded that an anchored Indo-1 AM loaded adipocyte could be achieved, the question of the techniques' interference with normal cell function had to be addressed. Since evidence strongly suggests an involvement of intracellular free calcium with insulin regulated glucose transport in adipocytes and this is an area of future research with the techniques developed in this thesis, it is imperative that this technique does not interfere with the normal insulin dependent glucose transport in adipocytes. To investigate any interference it was necessary to first establish a "normal" glucose transport response of the cells used. The glucose transport procedure was then modified according to the techniques and conditions congruous for the ACAS phase.

The following glucose transport studies were performed:

1. Normal glucose transport; and
2. Variations of glucose transport procedure:
   A. Indo-1 AM glucose transport studies;
   B. Collagen (microcarrier beads) glucose transport study;
   C. Indo-1 AM and collagen glucose study;
   D. Simultaneous determination of low and high response in Indo-collagen treated cells and untreated cells; and
   E. Study of other Indo-1 AM concentrations (1/5 & 5x).

A detailed description of each study is in the latter part of this chapter.

GENERAL PROCEDURES

Reagents and Materials

Krebs ringer phosphate working solution--aka KRP I

3.0 gm bovine serum albumin(Sigma-insulin free), 200 mg dextrose,90 mLs KRP stock (buffer), 10 mLs 14 Mm $CaCl_2$. Mix all reagents together in a beaker until dissolved, pH to 7.4. Place in 37°C water bath. Make daily.

Krebs ringer phosphate solution--aka KRP stock

Chemical	gm/3.6L	Final concentration in KRP I
NaCl	29.92 gm	128.0 mM
KCl	1.55 gm	5.2 mM
$MgSO_47H_20$	1.48 gm	1.4 mM
Na_2HPO_4	10.72 gm	10.0 mM

Combine all chemicals in 3.6 L water, adjust pH to 7.4. Stores indefinitely at room temperature.

14 mM $CaCl_2$

0.206 gm $CaCl_2$ H_20 dissolved in 100 mLs H_20. Refrigerate, make fresh every 2 weeks.

Krebs ringer albumin-pipes buffer--aka KRP:GT

1 gm bovine serum albumin (Sigma-insulin free), 757 mg PIPES, 90 mLs KRP stock buffer 10 mLs 14 mM $CaCl_2$ Mix all reagents together in a beaker until dissolved, pH to 7.4. Place in 37°C water bath. Make daily.

Collagenase

Sigma C-8897, Type VII Acid Soluble

Medium II -- (0.25 M sucrose, 10 mM Tris)

85.6 gm Sucrose, 20 mLs 500 mM Tris Buffer (pH 7.29). Dissolve reagents in H_20, QS to 1.0 L. Adjust pH to 7.4. Stores indefinitely in refrigerator (4-8°C). Warm needed amount in 37°C water bath.

Rat Adipocyte Extraction and Fractionation

The cell isolation method used was based on those described by Rodbell [22], and Mckeel and Jarret [23]. It was used for all cell isolation procedures.

Extraction

Untreated Sprague-Dawley male rats weighing 150-200 g were used. They were decapitated without use of any drugs. The epidyimal fat pads were immediately removed and placed in 37°C, 0.9 % saline solution. The saline washed pads were placed in a warm plastic weighing boat (kept over a beaker of 37°C water). Any veins and/or extra tissue were removed. The cleaned pads were cut into very small pieces with surgical scissors and placed in a 50 mL plastic erlenmeyer flask containing a 1 mg/mL collagenase-KRP I solution kept at 37°C. Normally four pads were placed in 5 mL of the collagenase solution. Keeping the flask in a 37°C waterbath bucket, the samples were transferred to a vibrating 37°C waterbath and shook at 120 cycles per minute. The total time in the collagenase solution was 30 minutes.

Fractionation

The collagenase treated cells were strained in the following apparatus: A plastic mesh was placed in a 50 mL plastic syringe which had an enlarged opening. The collagenase-cell mixture was carefully added to the syringe, rinsing the flask twice with 3 mL of 37°C KRP-I. The plunger was reinserted and gently pressed down to strain the cells. The cells were collected in 15 mL plastic conical tubes kept in a 37°C water beaker. The tubes were allowed to stand for approximately 2-3 minutes until most of the cells floated to the top. Excess liquid was removed by suction and the cells resuspended twice with KRP-I then twice with KRP-GT. A wooden stick was used to help facilitate the resuspension. For some of the ACAS experiments the second resuspension solution was with Medium II or KRP-I. Cells were always kept at 37°C. This procedure yielded a

cellular concentration of 75-90 adipocytes per 0.1 mm^3. A hemacytometer was used to perform cell counts.

ADHERENCE COMPOUND STUDY

A critical factor for the use of the ACAS is the ability of cells to become attached to a surface. Adipocytes will normally not attach well to glass or plastic slides, therefore an adherence compound must be used. Three compounds, formvar, collagen, and poly-L-lysine have been used successfully with other cells [24,25,26]. These three were evaluated for use with adipocytes.

Reagents and Materials

24 x 40 mm corning cover glass slides

Formvar: Prepare slide by dipping it in solution, air dry.

Collagen: Sigma type VII, acid soluble

Dissolve 1 mg collagen/ 1 mL boiling H_20, let solution cool.

Place 20 uL on a slide, use a pipette tip to spread to a thin layer, air dry.

Slides are good for at least four weeks stored at room temperature.

Poly-L-lysine: 0.1% and 1.0% solutions using H_20 were prepared.

Place 20 uL on a slide, use a pipette tip to spread to a thin layer, air dry.

Slides are good for at least four weeks stored at room temperature.

Procedure

All the slides were preheated to 37°C, a 20 uL drop of cell suspension was placed on each slide and the slide was inverted. Two slides were made for each compound

studied at each cell plating time. At the desired time excess cell suspension was gently shook from the slide and the slide was washed once with Medium II. Adherence was determined by observing the cells with a microscope using a 10x objective and gently shaking the slide. The following adherence grading pattern was used in Figure 1, based on the observations in 10 fields per slide:

0 No adherence

\+ 1-10 adipocytes attached per field - (25% field is filled)

++ Field ~50% covered with attached adipocytes

+++ Field tightly packed with attached adipocytes

cd Cell destruction

Results and Discussion

The results of the adherence compound and plating study are shown in figure 1. Of the compounds studied formvar was not suitable because adipocytes did not adhere to it. Collagen and poly-L-lysine appeared equal on the basis of adherence alone, with 0.1% poly-L-lysine having a slight advantage over 1.0% poly-L-lysine at the shorter times. When the slides were observed using the microscope the 1.0% poly-L-lysine slide had a slightly opaque background which made it less desirable for microscope work. Of the two remaining compounds, collagen and 0.1% poly-L-lysine, collagen gave a slightly "cleaner" i.e., less fluorescent background on the ACAS than did poly-L-lysine. For this reason collagen was chosen to be used in the rest of the study. It also should be noted that by 30 minutes after plating cell destruction began to occur on all slides.

Minutes Plated	Collagen	Formvar	1.0% p-L-l	0.1% p-L-l
5	++ to +++	+	++	++ to +++
10	++	+	++	+++
15	+++	+	++	++
20	++ to +++	+	++	++ to +++
30	+++	+	++ to +++	+++
	some cd	cd	some cd	some cd
45	+++	0	++	+++
	some cd	cd	some cd	some cd
60	++	0	++	na
	cd	cd		

FIGURE 1. Adherence Compound and Plating Time Study.

A 20 uL cell suspension was placed on a 37°C treated slide for each plating time, then washed once with Medium II. Grading is based on the average area covered by adhering cells in 10 fields (microscopically, 10 x objective).

0 = No adherence, + =~ 25% field covered, ++ = ~50% field covered; +++ = Field tightly packed; cd = Cell destruction; p-L-l = poly-L-lysine

Two slides were used for each plating time per compound. Procedure was done three times with collagen and 0.1% p-L-l, twice with formvar and 1.0% p-L-l.

INDO-1 AM LOADING STUDIES

These initial studies were to investigate the feasibility of using Indo-1 AM with adipocytes. Three objectives were to be determined:

1. If Indo-1 AM is capable of being loaded into adipocytes;
2. What concentration of Indo-1 AM should be used; and
3. The incubation time needed for Indo-1 AM to enter cells.

Reagents and Materials

INDO-1 AM

Stock 10 mM Indo-1 AM, is kept in 50 uL aliquots in -70°C freezer

Source: Molecular Probes Inc, P.O. Box 22010 Eugene, OR 97402.

Working solutions:

5 mM---dilute stock tube with 50 uL DMSO

1 mM---dilute 5 mM Indo-1 AM 1:5 with DMSO

Cover slides - Bionique-glass cover slides (Corning)

Loading Feasibility (Capability) and Concentration Study

Procedure

1. Keeping Indo-1 AM away from light, set up the following concentrations of Indo-1 AM in plastic snap tubes covered with aluminum foil (A-E). Adipocytes are in Medium II.

