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Myosin II is required for GLUT4 mediated glucose uptake in 3T3-L1 adipocytes. Previous studies from our lab have shown that myosin IIA and GLUT4 are stimulated to translocate to the plasma membrane from a perinuclear region upon insulin stimulation. We also know that upon insulin stimulation, myosin IIA colocalizes with filamentous actin (F-actin) near the site of GLUT4 fusion and that myosin IIB is localized at the cell cortex and is unaffected by insulin stimulation. Our study aimed to determine the role of myosin II in GLUT4 docking and fusion to the plasma membrane in adipocytes and also to determine which myosin II isoform is involved in this process.

Using fluorescence microscopy, we visualized the localizations of myosin IIA, GLUT4, syntaxin-4, and VAMP2 in adipocytes stimulated with insulin in the presence or absence of the myosin II specific inhibitor, blebbistatin. Our studies showed that by inhibiting myosin II, there was a 56% decrease in the localization of GLUT4 at the plasma membrane as well as a 31% decrease in the interaction between the GLUT4 vesicle and the membrane bound docking and fusion protein, syntaxin-4. We also showed that there was an accumulation of GLUT4 below the plasma membrane in cells treated with blebbistatin suggesting that inhibition of myosin II did not prevent insulin-stimulated GLUT4 translocation towards the plasma membrane but did prevent the GLUT4 vesicles from reaching and fusing with the plasma membrane.

Since 3T3-L1 adipocytes express two myosin II isoforms, we wanted to determine the roles of each isoform at the cell cortex. Using a plasma lawn assay and fluorescence
microscopy we examined the localizations of myosin IIA, myosin IIB, GLUT4, and myosin Va at the plasma membrane. Our studies demonstrated that the level of myosin IIA at the plasma membrane decreased by 53% in the presence of blebbistatin while the level of myosin IIB was not affected. However, myosin IIA and GLUT4 had a distinct punctate staining pattern that showed a clear separation between myosin IIA and GLUT4. Immunostaining for myosin IIB and GLUT4 showed that myosin IIB remained in a dense lawn across the plasma membrane while the localization of GLUT4 at the plasma membrane decreased in adipocytes treated with blebbistatin. The studies described here demonstrate that GLUT4 was prevented from fusing with the plasma membrane while the localization of myosin IIA at the plasma membrane was also affected when myosin II was inhibited. This study suggests that myosin II is required for the GLUT4 vesicle to access the plasma membrane. The information gained from this study will help to further our understanding of GLUT4 vesicle trafficking.
DETERMINING HOW MYOSIN II AFFECTS GLUT4 DOCKING AND FUSION TO THE PLASMA MEMBRANE IN 3T3-L1 ADIPOCYTES

by

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A Thesis Submitted to the Faculty of The Graduate School at The University of North Carolina at Greensboro in Partial Fulfillment of the Requirements for the Degree Master of Science

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CHAPTER I

INTRODUCTION

Insulin stimulated glucose uptake is a vital mechanism involved in maintaining whole body glucose homeostasis. The insulin responsive glucose transporter protein (GLUT4) is responsible for transporting glucose across the plasma membrane and into the cell upon insulin stimulation. GLUT4 is highly expressed in adipose tissue and skeletal muscle, two cell types that are known to take in excess glucose when blood glucose levels are high. In adipocytes, GLUT4 is stored in a perinuclear region in vesicles called GLUT4 storage vesicles (GSVs) and upon insulin stimulation, are mobilized to the plasma membrane (4). When insulin binds to its cell surface receptor on adipocytes, signaling pathways which include phosphatidylinositol-3-kinase (Class IA PI3K), mitogen activated protein kinase (ERK), and calcium are activated (4, 33). Together, these pathways will activate the translocation of the GSVs. To translocate, GSVs travel on microtubules toward the membrane where they eventually interact with an actin filament tether (4). In adipocytes, an actin cytoskeleton also acts as a barrier between the GLUT4 vesicle and the plasma membrane. To achieve vesicle fusion to the membrane, actin must be reorganized to provide a pathway for the vesicle to reach the plasma membrane. A model for actin reorganization at the cell cortex is shown in Fig. 1. Studies in our lab have shown that GLUT4 mediated glucose uptake is dependent upon the activation of myosin II through the phosphorylation of the regulatory light chain (RLC)
by myosin light chain kinase (MLCK) (2) and that myosin IIA and GLUT4 colocalize at the plasma membrane during insulin stimulation (3). The role of myosin II in GLUT4 vesicle trafficking still remains unclear. Therefore, our study examined the relationship between myosin II and the docking and fusion of GLUT4 to the plasma membrane.

![Figure 1. Myosin II and Actin Interaction](image)

**Figure 1. Myosin II and Actin Interaction.** A) Without insulin. B) With insulin.

Myosin II is an actin-binding motor protein which is important in muscle contraction. In non-muscle cells, myosin II is involved in regulating cell morphology and aiding in cell locomotion (17). Myosin II is a hexamer consisting of two heavy chains, two essential light chains (ELC), and two regulatory light chains (RLC) (6). The heavy chains contain a globular head which has actin and ATP binding sites to facilitate the motor activity of the protein (6). Myosin II is an abundant protein that can be found in almost all eukaryotic cells (16). Adipocytes express different isoforms of the myosin II protein which include myosin IIA and IIB (25). It has been shown that myosin IIA is
localized in a perinuclear region while myosin IIB is highly expressed at the cell cortex (2). Myosin II also plays a critical role in glucose uptake via GLUT4 fusion with the plasma membrane but no mechanism has been identified (3). Thus, there is a clear need to further examine the mechanism by which myosin II, and specifically the myosin II isoforms, mediate the fusion of GLUT4 to the plasma membrane.

An essential step in GLUT4 mediated glucose uptake is the docking and fusion of GLUT4 to the plasma membrane. After the cortical actin is reorganized, the GSV is carried along an actin tether where it first interacts with the exocyst complex. The exocyst complex is made up of eight proteins that anchors the GSV to the plasma membrane. Then, the GLUT4 vesicle interacts with the t-SNARE at the plasma membrane to mediate the fusion of the GLUT4 vesicle to the membrane. Multiple proteins constitute the SNARE complex and they include Syntaxin-4 and SNAP23 which makes up the t-SNARE. VAMP2 is located on the GSV which constitutes the v-SNARE (Fig. 2) (12). In order for this interaction to occur, the two membranes must be within a distance of 4-8 nm of each other (11). This complex brings the two membranes close enough to overcome energy barriers in a zipper like process resulting in membrane fusion (13, 14).
Figure 2. Model for GLUT4 Vesicle Docking and Fusion to the Plasma Membrane. The GLUT4 vesicle first interacts with the exocyst complex which anchors the GSV to the plasma membrane. The GSV then binds to syntaxin-4 via VAMP2 which will mediated the fusion of the vesicle to the plasma membrane.

Previous studies have shown that upon insulin signaling both GLUT4 and myosin IIA colocalize at the membrane (2). It is also known that myosin IIB remains present at the cell cortex and its localization remains unchanged after insulin stimulation (2). Studies in our lab have also shown that upon insulin stimulation, GLUT4 and F-actin colocalize at the site of GLUT4 vesicle fusion. This study will provide information about the roles of the myosin II isoforms in GLUT4 vesicle trafficking. This study furthers our understanding of the specific mechanisms involved in GLUT4 vesicle trafficking.
CHAPTER II
MATERIALS AND METHODS

Materials

Dulbecco’s Modified Eagle Medium (DMEM) (Cat # 11995-065) and fetal bovine serum (Cat # 26140-079) were obtained from Gibco (Grand Island, NY). Bovine calf serum (Cat # SH30072.03) was purchased from GE Life Sciences (Logan, UT). Dexamethasone (Cat # D-2915), 3-isobutyl-1-methyl-xanthine (Cat # I7018), GLUT4 antibody (Cat # PA5-19333), MyoIIA antibody (Cat # M8064), MyoIIB antibody (Cat # M7939) and horseradish peroxidase conjugated secondary antibodies were from Sigma (St. Louis, MO). Blebbistatin (Cat # ab120425) was purchased from Abcam (Cambridge, MA). Syntaxin-4 antibody (Cat # sc-101301), MyoVA antibody (Cat # sc-365988), and A/G-PLUS agarose beads (Cat # sc-2003) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). VAMP2 antibody (Cat # 13508S) was from Cell Signaling Technology (Danvers, MA). The enhanced chemiluminescence (ECL) (Cat # K-12045-D20) detection kit was from Amersham Bioscience (Piscataway, NJ).

Cell Culture

3T3-L1 pre-adipocytes were cultured in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 100 U/mL penicillin/streptomycin and 10% calf serum (CS). Media was replaced every two days and cells were passed when they reach
approximately 80% confluency. Two day post confluent 3T3-L1 cells were induced to differentiate in 10% FBS/DMEM containing 0.52 mM 3-isobutyl 1-methyl-xanthine (MIX), 1.7 μM insulin, and 1 μM dexamethasone (DEX) for three days. After three days, adipocytes were given 10% FBS/DMEM containing 0.872 μM insulin. Then, adipocytes were maintained in 10% FBS/DMEM and the media was replaced every two days.

**Insulin Stimulation Assay**

Day 7-10 adipocytes grown on coverslips were serum starved for 4 h under the following conditions: untreated (Basal), 0.1% DMSO (INS), or 100 μM blebbistatin (Blebb). After the 4h incubation, adipocytes were either left untreated (Basal) or stimulated with 100 nM insulin alone (INS) or with insulin and 100 μM blebbistatin (Blebb) for 30 min.