***

A.	5.0 mM	5.0 uL (5mM Indo) + 0 uL DMSO	+ 1 mL adipocytes
B.	2.5 mM	2.5 uL (5mM Indo) + 2.5 uL DMSO	+ 1 mL adipocytes
C.	1.0 mM	5.0 uL (1mM Indo) + 0 uL DMSO	+ 1 mL adipocytes
D.	0.5 mM	2.5 uL (1mM Indo) + 2.5 uL DMSO	+ 1 mL adipocytes
E.	0.25 mM	1.25uL (1mM Indo) + 4.75uL DMSO	+ 1 mL adipocytes

*** -- final concentration of Indo-1 AM in tube.

2. Incubate all tubes in the dark at 37°C for 45 minutes.
3. Wash cells 3x with Medium II, keeping a final volume of ~ 1 mL.
4. Apply 10 uL of cells to a collagen and a 0.1% poly-L-lysine coated slide, invert slide for 5 minutes, keeping it over a 37°C heating block.
5. Scan slide using the ACAS, observing fluorescence in cells and background.

Results

The adipocytes in slides D (0.5mM) and E (0.25mM) were weakly fluorescent in the first two trials, indicating that 1) all available dye entered the cells and that not enough dye was present in the solution or 2) a longer dye incubation time is needed to absorb and accumulate more dye in the cells at these concentrations. It was not desirable to increase the loading time since adipocytes are less viable at longer times. Slide A (5.0mM) had some background fluorescence in the first two trials, which required additional washing to remove. This additional manipulation increased the chance of cellular destruction and was not desirable. B (2.5mM) and C (1.0mM) showed good cellular fluorescence with minimal background fluorescence. B (2.5mM Indo-1) and C (1.0

mM Indo-1) concentrations were further investigated (see Concentration and Loading Time Study) to determine if a shorter loading time may be used. It was noted in this experiment that the poly-L-lysine slides had more background fluorescence then collagen slides, therefore collagen slides would be used in future studies.

Concentration and Loading Time Study

Procedure

Set up tubes B & C as listed in the objective I and II procedure, varying the incubation times (step 2) to 15, 30 and 45 minutes. There are two tubes for each concentration at each time.

Results

A 15 minute incubation time was moderately fluorescent for both concentrations during each of the two trial runs. There was no observable fluorescence difference between 30 and 45 minutes at either concentration indicating that indo-1 saturation occurred by 30 minutes. Both times were highly fluorescent and in an acceptable PMT range (see appendix for PMT definition). Slide B (2.5 mM) at 30 minutes was slightly more fluorescent then slide C (1.0 mM). Since there may be lot to lot variation in Indo-1 AM, and glucose transport studies using Indo-1 AM were still to be performed, a 2.5 uL of 5 mM Indo-1 AM per mL adipocyte was chosen. This higher concentration should augment any shift in glucose transport due to Indo-1 AM. If another lot results in greater cellular fluorescence an adjustment to a lower PMT setting could be used which is more desirable than adjusting a PMT setting higher which may result in photobleaching. Due to adipocyte viability the shorter incubation time was preferred. All future studies will be done using 2.5 mM Indo-1 AM (2.5 uL of a 5mM stock added to 1mL of cells suspension)

with a 30 minute incubation at 37°C. It should be noted that the 2.5 mM Indo-1 concentration refers to the loading solution concentration and not the Indo-1 concentration in the adipocyte which is unknown and not needed for determining the calcium concentration.

GLUCOSE TRANSPORT STUDIES

Reagents and Materials

The following reagents, previously presented in the General Procedure section, are used: Krebs ringer phosphate solution--stock, 14 mM $CaCl_2$, Krebs ringer phosphate working solution--aka KRP I, Krebs ringer albumin-pipes buffer--aka KRP:GT, Medium II. The following reagents are also used:

0.2 mM $HgCl_2$

450 mL KRP stock buffer, 50 mL $CaCl_2$, 2.715 gm $HgCl_2$

Mix all reagents together in a beaker until dissolved. Store in refrigerator until ready to use. Make weekly. This reagent is used cold.

Cytochalasin B--7.5 mM stock

7.2 mg cytochalasin B (Sigma) dissolved in 1.0 mL ethanol

Store tightly covered in freezer. Stores indefinitely.

Cytochalasin B--working

Make amount needed according to the following ratio each day:

2.0 uL Cytochalasin B stock + 13.0 uL KRP-GT

[^{14}C]D-glucose

Source: DuPont NEC-0428, 9.25 MBq (.25m Ci)

Make amount needed according to the following ratio each day:

1 uL [^{14}C]D-glucose + 66 uL KRP-GT

Insulin

Insulin source: Sigma # 116F-0402, 25.6 IU/mg, bovine pancreas

A stock of 10 IU/mL insulin solution was made per Sigma technical-dissolve ~2 mg/mL H_2O, adjust pH to <2.5 with 0.1 N HCl, then reconstitute to desired volume. Store 200 uL aliquots in plastic snap tubes at -70°C.

For glucose transport studies a tube was unthawed, then serial dilutions were made using KRP-GT buffer as follows.

		Final Concentration
100 uL (10,000 mU/ mL)	+ 900 uL KRP-GT buffer , mix	1,000 mU/mL
100 uL (1,000 mU/ mL)	+ 900 uL KRP-GT buffer , mix	100 mU/mL
100 uL (100 mU/ mL)	+ 900 uL KRP-GT buffer , mix	10 mU/mL
100 uL (10 mU/mL)	+ 900 uL KRP-GT buffer , mix	1 mU/mL
100 uL (1 mU/ mL)	+ 900 uL KRP-GT buffer , mix	0.100 mU/mL
100 uL (.100 mU/ mL)	+ 900 uL KRP-GT buffer , mix	0.010 mU/mL
100 uL (.010 mU/ mL)	+ 900 uL KRP-GT buffer , mix	0.001 mU/mL

Insulin-KRP:GT mix

Total transport tube (TTT)

275 uL KRP:GT + 25 uL appropriate insulin level.

Nonspecific background tube (NBT)

275 uL KRP:GT + 25 uL appropriate insulin level + 10 uL working cytochalasin B

To avoid tube to tube variation in pipeting at a given insulin level, a batch of insulin-KRP was mixed together for that level, then 300 uL was placed in each tube (TTT and NBT).

Scintillation cocktail

High flash cocktail: Safety-solve, Research Products International

Materials

10 x 100 mm plastic tubes, Ice bath, 37°C water bath, Pipettes and tips,
Large scintillation vials (20 mL-22 mm cap), Whatman filter apparatus
Glass filters, Containers for ^{14}C waste-solid and liquid
Gloves, forceps, for use with ^{14}C.

Normal (Untreated Cells) Glucose Transport Study

The purpose of this study is to establish a insulin dose response curve for untreated adipocytes which will function as a "normal" for comparisons with subsequent modifications of the glucose transport assay. A run is defined as the isolation of adipocytes from 3 to 5 rats, and immediate performance of the following glucose transport assay (procedure) on the isolated cells. All insulin concentrations and a basal level for the dose response curve are included in a run. Unless specified all studies will consist of three runs (i.e., the complete procedure including isolation of adipocytes will be done on three separate days). At the completion of the three runs, the value obtained

for each point on the dose response curve will be utilized for the determination of the composite insulin dose response curve for the study. A composite mean value for a given insulin level is thus the mean of the three individual runs mean values.

Procedure

1. In 10 x 100 mm plastic tubes set up the following:

   Each insulin concentration in triplicate (total transportation tubes=TTT), with a fourth tube for the cytochalasin-B blank (nonspecific transportation tube=NTB). Run a set of baseline insulin (0 insulin) tubes at the start and end of each experiment.
2. Add 300 uL of insulin-KRP mix to the appropriate tubes, (TTT & NBT) and 10 uL cytochalasin B to each NBT, keep on ice.
3. At timed intervals (i.e., 30 seconds), carefully add 200 uL of adipocytes to each tube, rinsing pipette tip 3x in the insulin-buffer mix. Place in a 37°C water bath. A new nonprimed pipette tip should be used for each tube.
4. After exactly 30 minutes and keeping the same timed interval, add 30 uL ^{14}D-glucose solution to each tube. Use a new pipette tip for each tube, rinsing the tip out 4x in the tube mixture. Keep in the 37°C water bath.
5. At exactly ten minutes and keeping the same timed interval, remove tube from the water bath and add 3 mL cold $HgCl_2$.
6. Using Whatman filter apparatus, carefully pour reaction mixture onto filter paper, rinse tube 3 times with $HgCl_2$, then rinse the filter paper once with $HgCl_2$.
7. Place filter in large scintillation vial.
8. Add 10 mL scintillation cocktail.

9. Using a scintillation counter, measure the amount of ^{14}C in the vial. Do duplicate counts. Use average counts per minute (CPM) as transport value.
10. Calculate insulin dependent glucose transport (IDGT)

    IDGT = TTT - NTB

    and report as a percentage of basal (0 level insulin)

    i.e., glucose transport at 1000 mU/mL insulin

    = [IDGT (1000 mU insulin)/IDGT (0 mU insulin)] x 100
11. Determine the run's percent basal response at each insulin concentration and by averaging the value obtained for the triplicate tubes.
12. Generate a dose response curve by plotting insulin concentration (x axis) versus percent basal response (y axis).

Results

Three runs of the experiment were conducted. The composite curve of these runs is presented in Figure 2, Normal (Untreated Cells) Glucose Transport. All glucose transport curves will be compared to this curve. A detailed discussion of the glucose transport curves is at the end of the chapter.