**Immunofluorescence**

Adipocytes were washed twice with cold 1X PBS and then fixed with 3.7% paraformaldehyde for 15 min. Cells were permeabilized with 0.25% triton X-100 for 5 min, washed three times with 1X PBS, then blocked in 5% donkey serum for 15 min. Cells were then incubated for 1 h with antibodies against either MyoIIA, GLUT4, Syntaxin-4, or VAMP2. Cells were washed and incubated with the appropriate secondary antibodies which were: anti-goat IgG conjugated to AlexaFluor 594, anti-rabbit IgG conjugated to AlexaFluor 488, and anti-mouse IgG conjugated to AlexaFluor 488. Cells were imaged using a Keyence BZ-X710 Fluorescence Microscope. The relative intensity
of immunofluorescence was quantified using ImageJ by drawing a line at the plasma membrane and measuring the mean gray value. The line was then moved into the cytosol where another measurement was made and a ratio of membrane to cytosol was used to quantitate change in fluorescence at the plasma membrane under each of the previously described treatments. Also using ImageJ, the width of the fluorescence band below the plasma membrane was measured by drawing a line starting at the plasma membrane and extending into the cytosol. The relative distances were normalized to basal.

**Protein Extraction**

Adipocytes (day 7) were subjected to the insulin stimulation assay as previously described. Adipocytes were washed twice in cold 1X PBS and lysed in 500 µl of a buffer containing 5.68 mM NaF, 28.4 mM HEPES, 114 mM NaCL, 1.14 mM EDTA, 1.62% NP-40, 1.14 mM NaPPi, and 2.27% glycerol. Whole cell lysates were rocked at 4° C for 20 min and then centrifuged at 6000 x g for 20 min. Protein extracts were then used for immunoprecipitation and western blot analysis.

**Immunoprecipitation**

Protein extracts were obtained as previously described above. A bicinchoninic acid (BCA) protein assay was used to determine the protein concentration. Whole cell lysates (500 µg) were incubated with 2 µg of antibody against GLUT4 and rocked overnight at 4° C. Twenty µl of A/G PLUS-agarose beads were added for an additional 3 h while rocking at 4° C. Samples were centrifuged at 2500 x g for 5 min then washed
with cold lysis buffer three times. The immunoprecipitated proteins were subjected to SDS-PAGE and immunoblot analysis.

**Western Blot Analysis**

Protein extracts were subjected to SDS-PAGE using either a 10% or 12% gel. Proteins were then transferred to an immobilon membrane and blocked in 4% BSA-1X TBST. Membranes were incubated with the specific primary and secondary antibodies as indicated and visualized using enhanced chemiluminescent (ECL). Protein bands were quantified by densitometry using ImageJ. All calculations were normalized using the protein used during immunoprecipitation (GLUT4).

**Plasma Lawn Assay**

Day 7-10 adipocytes were subjected to the insulin stimulation assay as previously described. Adipocytes were washed twice in cold 1X PBS and incubated in poly-D-lysine (0.5 mg/ml) for 1 min. Cells were then incubated in a hypotonic buffer (23 mM KCl, 10 mM HEPES, 2 mM MgCl2, 1 mM EDTA, pH 7.5) and sonicated in a buffer containing 70 mM KCl, 30 mM HEPES, 6 mM MgCl2, 3 mM EGTA, 1 mM dithiothreitol, 0.1 mM PMSF, pH 7.5. Plasma membrane sheets were incubated with antibodies against GLUT4, MyoIIA, MyoIIB, or MyoVA. During the plasma lawn assay, adipocytes are sheared through sonication which leaves a plasma lawn layer. Fluorescence was measured in ImageJ by drawing a line inside the plasma lawn and obtaining the mean gray value.
Statistical Analysis

Each experiment was replicated three times. Five images were taken from each independent experiment and five cells were quantified in each image (n = 25). Results are expressed as mean ± SEM. Statistical significance was determined using unpaired Student's t test, with P<0.05 considered significant.
CHAPTER III
RESULTS

Myosin II Inhibition Impairs GLUT4 and Myosin IIA Translocation to the Plasma Membrane

Previous studies in our lab have identified the importance of myosin IIA on GLUT4 mediated glucose uptake in adipocytes (2). Adipocytes have a layer of cortical actin just below the plasma membrane which must be reorganized to allow the GSV to access and fuse with the plasma membrane. Myosin II is a motor protein that possesses a globular head domain which contains an actin binding site (6). Therefore, if myosin IIA were to facilitate actin reorganization, it would have to localize at the cell cortex during insulin stimulation. To address this question, fully differentiated adipocytes were serum starved for 4 h and then left untreated (Basal) or stimulated with insulin alone (INS), or in the presence of blebbistatin (INS+Blebb). Myosin IIA and GLUT4 protein localization was visualized by immunostaining and fluorescence microscopy (Fig. 3A). The white arrows indicate protein localization at the plasma membrane in each of the three treatment groups. In the presence of insulin, myosin IIA fluorescence at the plasma membrane increased by approximately 39% when compared to untreated adipocytes (Fig. 3B). In adipocytes treated with insulin and blebbistatin, the effect of insulin was decreased by approximately 59% when compared to adipocytes treated with insulin alone (Fig. 3B). A similar trend was observed for GLUT4 localization. In adipocytes treated with insulin, GLUT4 fluorescence at the plasma membrane increased by 35% compared
to untreated cells (Fig. 3B). In adipocytes treated with insulin and blebbistatin, the insulin effect was decreased by 53% when compared to adipocytes treated with insulin alone (Fig. 3B). These data demonstrate that inhibition of myosin IIA with blebbistatin significantly decreased the levels of both myosin IIA and GLUT4 at the plasma membrane.