Indo-1 AM Glucose Transport Study

The purpose of this study is to determine what effect, if any, Indo-1 AM, at a concentration of 2.5 mM loaded for 30 minutes in a cell suspension, would have on glucose transport.

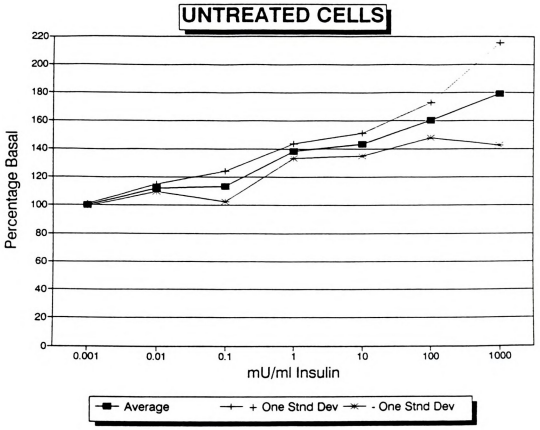

FIGURE 2. Normal (Untreated Cells) Glucose Transport.

Untreated adipocytes incubated (37°C) for 30 minutes in KRP:GT-insulin. D-glucose added for ten minutes. Reaction terminated with $HgCl_2$. Average of 3 runs.

mU/mL Insulin	% basal	STD	mU/mL Insulin	% basal	STD
0.001	100	1	10	143	8
0.01	112	3	100	161	12
0.1	113	11	1000	179	37
1.0	138	5			

Basal = 100% = average CPM cells incubated with no insulin.

Procedure

1. Isolate adipocytes per procedure described earlier. Retain 2 mL of cell suspension to use as a untreated blank. The final cell count of these untreated cells should be the same as the treated cells.
2. Add 2.5 uL of 5 mM Indo-1 AM, diluted with DMSO per 1 mL of cell suspension. This results in a 2.5 mM Indo-1 AM suspension.
3. Incubate cells 30 minutes in medium II in a 37°C water bath, keeping suspension away from light. Record suspension volume.
4. Wash cells once with 37°C medium II, then twice with 37°C KRP-GT buffer, retaining original volume of step 2 after final wash.
5. Run untreated cell glucose transport procedure using the Indo-1 AM incubated cells. Include a 0 mU insulin blank for both treated and untreated cells.

Results

Figure 3, Indo-1 AM Glucose Transport shows the composite of the three runs. Untreated cells (no INDO-1 AM or insulin) gave a transport value of 110, 94, 97 percent compared to the basal value of the treated cells for the three runs. A detailed discussion of the glucose transport curves is at the end of the chapter.

Collagen Bead Study

The purpose of this study was to devise a glucose transport experiment which would have the maximal number of adipocytes possible attached to a collagen coated surface in order to study the effect of the anchoring compound on glucose transport. Beads were chosen since they provide a sufficiently large surface area yet are compact

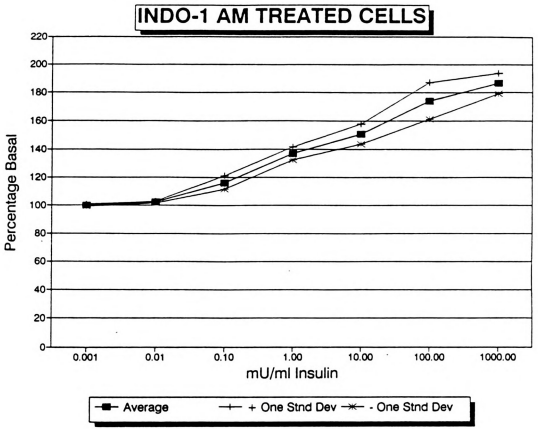

FIGURE 3. Indo-1 AM Glucose Transport.

Indo-1 AM treated adipocytes treated (37°C) for 30 minutes in KRP:GT-insulin. D-glucose added for ten minutes. Reaction terminated with $HgCl_2$. Average of 3 runs.

mU/mL Insulin	% basal	STD	mU/mL Insulin	% basal	STD
0.001	100	1.0	10	151	7
0.01	102	0.5	100	174	13
0.1	116	5.0	1000	186	7
1.0	137	5.0			

Basal= 100% = average CPM of Indo-1 AM treated cells incubated with no insulin.

in a test tube thus not increasing the volume of reagents needed for the glucose transport assay. The three main objectives were:

1. To determine if adipocytes will adhere to collagen coated microcarrier beads;
2. To determine the amount of beads to be used; and
3. To determine the type of test tube needed.

Materials

Collagen coated microcarrier beads: Polyscience Catalog # 19071, 150-210 microns, specific gravity 1.04

10 x 12 mm plastic test tubes

Miniscintillation vials, Research Products International

Adipocyte suspension (in Medium II)

Collagen microcarrier bead feasibility

Procedure

Weigh 5, 8, 10, 15, 20 and 25 mg collagen beads into labeled 10 x 12 plastic test tubes. Place in a 37°C water bath and add 200 uL of cell suspension to each tube, rotating the tube gently to facilitate adhesion. After 5 minutes add 300 uL KRP buffer. Check macroscopically and microscopically for adhesion.

Results

Amount collagen macroscopic observations

microscopic observations

5 mg thin cell-bead interface layer, most cells above beads

~ 33% of cells attached

8 mg thin cell-bead interface layer, most cells above beads

~ 33% of cells attached

10 mg moderate cell-bead interface layer, some cells above beads

~33-50% of cells attached

15 mg moderate-thick cell bead interface layer, few cells above beads

~33-50% of cells attached

20 mg thick cell-bead interface, no observable cell layer on top

~50+% of cells attached

25 mg thick cell-bead interface, no observable cell layer on top

~50+% of cells attached-no difference from 20 mg

Conclusion

The 20 mg and 25 mg collagen beads/tubes gave the highest percent cell attachment. Since there was no advantage in using the 25 mg tube over the 20 mg tube the 20 mg tube was chosen for further use.

There was a microcarrier bead "button" on the bottom of all the tubes, therefore not all the beads were used. Due to specific gravity the unattached beads fell to the bottom and the unattached cells rose to the top. The cell-bead complex fell midway. A tube with a greater bottom surface area would decrease the height of the cell-buffer mix and

increase the chance that the cells and beads come in initial contact and adhere with minimal mixing and damage to cells. The volume of the cell buffer mix is determined by the glucose transport assay and could not be changed. The effect of the different available tubes is examined next.

Test tube size determination

Materials

35 mM Petri dishes, Scintillation vials, Miniscintillation vials

Collagen coated microcarrier beads

Initial Observations

Three containers were investigated as possible reaction "tubes" - 35 mM petri dishes, scintillation vials and miniscintillation vials. The scintillation vials and petri dishes had too great a bottom surface area and were inappropriate for the glucose transport procedure due to three reasons: 1) The reaction volume would have to be increased to cover all the cells; 2) more radiation would be emitted into the room air when reaction vials were uncovered; and 3) a longer more vigorous mixing of added reagents would be needed thus increasing the chance of damage to the cells. The miniscintilation vials with approximately a 10 mm diameter were a possibility. There would be no need to increase the reaction volume and there would be less radiation emitted through the smaller top then the other two "tubes". A gentle rotation motion seemed sufficient for good mixing. Since the cells would be adhering to the collagen beads in solution there would be minimal contact of the cells with glass.

Procedure

Place mini scintillation vials, containing 15 mg, 20 mg, and 25 mg collagen beads in a 37°C water bath. Add 200 uL of cell suspension to each tube. Rotate the tube gently to facilitate adhesion. After 5 minutes add 300 uL KRP buffer. Check macroscopically and microscopically for adhesion.

Results

Amount collagen - macroscopic observations

microscopic observations

15 mg all cells appear attached

~50+% attached on slide

20 mg all cell appear attached

50-75% attached on slide

25 mg all cells appear attached

50-75% attached on slide

Collagen glucose transport studies will be performed using miniscintillation vials each containing 20 mg of collagen coated beads since this provides maximal attachment of adipocytes to collagen.

Collagen Glucose Transport Study

The purpose of this study is to investigate if adipocytes adhering to collagen interfere with glucose transport.

Procedure

1. Place 20 mg collagen coated microcarrier beads in miniscintillation vials, labeled as in untreated glucose transport procedure. Place in 37°C waterbath.
2. Add 200 uL cells to each tube at timed intervals (i.e., 30 seconds). allow at least 5 minutes for cells to attach.
3. Add appropriate insulin-buffer mix at the same time interval used in step #2.
4. Continue with normal (untreated) cell glucose transport procedure, step #4..

Results

Figure 4, Effect of Collagen on Glucose Transport, shows the composite curve of three runs. There is no difference between this curve and the normal (untreated cells) glucose transport curve. A discussion of the glucose transport curves is at the end of the chapter.

Indo-1 AM and Collagen Glucose Transport Study

This set of experiments was designed to determine if the combination of Indo-1 AM and collagen would effect the glucose transportation curve.

Procedure

Indo-1 AM loaded adipocytes were prepared as described in the section "Indo-1 AM Glucose Transport Study." The glucose transport procedure was done as described in the section "Collagen Glucose Transport Study."

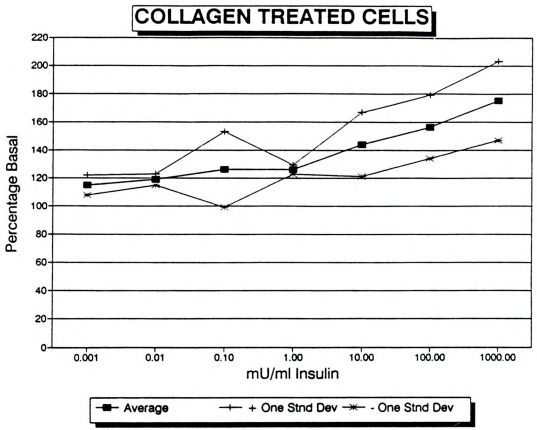

FIGURE 4. Effect of Collagen on Glucose Transport.

Adipocytes and collagen coated beads incubated (37°C) for 30 minutes in KRP:GT-insulin.

D-glucose added for ten minutes. Reaction terminated with $HgCl_2$. Average of 3 runs.

mU/mL Insulin	% basal	STD	mU/mL Insulin	% basal	STD
0.001	115	7	10	144	23
0.01	119	4	100	157	22
0.1	126	27	1000	175	28
1.0	126	3			

Basal= 100% = average CPM of collagen treated cells incubated with no insulin.

Results

Figure 5, Effect of Indo-1 AM and Collagen on Glucose Transport, shows the composite of three runs. There is a increase in glucose transport between this curve and the normal (untreated cells) glucose transport curve. A discussion of the glucose transport curves is at the end of the chapter.

Simultaneous High/Low Response Study

This set of experiments is designed to eliminate any between run variations which may have occurred when doing the insulin dose response curves for the untreated cells versus the Indo-1 AM and collagen treated cells. The basal (low) and high insulin dose responses of the Indo-1 AM and collagen treated cells and the untreated cells are determined simultaneously. Three test tubes are set for each dose to check within run precision. Any variation of the magnitude of the response between the two groups is attributed to the Indo-1 AM and collagen technique.

Procedure

Treated cells:

Indo-1 AM loaded adipocytes were prepared as described in the section "Indo-1 AM Glucose Transport Studies". The glucose transport procedure was done as described in the section "Collagen Glucose Transport Study".

Untreated cells:

Indo-1 AM loaded adipocytes were prepared as described in the section "Indo-1 AM Glucose Transport Study" except no Indo-1 AM was added. The glucose transport

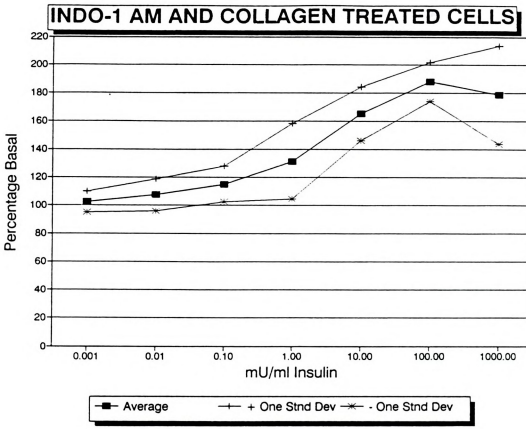

FIGURE 5. Effect of Indo-1 AM and Collagen on Glucose Transport.

Indo-1 AM adipocytes and collagen coated beads incubated (37°C) for 30 minutes in KRP:GT-insulin. D-glucose added for ten minutes. Reaction terminated with $HgCl_2$. Average of 3 runs.

mU/mL Insulin	% basal	STD	mU/mL Insulin	% basal	STD
0.001	102	7	10	165	19
0.01	107	12	100	188	14
0.1	115	13	1000	179	35
1.0	131	27			

Basal = 100% = average CPM of Indo-1 AM & collagen treated cells incubated with no insulin.

procedure was done as described in the section "Collagen Glucose Transportation Study" except no beads were added to the tubes. Except for the addition of Indo-1 AM and microcarrier beads to the treated cell system both groups were treated identically in any cell manipulations, temperature or times involved.

Results

Three runs of the experiment were done. Figure 6, High and Low Responses, provides a summary of the data. The treated cells had a slightly higher response then the untreated cells, which was not evident in the earlier dose-response curves. One explanation is that the Indo-1 AM collagen microcarrier bead system does enhance the response. Another explanation which is the most plausible is that even though both treated and untreated tubes had approximately the same number of cells, the microcarrier system allowed for more cell contact and dispersion in the reaction medium. It had a higher "working" cell count, hence more transport. This would explain why both the low and high values for the treated cells are increased in comparison to the untreated cells.

	Low (0 mu/mL insulin)		High (1,000 mu/mL insulin)	
	% BASAL*		% BASAL*	
	Untreated	Treated	Untreated	Treated
	96	108	250	317
	103	104	168	312
RUN 1	102	109	296	284
	97	112	219	285
	103	97	202	219
RUN 2	**	**	210	226
	104	111	226	230
	96	119	149	195
RUN 3	**	**	192	206

% BASAL = The average ^{14}C count of the untreated low cells for each run is used as the basal count and is equal to 100%. All other columns are comparisons to this count.

FIGURE 6. Simultaneous High/Low Response Study.

Untreated and treated cells (Indo-1 AM, collagen) are stimulated with low (0 mu/mL) and high (1,000 mu/mL) insulin doses in a glucose transport assay: cells incubated (37°C) for 30 minutes in KRP:GT-insulin. D-glucose added for ten minutes. Reaction terminated with $HgCl_2$. Results of 3 runs. ** Insufficient cells for these tubes or lab error.

Impact of Other Indo-1 AM Concentrations on Glucose Transport

This study examines if 2.5 mM Indo-1 AM had any effect on glucose transport by using a lower and higher concentration of Indo-1. It was to test if any change in transport would be enhanced at a different concentration. Glucose transport assays using 0.5 mM Indo-1 AM and 12.5 mM Indo-1 AM were done (1/5 and 5x the amount used in the preceding experiments). A 10 mM Indo-1 AM stock was used to minimize the amount of DMSO needed at the higher concentration.

Procedure

The cells were prepared according to the procedure listed in "Indo-1 AM Glucose Transport Study," with the variation being the amount of Indo-1 AM used. There was no other variation.

Results

Figures 7a and 7b, Other Concentrations of Indo-1 AM on Glucose Transport, shows the composite curves. A discussion of the glucose transport curves is at the end of the chapter.

CHAPTER SUMMARY

The objective of this chapter was to determine if adipocytes could be anchored to a slide and loaded with Indo-1 AM. Once this was accomplished, the question of whether normal cell function would be altered by Indo-1 AM and/or adherence compounds was

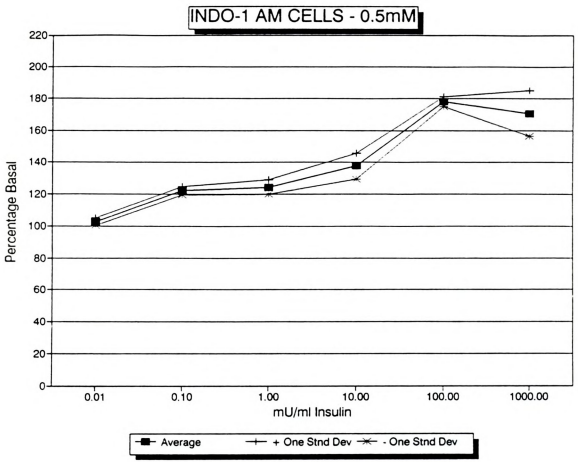

FIGURE 7 a. Other Concentrations of Indo-1 AM on Glucose Transport.

Indo-1 AM treated adipocytes (0.5 mM Indo-1 AM) were incubated in KRP:GT-insulin. D-glucose added for ten minutes. Reaction terminated with $HgCl_2$. One run.

mU/mL Insulin	% basal	STD	mU/mL Insulin	% basal	STD
0.01	103	2.5	10	138	8
0.1	122	2.6	100	178	3
1.0	124	4.5	1000	171	14

Basal= 100% = average CPM of Indo-1 AM treated cells incubated with no insulin.

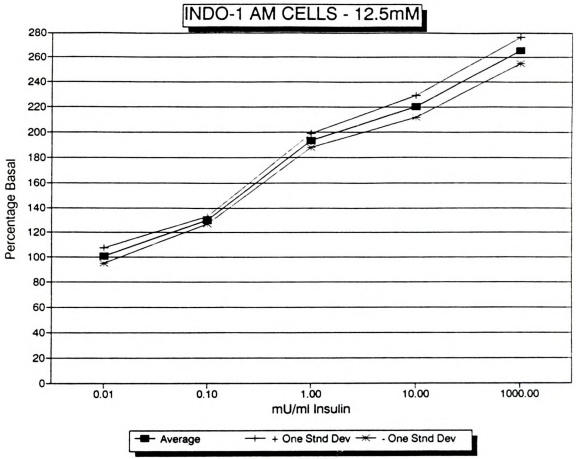

FIGURE 7 b. Other Concentrations of Indo-1 AM on Glucose Transport.

Indo-1 AM treated adipocytes (12.5 mM Indo-1 AM) were incubated in KRP:GT-insulin.