Further analysis of the immunofluorescence images of the blebbistatin treated adipocytes showed an increase in the fluorescence just below the plasma membrane. To quantify the accumulation of myosin IIA and GLUT4 below the plasma membrane, the fluorescence band width was measured as described in the Materials and methods. In adipocytes treated with insulin and blebbistatin, there was an approximate 211% increase in the myosin IIA fluorescence band width compared to cells treated with insulin alone (Fig. 3C). The GLUT4 fluorescence band width in adipocytes treated with insulin and blebbistatin increased by approximately 207% compared to adipocytes treated with insulin alone (Fig. 3C). These findings suggest that the GSV is still stimulated by insulin to translocate to the plasma membrane but is unable to fuse with the plasma membrane thus causing the accumulation of a diffuse signal directly below the plasma membrane.
Figure 3. Inhibiting Myosin II Affects the Localization of GLUT4 and Myosin IIA in Adipocytes. 3T3-L1 adipocytes were serum starved for 4 h and then either left untreated (Basal), treated with 100 nM insulin (INS), or treated with 100 nM insulin and 100 mM blebbistatin (INS+Blebb). (A) Fluorescence microscopy was used to determine the localization of myosin IIA and GLUT4 as described in the Materials and methods. White arrows indicate fluorescence at the plasma membrane in adipocytes. The results are representative of three independent experiments. Images were taken at a magnification of 40x. (B) The percentage of either GLUT4 or myosin IIA present at the plasma membrane under the three treatment conditions was quantified as described in the Materials and methods. (C) The percent difference in the width of the fluorescence band just below the plasma membrane was measured as described in Materials and methods. All measurements were normalized to basal. The percent difference was determined by comparing basal to INS and INS to INS+Blebb. Results are the means ± SEM of three independent experiments. *p < 0.05

The Localization of GLUT4 and Syntaxin-4 Decreases with Myosin II Inhibition

During GLUT4 vesicle trafficking, an important step in the process involves the interaction of the GSV with the syntaxin-4 protein at the plasma membrane (4). This interaction is necessary for the vesicle to properly fuse to the plasma membrane. If there is less localization between GLUT4 and syntaxin-4 when myosin II is inhibited, it would provide further evidence as to the importance of myosin II in GLUT4 fusion to the plasma membrane as well as our observation that neither GLUT4 nor myosin IIA are
present the plasma membrane. Therefore, to determine if inhibiting myosin II has an
effect on GLUT4 fusion we examined its localization with syntaxin-4 and GLUT4.
Adipocytes were serum starved for 4 h and then either left untreated or stimulated with
insulin alone or insulin and blebbistatin (Fig. 4A). The white arrows indicate protein
localizations at the plasma membrane of adipocytes in each of the treatment groups.
Analysis of syntaxin-4 fluorescence at the plasma membrane showed that there is no
statistically significant change across the three treatments (Fig. 4B). This is expected
since syntaxin-4 is a membrane bound protein, which would be unaffected by insulin
stimulation or the inhibition of myosin IIA (26). Consistent with our previous
experiment, there was a 35% increase in GLUT4 fluorescence at the plasma membrane in
adipocytes that were stimulated with insulin compared to untreated cells (Fig. 4B). We
also observed that the effect of insulin was decreased by 56% in adipocytes treated with
insulin and blebbistatin compared to adipocytes treated with insulin alone (Fig. 4B).
Therefore, by inhibiting myosin II, there is an overall decrease in the localization of
GLUT4 but not syntaxin-4 at the plasma membrane. These results also provide evidence
that GLUT4 did not reach the plasma membrane when myosin II was inhibited.
Figure 4. Myosin II Inhibition Impairs GLUT4 Vesicle Fusion at the Plasma Membrane. 3T3-L1 adipocytes were serum starved for 4 h and then either left untreated (basal), treated with 100 nM insulin (INS), or treated with 100 nM insulin and 100 mM blebbistatin (INS+Blebb). (A) GLUT4 and syntaxin-4 localization was observed by fluorescence microscopy as described in the Materials and methods. The white arrows indicate syntaxin-4 and GLUT4 localization at the plasma membrane in adipocytes. The results are representative of three independent experiments. The magnification used to take images was 40x. (B) The percentage of each protein present at the plasma membrane.
under the three treatment conditions was quantified as described in the Materials and methods. Fluorescence intensity at the plasma membrane and cytoplasm was measured and a ratio was obtained by comparing the intensity at the two locations. The percent difference was determined by comparing basal to INS and INS to INS+Blebb. Results are the means ± SEM of three independent experiments. *p < 0.05.