D- glucose added for ten minutes. Reaction terminated with $HgCl_2$. One run.

mU/mL Insulin	% basal	STD	mU/mL Insulin	% basal	STD
0.01	101	6.1	10	221	9
0.1	130	3.0	1000	266	10
1.0	194	5.5			

Basal= 100% = average CPM of Indo-1 AM treated cells incubated with no insulin.

investigated. Adipocytes adhered to both collagen and poly-L-lysine coated slides, but not to formvar slides. Collagen had a slightly less fluorescent background than poly-L-lysine and was chosen for further study. Poly-L-lysine in lower concentrations (<0.1 %) should not eliminated from consideration as a suitable adherence compound.

It was found that Indo-1 AM is capable of entering the adipocytes. A 30 minute incubation (37°C) of a 2.5 mM Indo-1 AM loading solution concentration achieved a good fluorescence level in adipocytes. The Indo-1 appeared distributed evenly throughout the cell.

The question of the Indo-1 AM and collagen effect on adipocytes was addressed in a series of glucose transport assays. Figure 8 is a summary graph of the glucose transport curves. The shape and magnitude of these dose response curves together with the results of the simultaneous high/low response study indicate that there is no alteration of glucose transport in adipocytes treated in the manner described compared to untreated cells. Each curve represents the average of three different runs, each run being done on a separate day with 3 test tubes per dose to check within run precision. It should be noted that other authors have obtained higher fold maximum responses (i.e., 5 fold or higher) [1] in adipocyte stimulation with insulin. They used smaller, 120-150 gram Sprague-Dawley rats as compared to the 150-200 gram rats available for this study. This size variation indicates that younger rats may have been used for the other studies. Since insulin response decreases with the age this is the most probable cause of the different maximum response. The rats in this study were part of a pharmacology control group and needed to be a minimum of 150 grams. With increased emphasize on limiting animal experiments, coordinating this research with pharmacology research was essential. Cells used in this study were collected at one site, placed in a 37°C collagenase

solution and transported by car to another building. Due to uncontrollable external factors this additional specimen handling was not always uniform in time or outside temperature, and may have resulted in 1) decreased cell function due to decreased cell viability or 2) increased basal transport levels. Either factor would account for some of the variability seen in response ranges. Insulin lot variability has also been suggested as a source of maximum response fluctuation. All experiments done in this study were done using a single lot of insulin. The bottle was reconstituted and frozen aliquots were stored at -70°C until needed. Lot variation is a possible explanation for the lower maximum response seen in this study as compared to other studies, but would not explain fluctuations within this study. It has been reported that the medium glucose concentrations are associated with changes in basal rates, maximal insulin stimulation and insulin sensitivity in adipocytes [27]. The variability of glucose concentrations used among researchers accounts for some of the curve differences. The potential number of variables among researchers that affect magnitudes of dose-response curves for glucose transport is large. Selecting a set of working parameters and having only the desired set of variables within a set of glucose transport assays is the most critical factor.

Analysis of the curves in Figure 8 suggests a left shift of glucose transport at the 10.00 mU/mL and 100.00 mU/ml insulin concentrations induced by Indo-1. There is an apparent increased level of glucose transport at these insulin levels when compared to the normal glucose curve. However if one examines the mean percentage basal values and their standard deviation, Indo-1 at 10.00 mU/mL insulin = 151% +/- 7 versus untreated 143% +/- 8, and Indo-1 at 100.00 mU/mL = 174% +/-13 versus untreated = 161% +/- 12, there is an overlap of their values. Considering the precision range, as expressed by standard deviations, of the method, the Indo-1 versus untreated cells

difference is not enough to state that there is a definite left shift. The microbead effect of producing a higher working cell count and the precision range of the assay would explain differences in the collagen and Indo-1 and collagen curves versus the untreated cell curve. There is not a large enough difference between the values on the curve to conclude that glucose transport is effected by the 2.5 mM Indo-1 AM and collagen. Due to the small sample size (three mean values for each point), it is inappropriate to use inferential statistical analysis, and only descriptive statistics are reported. The 12.5 mM Indo-1 study does indicate that a left shift may occur at this concentration. This was considered a "pilot study" and was done one time, therefore no firm conclusions can be made. However, if further study confirms the left shift it suggests a mechanism in which decreasing the amount of intracellular calcium signals an increase in glucose transport. Large amounts of other calcium dyes such as Quin 2 decrease glucose transport by binding to calcium, since Indo-1 also binds to calcium one would expect a similar effect. The difference between the two dyes may be the magnitude of the calcium binding, Quin 2 shuts down some systems whereas Indo-1 with a slight binding imitates a cellular event and releases or enhances a second messenger system without shutting down systems.

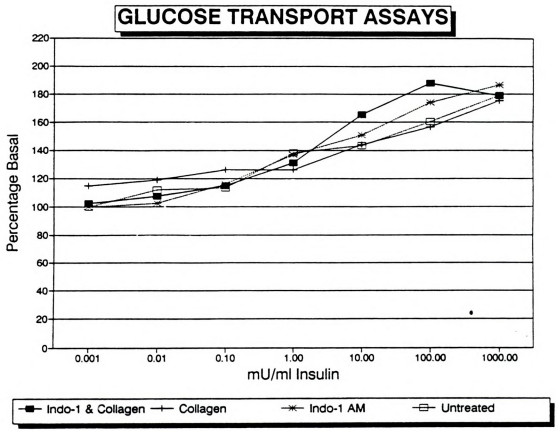

FIGURE 8. Summary-Glucose Transport Curves.

This graph presents the mean values from Figures 2, 3, 4, and 5.

CHAPTER III

ACAS STUDIES

The goal of this thesis is to develop a technique using Indo-1 AM in conjunction with the ACAS 470 to study free calcium in adipocytes. At present the function of free calcium in insulin stimulated adipocytes is unclear. Researchers have suggested various roles including that of a second messenger for release of the glucose transport protein or as a component in a protein kinase C system. Chapter Two of this thesis has demonstrated two factors: 1) that Indo-1 AM may be loaded into the adipocytes; and 2) the Indo-1 AM/collagen system does not affect glucose transport in adipocytes at a 2.5mM concentration and thus could be a valid tool in the study of free calcium in the insulin/glucose transport response. As a preliminary step in this study the free calcium level in unstimulated (basal) adipocytes was measured. These cells were then stimulated with insulin to determine if the free calcium levels changed. A detail explanation of these studies will be presented in the latter part of this chapter. The first part of the chapter will explain the calcium calibration curve.

CALCIUM CALIBRATION CURVE

The calcium calibration curve is based on a method described by Grynkiewicz et al [17] to measure cytosolic free calcium. This method utilizes the ratio of the fluorescent intensities of free indo-1 and calcium bound indo-1 at their emission wavelengths. Grynkiewicz et al [17] limit the applicability of the method to these assumptions: 1) Indo-1 forms a simple 1:1 complex with Ca++; 2) it behaves in cells as it does in calibration

media; 3) it is sufficiently dilute for fluorescence intensity to be linearly proportional to the concentration of the fluorescent species. When these conditions are met calcium measurements are independent of dye content. It should be noted that assumption one involves forming a Indo-1: calcium complex. As a corollary then only calcium in areas of cells where Indo-1 is present is being measured. In very lipophilic areas such as fat droplets little or no dye may be present, hence calcium is not being measured in these areas.

A calcium concentration curve is generated by measuring the fluorescent intensity of calcium bound Indo-1 and free Indo-1 at known concentrations of calcium To achieve nanomolar (nM) concentrations of calcium, EGTA is used in the calibration procedure [28]. The EGTA and calcium standard solutions are prepared in 15% ethanol (V/V with H_2O) to compensate for cytosolic polarity as reported by Popov et al. [29]. A detailed explanation of calcium curve formula derivation is included in the appendix.

Reagents and Materials

Bionique Chamber/Dish [30]

Made by Bionique, P.O. 535, Lake Placid, NY 12946

Pipettes -1 mL and 20 uL

Indo free acid: Pentapotassium salt, cell impermeant

Molecular Probes In, Box 22010, Eugene, OR 97402

1 mM stored in 50 uL aliquots and kept at -80°C until needed

Calcium Stock Standard - Orion 0.100 M standard

Stock 20 mM Na/ 115 mM K solution

8.574 gm KCL, 1.169 gm NaCl

Dissolve in 100 mL H_20, store in refrigerator

EGTA-500 mM

500 mM EGTA (0.01902 gm); 115 mM K, 20 mM Na (10 mL stock Na/K solution);

10 mM HEPES (0.2383 gm). Dissolve in 15% ethanol solution. pH to 7.02.

5 mM Ca Standard

5 mM Ca (5 mL stock standard); 115 mM K, 20 mM Na (10 mL stock Na/K solution);

10 mM HEPES (0.2383 gm). Dissolve in 15% ethanol solution. pH to 7.02.

ACAS Parameters

Each set of PMT (photomultiplier tube) settings requires a calibration curve. Therefore to avoid errors which may be inherent when using several curves it is ideal to use the same PMT settings for all experiments so that the same curve may be used. The curve should be made the same day as the experiments are run to minimize possible instrument fluctuation.