In a complimentary assay, we utilized immunoprecipitation to determine if myosin II inhibition altered the insulin induced interaction between the GLUT4 vesicle and syntaxin-4. In adipocytes that were treated with insulin, there was a 49% increase in the amount of syntaxin-4 complexed with GLUT4 when compared to adipocytes that were untreated (Fig. 5B). In adipocytes that were treated with insulin and blebbistatin, the level of syntaxin-4 complexed with GLUT4 decreased by 31% when compared to insulin treated cells (Fig. 5B). This decrease was not considered to be statistically significant. However, there is an obvious trend that occurs when you compare this data with the data gathered from the immunofluorescence images. Together, our results suggest that inhibition of myosin II impaired the localization of GLUT4 and syntaxin-4.

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![Immunoprecipitation and Immunoblotting](image)
Figure 5. The Interaction Between Syntaxin-4 and GLUT4 Requires Myosin II.
Adipocytes were serum starved for 4 h and then either left untreated (basal), treated with 100 nM insulin (INS), or treated with 100 nM insulin and 100 mM blebbistatin (INS+Blebb). Whole cell lysates from each treatment were subjected to the immunoprecipitation protocol described in the Materials and methods. A) Immunoprecipitated proteins were subjected to 10% SDS-PAGE and then immunoblotted using antibodies against GLUT4 or syntaxin-4. B) Immunoblots were quantified by densitometry. Values were normalized using the band for the protein which was immunoprecipitated (GLUT4). Results are the means ± SEM of three independent experiments. *p < 0.05

Inhibition of Myosin II does not Alter Insulin-induced VAMP2 Localization at the Plasma Membrane

Next, we examined the localization of VAMP2 and syntaxin-4 in adipocytes treated with blebbistatin during GLUT4 exocytosis. It is known that the GLUT4 vesicle contains the VAMP2 protein which is responsible for mediating the interaction between the vesicle and syntaxin-4 (4). This interaction allows for the fusion of the vesicle with the plasma membrane. Therefore, if the localization of VAMP2 is altered when myosin II is inhibited, we can use this information to draw conclusions about the role of myosin II in GLUT4 vesicle docking. Fully differentiated 3T3-L1 adipocytes were serum starved
for 4 h in the presence of blebbistatin, then stimulated with insulin or left untreated.

Similar to our previous experiments, syntaxin-4 fluorescence at the plasma membrane showed no significant change when comparing each of the treatments (Fig. 6B). In adipocytes treated with insulin, there was a 22% increase in VAMP2 fluorescence at the plasma membrane compared to adipocytes that were left untreated (Fig. 6B). In adipocytes treated with insulin and blebbistatin, the effect of insulin decreased by 50% compared to cells treated with insulin alone (Fig. 6B). However, this decrease is not considered to be statistically significant. The 50% decrease in VAMP2 localized at the plasma membrane is consistent with the approximately 50% decrease in the other proteins that we studied including GLUT4 and myosin IIA. These results show that the inhibition of myosin II did not affect the amount of VAMP2 present at the plasma membrane but the trend was similar to our other findings.
Inhibiting Myosin II did not Affect VAMP2 Localization. 3T3-L1 adipocytes were serum starved for 4 h in the presence of blebbistatin and then either left untreated (basal), treated with 100 nM insulin (INS), or treated with 100 nM insulin and 100 mM blebbistatin (INS+Blebb). (A) VAMP2 and syntaxin-4 localization was determined as described in the Materials and methods. The white arrows indicate VAMP2 and syntaxin-4 localization at the plasma membrane within adipocytes. The results are representative of three independent experiments. The magnification used was 40x. (B) The percentage of each protein present at the plasma membrane and cytoplasm under the three treatment conditions was quantified as described in the Materials and methods. Fluorescence intensity at the plasma membrane and cytoplasm was measured and a ratio was obtained by comparing the intensity at the two locations. The percent difference was determined by comparing basal to INS and INS to INS+Blebb. Results are the means ± SEM of three independent experiments. *p < 0.05.

Myosin IIA and GLUT4 do not Localize in the Same Place at the Plasma Membrane

To gain further insight into the effect of myosin IIA on GLUT4 trafficking, we utilized a method to generate plasma lawns which allows for the examination of events near the plasma membrane in more detail. Fully differentiated adipocytes were serum starved for 4 h then left untreated, stimulated with insulin, or stimulated with insulin and blebbistatin for 30 min. Fluorescence microscopy was used to examine protein locations on the plasma lawns for each of the three treatment groups. In Fig. 7B, insulin stimulation
increased the presence of myosin IIA at the plasma membrane by 36% compared to levels in untreated adipocytes. In adipocytes stimulated with insulin in the presence of blebbistatin, the presence of myosin IIA at the plasma membrane decreased by 53% compared to adipocytes treated with insulin alone (Fig. 7B). When adipocytes were stimulated with insulin, there was a 34% increase of GLUT4 at the membrane compared to adipocytes that were left untreated (Fig. 7B). When cells were treated with blebbistatin, the GLUT4 present at the plasma membrane decreased by approximately 53% when compared to adipocytes treated with insulin alone (Fig. 7B). These results show that blebbistatin affects the ability of myosin IIA to translocate to the plasma membrane during insulin stimulation. By inhibiting myosin II, GLUT4’s ability to translocate and fuse with the plasma membrane is impaired. Further analysis of the fluorescence images showed punctate staining of both myosin IIA and GLUT4 on different locations on the plasma membrane sheets (Fig. 7A). This shows that myosin IIA and GLUT4 are not in the same location based on the distinct separation of fluorescence in the composite images.
Figure 7. Myosin IIA and GLUT4 do not Translocate to the Same Position on the Plasma Membrane. 3T3-L1 adipocytes were serum starved for 4 h and then either left untreated (basal), treated with 100 nM insulin (INS), or treated with 100 nM insulin and 100 mM blebbistatin (INS+Blebb) as described in the Materials and methods. Plasma lawns were then obtained following the procedure in the materials and methods. A) GLUT4 and myosin IIA were detected using fluorescence microscopy. The white arrows indicate the presence of these two proteins at the plasma membrane. The results are representative of three independent experiments. The magnification used to obtain
images was 40x. B) The fluorescence of each protein present on the plasma lawn under the three treatment conditions was quantified as described in the Materials and methods. The percent difference was determined by comparing basal to INS and INS to INS+Blebb. Results are the means ± SEM of three independent experiments. *p < 0.05

**Blebbistatin Treatments had no Effect on Myosin IIB Localization in Adipocytes**

To distinguish between the two isoforms of myosin II and their role in GLUT4 exocytosis, myosin IIB was visualized on plasma membrane sheets to determine if blebbistatin affected its localization in relation to GLUT4. The localization of myosin IIB and GLUT4 were determined using immunostaining and fluorescence microscopy. In adipocytes stimulated with insulin, there was a 7% increase in myosin IIB at the plasma membrane (Fig. 8B). When adipocytes were treated with insulin and blebbistatin, there was a 2% decrease in myosin IIB at the plasma membrane compared to cells treated with insulin alone (Fig. 8B). This decrease is not statistically significant. GLUT4 levels at the plasma membrane were consistent when compared to previous experiments. We showed that GLUT4 fluorescence at the plasma membrane increased by 35% in adipocytes that were treated with insulin compared to untreated cells (Fig. 8B). In adipocytes treated with insulin and blebbistatin, the effect of insulin decreased by 48% compared to adipocytes treated with insulin alone (Fig. 8B). These results show that while insulin stimulation has only a small effect on myosin IIB presence at the plasma membrane, treating adipocytes with blebbistatin does not affect the localization of myosin IIB. This indicates that myosin IIB is present at the cell cortex and its presence does not have a direct effect on GLUT4’s ability to fuse with the plasma membrane. When examining the fluorescence images, myosin IIB was in a dense sheet across the plasma membrane in each of the
treatments. In insulin treated adipocytes, the immunofluorescence images clearly show an overlap of signal of myosin IIB and GLUT4 which suggests that they are both localized at the plasma membrane. This suggests that the two proteins may overlap in the same place.

**Figure 8. Myosin II Inhibition does not Alter Myosin IIB Localization.** 3T3-L1 adipocytes were serum starved for 4 h and then either left untreated (basal), treated with 100 nM insulin (INS), or treated with 100 nM insulin and 100 mM blebbistatin.
(INS+Blebb). Plasma lawns were then obtained following the procedure outlined in the Materials and methods. A) GLUT4 and myosin IIB were detected using fluorescence microscopy as outlined in the Materials and methods. The white arrows indicate the presence of these two proteins on the plasma lawns. The results are representative of three independent experiments. The magnification used in imaging was 40x. B) The fluorescence of each protein present on the plasma lawn under the three treatment conditions was quantified as described in the materials and methods. The percent difference was determined by comparing basal to INS and INS to INS+Blebb. Results are the means ± SEM of three independent experiments. *p < 0.05

Inhibition of Myosin II Decreased GLUT4 Localization at the Plasma Membrane While Myosin Va was Absent

After examining fluorescence images from previous plasma lawn assays, we were curious to determine how much of the plasma membrane and associated structures were being left on the coverslip after sonication. To address this, we stained plasma lawns for GLUT4 and myosin Va. Myosin Va is an actin motor protein that is involved in GLUT4 vesicle trafficking during insulin stimulation. Studies suggest that before the GLUT4 vesicle reaches the plasma membrane, it is carried along an actin tether via myosin Va (4). This actin tether extends into the cytoplasm and is anchored at the plasma membrane (4). The first point of contact between the GLUT4 vesicle and the actin tether is through its interaction with myosin Va. Therefore, if GLUT4 localizes with myosin Va on the plasma lawn, it will provide information as to how close proteins are to the plasma membrane.