Amount of free Indo to use

It is impossible and not necessary to know the exact amount of Indo-1 AM that has entered a cell. However it is helpful to estimate the amount to know what concentration

range should be included in calibration curve. There are several factors to consider when doing the estimation. These are:

1. Observe the color values obtained for the cells, if they are in the 2000-3000 color range for detector 1, and the 1000-2000 color value range for detector 2, these values should be duplicated on the working part of the curve. The ratios obtained for the cells should be approximately the same ratios obtained on the curve for those values.
2. Estimate what percentage of the Indo-1 AM may have entered the cell. Observe an unrinsed Indo-1 AM incubation cell and the slide background. Is the cellular fluorescence less than, equal to, or greater then the background? If there is an equal distribution of fluorescence, try using the Indo-1 AM incubation concentration for the free Indo at the zero calcium point. If the background is greater use a lesser amount of free Indo.
3. It may be impossible to obtain a zero calcium point for the curve. Physiologically one does not see a zero nM calcium level (100% free Indo-1) in cells but both free and calcium bound forms. The 100% free form used in the standard curve may record in the 4000+ color value range of detector one, yielding an invalid ratio (it is also not ideal for the optics or electronics to scan in this range). There are two possible solutions: 1) A starting value of 25 nM or 49 nM calcium may be needed to obtain a desired color value range for detector 1; or 2) decrease the amount of free Indo used. Whichever method is chosen refer to factor 1.
4. Since the standard curve solution does not exactly duplicate the cell's interior, it has been reported that ethanol helps minimize errors inherent when using an aqueous

solution. There may be future developments in this area. The adipocyte with its high lipid content may pose its own fluorescence curve potential errors.

Procedure

1. Place 1.0 mL EGTA and 0 uL Ca^{2+} solution onto a slide, and scan the slide. Refer to factor 3 in above text.
2. Add the next listed aliquot of Ca^{2+} (5 mM Ca Standard) to obtain the desired nM calcium. Mix completely between each addition rinsing out pipette tip. Use a new pipette tip for each addition. Scan the slide. Detector 1 and 2 values are recorded. An example of a curve is shown in Figure 9. The color value of Detector 1 (free Indo) will decrease as the color value of Detector 2 (calcium bound Indo) increases forming a sigmoid shaped curve.
3. Repeat step 2 until a plateau is achieved (top of sigmoid curve).

BASAL STUDIES

The free available calcium concentrations in basal (unstimulated) adipocytes were defined and measured under two different conditions: 1) in adipocytes incubated in a glucose free medium (KRP-glucose transport buffer); and 2) in adipocytes incubated in a glucose medium (KRP-I, 200 mg/100 mL dextrose). The adipocytes were obtained by the method described in Chapter Two and placed in the appropriate buffer for the Indo-1 AM incubation period and washes. The following chart summarizes the results.

Dextrose	#cells	nM free Ca^{2+}	std dev
0 mg%	31	212	+-34 nM
200 mg%	20	223	+-46 nM

Added uL Ca^{2+}	nM Ca^{2+}	Ratio
0	0	0.000
5	25	0.198
5	49	0.252
5	98	0.317
5	123	0.403
5	146	0.547
5	169	0.656
5	192	0.740
5	215	0.831
5	238	0.962
10	275	1.225
10	327	1.489
5	350	1.716
5	370	1.881
5	391	1.891

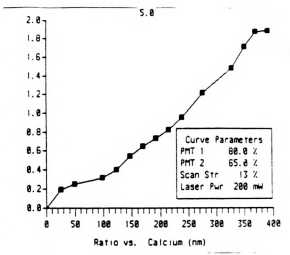

FIGURE 9. Example of a Calcium Calibration Curve.

1.0 mL EGTA solution is placed into a Bionique chamber dish, 5 uL aliquots of Ca^{2+} (5 mM Ca Standard) are added to obtain the nM calcium shown. Ratio = Detector 2 value/Detector 1 value. nM is printed as nm on the ACAS.

There was no significant difference between the two groups. This confirms the premise that any free calcium changes in an insulin/glucose transport system would be caused in conjunction with insulin not by glucose (dextrose) alone. Other researchers have obtained calcium values of 45-60 nM. Their measurements were done using Fura-2 loaded cell suspensions lysed in a test tube [1], not individual cell measurements as reported here. Further investigation of both methods is needed to determine if both are measuring total free available cytosolic calcium. The high lipid content of the adipocyte may affect measurements of either method. According to one report, calibration curves obtained using aqueous solutions lead to an overestimation of cytosolic calcium concentration [29]. It suggested use of a calibration solution containing 22-32 percent ethanol or glycerol. A 15 percent ethanol calibration solution was used for curves done in this thesis. Verification of the validity of the calcium curve duplicating cytosolic conditions in adipocytes for both Fura-2 and Indo-1 AM methods needs to be done.

INSULIN STIMULATION STUDIES

Insulin stimulation studies were done on both types of single cells, those incubated in the presence of 200 mg/100 mL dextrose and those incubated without dextrose. Initially the cells were scanned for five minutes to test for any photobleaching effects and to monitor calcium fluctuations in unstimulated cells. Calcium levels were found to vary by as much as +/- 10 nM from the 0 second level during the five minute time period. Larger fluctuations (greater than 200 nM) in calcium levels were found under these conditions: the cell moved out of the scanning field; another cell or artifact moved into the field; or the cell disintegrated. It is therefore critical when analyzing the scans to save the scan images and refer back to them (see Figure 10). Insulin stimulation studies were done by

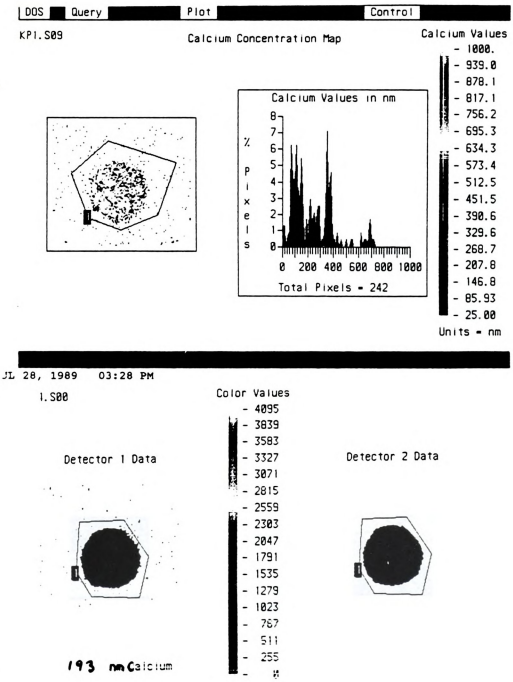

FIGURE 10 a. Basal Adipocyte.

Bottom shows ACAS image during experiment. Top shows calcium distribution.

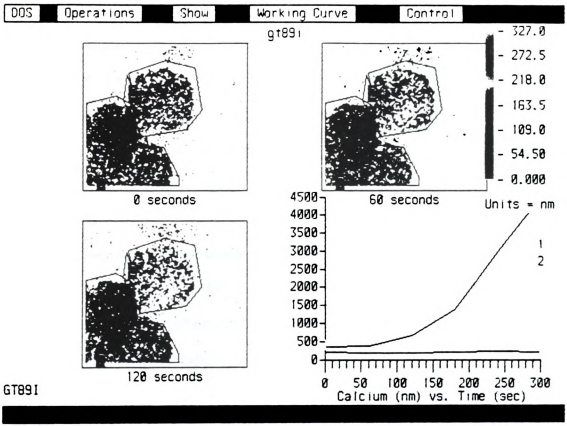

FIGURE 10 b. Ruptured Basal Adipocyte

Basal adipocyte in a glucose free media (0 Seconds). As cell ruptures calcium levels appear to increase. Demonstrating the need to correlate all data with optical image.

monitoring the basal calcium levels for one minute to check for gross fluctuations. Images were saved as 1000 mU/mL insulin was injected. 1000 mU/mL insulin was chosen because it produces the maximum glucose transport response. Figure 11 shows a typical response of single cells from each group. There is initially a slight decrease in calcium within the first 60 seconds followed by an increase in calcium compared to the unstimulated cell. There are several explanations for the decrease observed within the first 60 seconds. One, it is an instrument/technique artifact, or two, it reflects an intracellular event. It is probably not an artifact since it is consistently a decrease, instrument/method variation should result in a mix of lower and higher values being observed. Baseline studies have shown no photobleaching at the PMTs or time frames used. To test the effect of the buffer, insulin free KRP-I was added. There was no observed calcium change. Therefore, the calcium decrease cannot be caused by the buffer or injection technique. The other explanation is that the calcium decrease represents the cell using intracellular available free calcium. The subsequent increase may 1) reflect calcium entering from outside the cells or 2) calcium being made available from an internal source not accessible to Indo-1. Studies have shown that Ca^{2+} blocking agents will decrease glucose transport in adipocytes, presumably by preventing calcium from entering the cells. It has been theorized that free Ca^{2+} may increase 10 fold within 1 second after exposure to insulin [1]. Other researches could not detect effects of insulin between 15-90 seconds on free cytosolic calcium, but noticed an increase after 90 seconds up to 12 minutes. What the ACAS calcium experiment may show is an initial utilization of intracellular free calcium (first the decrease) which triggers a message for increased calcium, hence increasing the calcium influx from outside the cell. Several researchers have suggested a need for extracellular calcium in the glucose transport process. This

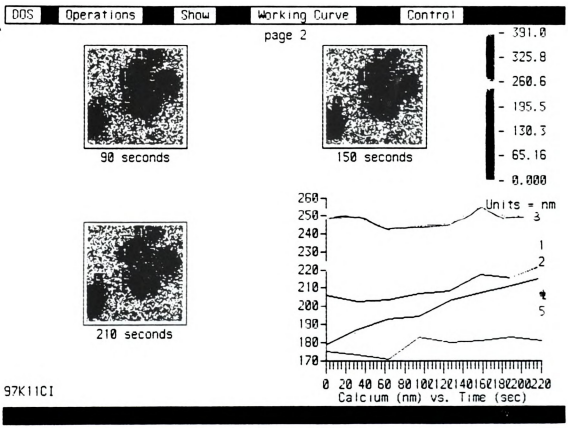

FIGURE 11. Insulin Stimulation of an Adipocyte.