Utilizing fluorescence microscopy, we determined the localization of GLUT4 and myosin IIB on plasma lawns. We demonstrated that myosin Va fluorescence was absent in each of the three treatment groups (Fig. 9A). However, GLUT4 localization was consistent with previous experiments. In adipocytes treated with insulin, there was a 43%
increase in GLUT4 fluorescence at the plasma membrane compared to adipocytes left untreated (Fig. 9B). In adipocytes treated with insulin and blebbistatin, the insulin effect was inhibited by approximately 68% when compared to adipocytes treated with insulin alone (Fig. 9B). These results show that on plasma lawns, GLUT4 and myosin Va do not localize with each other using this technique. Coupled with our previous results, we can conclude that both myosin IIA and myosin IIB are localized close enough to the plasma membrane that they are not affected by sonication.
Figure 9. Absence of Myosin Va on Plasma Lawns Determines its Proximity to the Plasma Membrane. 3T3-L1 adipocytes were serum starved for 4 h and then either left untreated (basal), treated with 100 nM insulin (INS), or treated with 100 nM insulin and 100 mM blebbistatin (INS+Blebb). Plasma lawns were then obtained following the procedure outlined in the Materials and methods. A) Fluorescence microscopy was utilized to detect GLUT4 and myosin VA at the plasma membrane as outlined in the Materials and methods. The white arrows indicate the presence of these two proteins on the plasma lawns. The results are representative of three independent experiments. Images were obtained using 40x magnification. B) The fluorescence of each protein was quantified as described in the materials and methods. The percent difference was determined by comparing basal to INS and INS to INS+Blebb. Results are the means ± SEM of three independent experiments. *p < 0.05
CHAPTER IV
DISCUSSION

While adipocytes don’t have an extensive actin cytoskeletal network, they do have an actin barrier that resides below the plasma membrane. The importance of this actin barrier in GLUT4 exocytosis has also been noted in previous studies (32). These studies show that the remodeling of the actin network is necessary for GLUT4 mediated glucose uptake (23). Previous studies in our lab have identified that during insulin stimulation, GLUT4 and myosin IIA are both translocated to the plasma membrane but GLUT4 is recruited before myosin IIA (3). We also showed that GLUT4 mediated glucose uptake was negatively affected when myosin II was inhibited (3). Given what is known about actin remodeling and the importance of myosin IIA in GLUT4 mediated glucose uptake, we wanted to determine the role of myosin IIA in the fusion of the GSV to the plasma membrane during insulin stimulation. The presence of GLUT4 and myosin IIA at the plasma membrane during insulin stimulation (Fig. 3) was consistent with previous studies (27). However, while analyzing these fluorescence images, we observed that there was an accumulation of GLUT4 and myosin IIA signal at the cell cortex in adipocytes treated with blebbistatin below the plasma membrane. To address this further, we measured the width of the GLUT4 and myosin IIA fluorescence band in adipocytes treated with insulin and blebbistatin and insulin alone (Fig. 3C). This measurement would determine how far each protein translocated toward the plasma membrane. In adipocytes
treated with insulin, the GLUT4 signal was much more localized and compact due to the proper actin reorganization and fusion of the vesicle with the plasma membrane (Fig. 3). The GLUT4 fluorescence band was much more diffuse near the plasma membrane in the adipocytes treated with insulin and blebbistatin (Fig. 3C). This suggests that while the GSV was being stimulated by insulin to translocate to the plasma membrane, the inhibition of myosin II was preventing cortical actin remodeling preventing the GSV from reaching the plasma membrane.

To determine the exact stage in the translocation process that was impaired we examined the final stage, GLUT4 docking and fusion to the plasma membrane. We examined the localization of syntaxin-4 and GLUT4 in adipocytes treated with blebbistatin. Our present study showed that while inhibiting myosin II, there was less interaction between syntaxin-4 and GLUT4 (Fig. 5) meaning that while myosin II is inhibited, GLUT4 is not able to form the SNARE complex which is required for vesicle fusion. Without successful assembly of the SNARE complex, GLUT4 mediated glucose uptake will be negatively affected. When quantifying the protein bands on the western blots, you can see that the amount of GLUT4 varied slightly in each treatment (Fig. 5A). There could be a number of reasons for this which could be attributed to procedural errors. For instance, the amount of protein that was bound to the A/G-PLUS agarose beads may have been less in blebbistatin treated cells or some beads could have been lost during washing. Since we normalized our results to the protein we immunoprecipitated (GLUT4), it does not have an effect on our overall results. As a complimentary assay, we examined the localization of VAMP2 in relation to syntaxin-4. Since VAMP2 is located...
on the GSV and mediates vesicle fusion by binding to syntaxin-4, we would expect to see a similar pattern of localization as GLUT4. However, the same pattern was not observed. While there was a significant increase in VAMP2 at the plasma membrane in adipocytes treated with insulin, there was not a significant decrease when comparing adipocytes treated with insulin and blebbistatin. A possible explanation for this is that VAMP2 is an abundant protein that exists on other types of secretory vesicles that are translocating to the plasma membrane dependent and independent to insulin signaling. For example, when adipocytes are stimulated by insulin, transferrin receptor-1 vesicles (TfR1) translocate to the plasma membrane (30). When TfR1 binds to the plasma membrane, an iron carrying transferrin molecule can be endocytosed (30). Another example of a protein that translocates to the plasma membrane under insulin stimulation is GLUT1 (31). Similar to GLUT4, GLUT1 also functions to transport glucose into adipocytes (31). While insulin stimulation did increase the presence of VAMP2 at the plasma membrane (Fig. 6B), the effect of inhibiting myosin II could have been minimal due to the overall level of VAMP2 in general being stimulated to translocate to the plasma membrane. There are also secretory vesicles in adipocytes that translocate to the plasma membrane that are independent of insulin signaling. For instance, adipocytes express the long form of the leptin receptor (LRb) which when fused to the plasma membrane binds to leptin at the cell surface (34). This insulin independent process of vesicle fusion to the plasma membrane, among others, could have affected the levels of VAMP2 at the plasma membrane. However, the 50% decrease observed in the amount of VAMP2 at the plasma membrane...
membrane is consistent with the other proteins we examined whose translocation to the plasma membrane is dependent upon insulin stimulation