The basal calcium level of adipocytes in a 200 mg% glucose is measured (0 sec). Insulin (1000 mU/mL) is added at 1 second. Slide is scanned every 30 seconds and calcium change determined.

increased intracellular calcium is in response to insulin, and is not dependent on the presence of extracellular dextrose as it occurs in cells incubated with and without dextrose.

One set of future experiments are those that would identify the source and mechanism of the calcium increase. By modifying the above experiment using a calcium free medium, the cell becomes the only source of calcium. If any calcium increase occurs its source must be intracellular. If there is no calcium increase then an extracellular (ie medium) source is indicated. By use of various calcium blockers to inhibit extracellular calcium movement the exact mechanism of calcium entry into the cell, such as a voltage dependent or ATPase dependent channel, may be determined. A second set of future experiments concerns use of a decreased time frame (less than 30 seconds) between scans. This is needed to determine the exact time lapse and magnitude of the calcium curve. The thirty second value obtained in the experiments may not be the true lowest calcium value. It may actually occur before or after 30 seconds and at a much greater magnitude. The size of the adipocytes prevented smaller time frames at the given speed. A higher scan speed may have sufficient cellular resolution to permit smaller time frames, although when tried there was occasional 'sticking' of the slide to the stage which resulted in streaked images. Other scanning options include doing sections of adipocytes instead of the whole cell or doing cross-sectional line scans to permit smaller time frames. The question then arises as to how these scans relate to the total cellular event. No cellular structure was evident on the basal adipocyte scans suggesting that calcium is uniformly distributed throughout non lipophilic areas of the cell, therefore a small section or line scan may be valid. However, some cells did exhibit a high calcium concentration around parts of the cell membrane. The calcium then appeared to diffuse

through the cell. Additional studies are needed to determine the nature of these calcium "hot spots". They may be part of a second messenger system. A fourth set of experiments would be to correlate the calcium change, both time and magnitude, with the insulin dose. It should be investigated whether lower insulin doses would cause an identical calcium shift or if the shift is dose dependent.

CHAPTER IV

SUMMARY AND CONCLUSIONS

The goal of this project was to determine the feasibility of measuring intracellular available calcium in adipocytes using Indo-1 AM and the ACAS 470. Collagen is the preferred adherence compound although poly-L-lysine also may be used. A 2.5 mM Indo-1 AM adipocyte solution with a 30 minute incubation at 37°C results in adequate cellular fluorescence for use on the Acas 470. It has been reported that incomplete hydrolysis of intracellular indo-1 AM in endothelial cells [31] and of Fura-2 [32,33] in leukocytes has led to errors in calcium concentration. The possibility of a similar occurrence in adipocytes is not considered in this paper.

The glucose transport assay studies conducted as a part of this project indicated that 2.5 mM Indo-1 AM and collagen did not alter the insulin stimulated glucose transport in adipocytes and may be used to study intracellular calcium levels in insulin stimulated adipocytes. A concentration study did indicate a possible left shift in glucose transport curves when using 12.5mM Indo-1 AM. To study the effect of collagen on glucose transport a method utilizing collagen coated microcarrier beads was developed. This method allows for more cell contact with a reaction medium than does the conventional glucose transport method. In the conventional method adipocytes float to the top of the reaction medium and often stick together. Thus the number of cells or cell surface area in contact with the reaction medium varies with tube diameter and mixing technique. Mixing of the reaction tube may cause cellular destruction and differs among researchers in intensity especially if hand mixing is involved. This mixing also results in fluctuation in

the number of viable cells. The microcarrier beads diminish the above fluctuations by permitting a more consistent dispersion of cells which have maximal contact with the reaction medium with minimal mixing to keep cells dispersed and therefore less cell destruction. With less cell destruction and more uniform cell/reaction media contact the precision of the transport procedure within and between test runs is improved.

It would be interesting to observe the Indo 1-AM entering the cell and its dispersion pattern to determine if any substructure is visible. Two possible approaches are possible: 1) incubate the cell solutions with Indo-1 AM, and at timed intervals take some cells, remove excess Indo-1 from the surrounding solution and observe them; or 2) place some untreated cells on a slide and inject them with Indo-1 AM. The latter is a " filling in a black hole" approach which requires predictive knowledge for good scan placement and PMT settings or the ACAS screen become a "white out" indicating too much fluorescence.

The second part of this project was to utilize the Indo-1 AM adipocyte loading methodology with the ACAS 470. The basal calcium level of adipocytes incubated with or without glucose in their medium was measured. No significant difference existed between the two groups. Stimulation of these basal adipocytes led to an initial decrease, followed by an increase in intracellular calcium, supporting the theory that calcium is involved in the insulin-glucose transport process. The source of this increased calcium is most likely extracellular. If the left shift in the glucose transport curve at 12.5 mM is verified it is possible that at this concentration Indo-1 is binding up low concentrations of calcium and binding is being interpreted by the cell as part of the initial calcium decrease. It thus enhances a signal for increased transport which is related to the observed increased calcium. The magnitude of the initial decrease calcium may be related to the magnitude of the response-the glucose transport. Future experiments are needed to

investigate both the source and the mechanism of the calcium increase. Additionally, the function of the increased calcium needs to be studied. Since the effect of insulin on adipocytes is pleiotropic a change in calcium levels may not all be associated with glucose transport. If "mutant" strains of adipocytes exist or may be developed in cell culture, comparing them to "normal" adipocytes could lead to insights on the role of calcium in the insulin interaction.

It is now possible to monitor calcium levels in individual adipocytes as they respond to insulin/insulinomimetic agent stimulation in the presence of various calcium blockers. By individual or combination use of these agents, and correlating the results to known effects of insulin and insulinomimetic agents placement of calcium in a cascade or bifurcating insulin pathway may be established.

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APPENDIX I

SAFETY

The major safety concerns with the ACAS-470 are the electrical power system and the laser system which includes both visible (blue-green) and invisible (ultra-violet) radiation. User safety during operation is incorporated in the ACAS-470 design. By following correct operating procedures and using common sense safety methods there should be no personal hazard. Basic rules insuring personal safety are:

1. Do not attempt any maintenance or adjustment unless specifically trained.
2. Always keep safety shields down.
3. Use UV absorbing safety glasses when adjusting the light beams.
4. Never view the lights directly.
5. Do not wear reflective jewelry near areas illuminated by the laser.
6. Do not place anything in the laser light path.

APPENDIX II

CALCIUM EXPERIMENTAL PROTOCOL

An experiment on the ACAS consists of four phases, 1) cell preparation; 2) ACAS-cell data generation 3) Calcium curve generation and 4) data analysis. Phase 1, cell preparation, is done away from the ACAS workstation and is described in earlier chapters. Phases 2 and 3, ACAS cell data and calcium curve generation, are done in on the ACAS workstation on a prescheduled signup basis. Phase four is done on the DASY computer located in another room although some data is examined while on the ACAS to verify proper data storage and technique usage. Explanations of the procedures involved follows.

CELL DATA GENERATION AND CALCIUM CURVE GENERATION

ACAS-470 START UP/SHUT DOWN PROCEDURES

Start-up Procedure

To measure calcium, the ACAS-470 must have the UV optics in place. Currently this adjustment is done by assigned ACAS personnel on a predetermined schedule. The following is the routine start up procedure performed by the user thirty minutes before use:

1) Visual check of instrument, objective lens of microscope should be down.
2) Turn cooling water on.
3) Turn master power switch on: the computer and interface unit are "ON", floppy disk drive door is open. Computer will boot and ACAS 470 control program will execute. The Meridian logo page will appear on the screen when the program is ready.
4) Laser start up-done by computer entry command.

Shut Down Procedure

1) Exit ACAS program.
2) Check that data are stored on the disk, remove disk, leave drive door open.
3) Turn master power switch to "OFF."
4) Run cooling water for at least five minutes after laser is turned off.

Microscope Instructions

The ACAS-470 utilizes an Olympus IMT-2 inverted microscope modified with a motorized stage, detector housing, optical mount and filter holder assembly. Several objective lenses are available. The motorized X and Y axis microscope stage, is externally referenced when the ACAS is turned on. The stage is then automatically returned to a centered position and is internally referenced from that point. The user may control movements in 0.25 microns increments along each axis with a speed range of 0.25 to 20.0 mm/ second. This permits a rapid and accurate repositioning of the specimen to allow for sequential measurements.