Next, we were interested in distinguishing between the roles of the myosin II isoforms to determine their role in GLUT4 docking and fusion to the plasma membrane. It is known that adipocytes express different isoforms of myosin II and that myosin IIA and GLUT4 colocalize at the cell cortex in the presence of insulin (2). It is also known that myosin IIB is localized at the cell cortex and the localization is unaffected by insulin stimulation (2). Previous studies have also demonstrated that inhibiting myosin II with blebbistatin or reducing calcium levels impairs F-actin from co-localizing with GLUT4 at the plasma membrane (27). However, it is still unclear if myosin IIB plays a role in actin remodeling and the subsequent fusion of GLUT4 to the plasma membrane. To address this, we used the plasma lawn assay to examine protein localizations. The plasma lawn assay has its benefits over staining whole cells. During the process of preparing plasma lawns, the adipocyte is sheared using sonication. Through the process of sonication, only proteins that are present or adjacent to at the plasma membrane will be detected by immunostaining. This also removes cellular proteins that may cause interference by non-specific binding of antibodies. In our present study, plasma lawns were stained for myosin IIA and GLUT4. The results were consistent with our staining of whole adipocytes showing that both proteins localize at the plasma membrane during insulin stimulation (Fig. 7). Inhibiting myosin II with blebbistatin caused a decrease in the presence of myosin IIA and GLUT4 at the plasma membrane (Fig. 7). This can be attributed to the inability of myosin IIA to remodel actin filaments, thus not allowing
GLUT4 to reach the plasma membrane. In the fluorescence images, there is distinct punctate staining pattern which shows the separation of myosin IIA and GLUT4. This suggests that myosin IIA may contract actin during actin filament remodeling though more studies need to be done to confirm this finding.

Another myosin II isoform, myosin IIB, was examined in relation to GLUT4. During insulin stimulation, we saw there was a slight increase in myosin IIB presence at the plasma membrane (Fig. 8). This increase of 7% was minimal especially when compared to the approximate 35% increase in myosin IIA localization at the plasma membrane in adipocytes stimulated with insulin. Since we know that there is an increase in actin at the cell cortex during insulin stimulation (27), this could account for the increase in myosin IIB. Also, we showed that inhibiting myosin II did not affect the amount of myosin IIB at the plasma membrane while it did have an effect on GLUT4 that is consistent with previous findings. The fluorescence images obtained using the plasma lawn assay shows a dense field of myosin IIB at the plasma membrane. This may suggest that the relationship between myosin IIB and actin is more structural.

While the plasma lawn assay has its benefits, there are also some drawbacks. For example, after sonicating adipocytes, it was unclear as to how much of the interior of the cell was left. Depending on how much of the cell was left would affect which proteins we would be able to detect using immunofluorescent staining. It is an important determination to make because we are interested in examining proteins that are at or directly below the plasma membrane. To do this, we stained for myosin Va and GLUT4.
We know that myosin Va transports the GLUT4 vesicle on the actin tether during GLUT4 vesicle trafficking (4). Myosin Va in the first motor protein to interact with GLUT4 before it is transferred to myosin Ic which is responsible for transporting GLUT4 to the exocyst complex (4). In the plasma lawn assay, we do not detect myosin Va in any of our treatments (Fig. 9). This suggests that after sonication, only proteins that have fused with the plasma membrane such as GLUT4 or proteins that are within nanometers of the plasma membrane (MyoIIA/B) are present.

In summary, our study shows that during insulin stimulation, myosin IIA localization is affected by the inhibition of myosin II while the localization of myosin IIB is unaffected. Analysis of the fluorescence images in the plasma lawn assay show that the localization of myosin IIA is distinct from GLUT4 which suggests that myosin IIA is contracting actin to allow GLUT4 to access the plasma membrane via the actin tether. In each of our experiments the presence of GLUT4 at the plasma membrane decreased when adipocytes were treated with insulin and blebbistatin. This suggests that by inhibiting myosin II, GLUT4’s ability to reach and fuse with the plasma membrane is impaired. Since myosin IIA localization is also affected by the inhibition of myosin II, this is further evidence that myosin IIA is the myosin isoform that is responsible for actin remodeling at the cell cortex.
REFERENCES