Instructions for Cell Placement for Calcium Analysis

1) Place the slide or Bionique chamber dish, containing indo-1 AM loaded cells, into the correct size slide holder and mount it on the microscope.
2) Rotate the desired objective lens into place and focus cells.
3) If cells will not focus properly refer to the ACAS training manual pages 13-15 which describe microscope alignment, including eyepiece adjustment, bulb filament alignment condenser alignment and phase ring adjustment.

4) Stage movement is accomplished by use of the mouse (click both buttons at once then move mouse to move stage, click left button to stop movement).

Microscope Preparation for Laser Illumination of the Cell

Once the desired cell is centered and the calcium program is engaged one can use the laser to excite the cell. The microscope components are readied by the following steps:

1) Place detector hood down to eliminate interference from the fluorescent ceiling light.
2) Turn light off (button on left side of microscope).
3) Push shutter out (lever on right side of microscope) to prevent stray light from entering the eyepiece.
4) Turn laser on using the mouse (click right button) or by the keyboard, a "laser on" message appears on the screen.
5) Check laser alignment -- a bluish light is near or on the crosshairs.
6) If the laser is not aligned see assigned ACAS personnel.
7) Neutral density filters are inserted in the back filter holder assembly if needed to adjust for brightness. To minimize photobleaching the intensity should be as low as possible while maintaining the desired signal to noise performance.

COMPUTER INSTRUCTIONS

Usage/Data Storage

1) The computer uses DOS, with entries done by either keyboard or mouse. Bernoulli disks are used for individual data storage.

2) The MERIDIAN logo page, which appears when the ACAS program is initially booted includes the master program main menu selections which is displayed on the top line of the screen.

3) Master program main menu selection titles are:

DOS ANALYZE LIST SORT UTILITIES OPTIONS TEST

When a title is chosen by use of either the keyboard (press first letter of title then enter) or by the mouse (Point arrow to title and press both buttons) the options contained in each title will be displayed for additional choices.

4) To return to the master program main menu selection page enter **CONTROL-** cancel.

Bernoulli Disk Usage for the ACAS

1) When the MERIDIAN logo page appears on the screen, place formatted bernoulli disk in the disk drive, close door, allowing boot up. Change default to this drive for data storage on this disk.

2) To format a disk go to master program main menu selection-option: DOS-Init diskette.

3) To change default go to master program main menu selection-option: DOS-change default change to E drive (type e:), then make a subdirectory and again change the default (type e:****** [up to 8 characters are allowed for a subdirectory name]) to store data in this subdirectory.

4) If default is not changed all data will be stored in the main directory (drive C).

5) To display files go to master program main menu selection-option: DOS-file utility.

Initiation of the Calcium Program

1) Go to the MERIDIAN logo page's master program main menu selections, enter title **ANALYZE**, option-ratio.
2) Calcium, Ph, and generic appear on screen -- enter calcium.
3) The main ratio program selection will appear on the top line. The options appear when a selection is chosen. The selections and options are:

DOS	**SCAN**	**ANALYZE**	**WORKING CURVE**	**CONTROL**
Quit	Point	Point	Build Curve	Cancel
Change Default	Line	Line	Edit Curve	
	Image	Image	Select Curve	

To Scan a Cell - Parameter Definitions

When scan option is chosen, a parameter page appears on the screen. As an example when an image scan is chosen these parameters need to be set:

Filename	Sequence of 1 to 8 characters to identify data.
PMT 1	(0-100%) The percentage of the maximum allowable high voltage applied to the on-axis detector.
PMT 2	(0-100%) The percentage of the maximum allowable high voltage applied to the off-axis detector.
Step size	Number of microns between data points.
Scans	Number of times data should be collected.
Delay	Time interval between the start of a scan #1 and the next scan
X points	The number of data points in the X-direction.
Y points	The number of data points in the Y-direction.

Speed	Maximum stage speed in millimeters per second.
Scan strength	(0-100%) The percentage of the available laser light directed at the target which prescribes the proper intensity for fluorescent measurements.
Laser power	Approximate output power of the laser in milliwatts. The amount of light at the target depends on the laser power setting, blast or scan strength, losses in the optical path and laser wavelength.
Save size	8 or 12 bits.

Example of Parameter Settings for an Adipocyte-ACAS Experiment

F1	Filename	adp12
F2	PMT 1	70
F3	PMT2	60
F4	Step size	0.25um
F5	Scans	6
F6	Delay	20.0 sec
F7	X points	120
F8	Y points	110
F9	Speed	0.25 mm/sec
F0	Scan str	20%
S1	Laser power	200 mW
S2	Save size	12 bit

Scanning a Cell

Once parameters are set, click bottomline to initiate scanning. Profiles from both detectors will be displayed on the screen during scanning with a color bar in the middle of the screen. The initial desired color range is in the center of the bar for both detectors. By adjusting the PMT values, scan strength or use of a neutral density filter this range is be achieved. Since the working curve is dependent on the PMT values, choose one set of values appropriate for all experiments if possible. Scan strengths can vary without changing the curve. The following are some commands that may be needed during a scan:

1) To abort an in progress scan: hit Esc key.

   Abort scans whose intensity is too high -- that is, they appear white.

2) To save or cancel data; after scanning is done a message appears on the bottom line of the screen, click bottom line to save data or use cancel option if data is not to be saved.

3) To continue: A prompt to do another experiment will appear, if yes sequence will repeat starting with the parameter settings. If no, program will return to main ratio program selection page.

Calcium Standard Curve Generation

A standard curve must be made for each set of PMT values used. Ideally the curve is made the same time as the experiments are performed. Details of the solutions and procedure for the calcium calibration curve are presented in Chapter Three. To make a curve, perform the following steps:

1) Go to main ratio selection page : **CURVE**-build curve
2) Assign a title
3) Assign a unit of measure
4) Select direction of ratio: 2:1
5) Select parameters (match experiments)
6) Scan slide - containing desired concentration of calcium
7) Enter curve value, or Esc to cancel scan
8) Steps 5-7 will repeat, until all desired points are measured

Definitions

The parameters used for an image scan are defined on page 68. These are additional definitions of terms used for operating the ACAS.

Blast strength	(0-100%) The percentage of the available laser light directed at the target for photon destructive purposes such as cell surgery, photobleaching.
PMT	(Photomultiplier tube) Detects light along each optical detection axis, PMT 1 is the on-axis detector, PMT 2 is the off-axis detector.
Smoothing	The averaging of a point with the two points on either side to reduce apparent noise.
AOM	Acousto-optic Modulator.
Number points	Number of data point in a line scan.

DATA ANALYSIS

Most data analysis is done on the DASY 9000 (data analysis system) located in another area. The DASY 9000 includes all the ACAS software needed to analyze data, thus enabling the ACAS to be available for data generation. The program and commands are identical to those on the ACAS-470 workstation. Using the Analyze menu and working curve the data may be looked at in a variety of ways. Calcium concentration may be determined for the whole cell, a region of it, a line through it or by points. A histogram will show the distribution of the calcium levels on a point by point basis. Calcium versus time and ratio versus time graphs are available for experiments done over a time period. Either single regions (or cells) or multiple (cells) regions in a scan may be analyzed in the same scan frame.

APPENDIX III

CALCIUM CURVE - FORMULA DERIVATION

A calcium measurement in a viable cell is approached by creating a calibration solution that resembles the biophysical properties of the cell. Since there exists a dynamic equilibrium between Indo in dye containing compartments, calcium, and calcium sequestering systems in the cell an absolute calcium measurement is not achieved. What is measured by the ACAS-470 is a measurement of the fluorescent emissions of the calcium bound and free Indo in the cellular compartments permeable to the dye in a given time frame. The ratio of these signals is then translated to available calcium by use of a calibration curve derived from calibrating solutions.

A major factor in preparing calibrating solutions is that even "pure" water may have calcium contamination at micromolar levels higher then calcium levels found in cells. Achieving a calibration solutions commensurate with the amounts of subcellular calcium is accomplished by use of the nonfluorescent calcium chelator EGTA. EGTA is present in the calibrating buffer at a sufficient concentration to determine the amount of Ca^{2+} ions available for binding to indo. Both the EGTA-Ca and indo-Ca complex are in equilibrium with each other and the uncomplexed Ca^{2+}. A cubic formula accounting for the EGTA and indo calcium complex and their interaction has been determined and is listed below:

$AY^3 + BY^2 + CY + D = 0$

$A = 1$

$B = L_1 + L_2 + K_{d1} + K_{d2} - Ca_T$

$C = (K_{d1} \cdot K_{d2}) + K_{d1}(L_2 - Ca_T) + K_{d2}(L_1 - Ca_T)$

$D = -K_{d1} \cdot K_{d2} \cdot Ca_T$

Y = Free calcium ($[Ca^{2+}]_{cyt}$)

and Ca_T = total calcium, L_1 = total EGTA concentration, L_2 = total indo concentration, K_{d1} = EGTA dissociation constant, K_{d2} = indo dissociation constant

Therefore when using this method the amount of calcium available for chelation is being measured.