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# Coordinated oscillations in glucose-stimulated insulin secretion and protein phosphorylation in clonal pancreatic beta-cells: exploring metabolic control of exocytosis

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BOSTON UNIVERSITY

ARAM V. CHOBANIAN & EDWARD AVEDISIAN SCHOOL OF MEDICINE

Thesis

**COORDINATED OSCILLATIONS IN GLUCOSE-STIMULATED INSULIN  
SECRETION AND PROTEIN PHOSPHORYLATION IN CLONAL PANCREATIC  
BETA-CELLS:  
EXPLORING METABOLIC CONTROL OF EXOCYTOSIS**

by

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B.S., King Saud University, 2017

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requirements for the degree of  
Master of Science

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## **DEDICATION**

This work is dedicated to all individuals affected by diabetes worldwide.

## **ACKNOWLEDGMENT**

I am grateful to my family and friends for their emotional support throughout my research journey. I would like to express my sincere gratitude to my primary investigator, Dr. Deeney, for his guidance and direction. I also appreciate the help and support of the program director, Dr. Moore. I am grateful to Dr. Pickering and Yuhan for their assistance with materials, and to Dr. Tornheim and Dr. Corkey for serving as my second and third readers. I also thank my laboratory colleagues, Claire, Fatemeh, Anam, Princess, and Nazli, for their collaboration and support. I also extend my thanks to my classmates, Rawan, Anoushka, Nabela, and Khalifa, and to my friends Ziyad, Naif, Shehab, Abdulaziz, Fahad, Omar, Khalid, and Mohammad, for their encouragement and support.

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**ABSTRACT**

**Introduction:**

Diabetes Mellitus affects 415 million people worldwide. It causes hyperglycemia due to impaired insulin production or action. It has been known for a long time that insulin secretion oscillates in vivo and in vitro. These oscillations in insulin release are impaired in diabetic patients. Oscillations in insulin secretion are driven by oscillations in metabolic coupling factors including the ATP/ADP ratio and intracellular  $Ca^{2+}$ . The Aim of this thesis is to determine whether phosphorylation of proteins regulating beta-cell lipid metabolism correlates with oscillations in insulin secretion.

**Methods:**

INS-1 cells were cultured in 4 and 11 mM glucose in 48-well plates. Insulin secretion was initiated with 12 mM glucose at timed intervals to generate an oscillation profile over 22 min. Media was collected and insulin was assayed by fluorescence based HTRF insulin assay. Cell protein was extracted with SDS-PAGE sample buffer, separated by electrophoresis and transferred to PVDF membrane for western blotting after SDS PAGE electrophoresis. Phosphorylated

and unphosphorylated acetyl-CoA carboxylase (ACC) and AMP-activated protein kinase (AMPK) were detected with specific rabbit antibodies (Cell Signaling). Protein bands were detected on a GE LAS-4000 gel imager using enhanced chemiluminescence. Bands were analyzed using ImageJ software (Schneider, Rasband and Eliceiri 2012).

### **Results:**

Insulin oscillations were detected over the 22 min time course with at least three resolved peaks of insulin secretion for cells cultured in either 4 or 11 mM glucose. The oscillations were of a 5 min period under both culture conditions while the amplitude was 10-20 fold higher in 4 mM glucose cells. The amplitude was dependent on the insulin content of the cells such that when normalized to insulin content the average insulin secretion was well matched between the high and low glucose conditions.

Oscillations in pACC/ACC and pAMPK/AMPK ratios were detected in cells cultured in both 4 mM and 11 mM glucose. In cells cultured at 4 mM glucose the pACC/ACC ratio oscillated with a similar period to insulin but was slightly left shifted such that pACC peaked before insulin. This correlation was not as strictly adhered to in cells cultured at high glucose. Oscillations in pAMPK/AMPK tracked well with those of pACC/ACC in cells cultured at both 4 and 11 mM. pAMPK/AMPK peaks were left-shifted relative to peaks in insulin secretion in cells cultured at 4 mM glucose while they seemed to be coincident with insulin peaks in cells cultured at 11 mM glucose.

**Conclusion:**

Oscillations in insulin secretion are accompanied by oscillations in ACC and AMPK phosphorylation to regulate lipid signals that amplify normal glucose-stimulated insulin secretion. Chronic excess nutrients may alter changes in ACC and AMPK phosphorylation resulting in impaired oscillations in insulin secretion. Regulation of lipid signals in the pancreatic beta-cell may provide therapeutic benefit in the treatment of hyperinsulinemia, insulin resistance and Type 2 diabetes.

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## LIST OF ABBREVIATION

ACC.....	Acetyl-CoA Carboxylase
ACL.....	ATP Citrate Lyase
AMPK.....	AMP-Activated Protein Kinase
BME.....	$\beta$ -Mercaptoethanol
BSA.....	Bovine Serum Albumin
CPT-1.....	Carnitine Palmitoyltransferase-1
DM.....	Diabetes Mellitus
ECL.....	Enhanced Chemiluminescence
ETC.....	Electron Transport Chain
FFA.....	Free Fatty Acid
FBS.....	Fetal Bovine Serum
GLT.....	Glucolipototoxicity
GSIS.....	Glucose-Stimulated Insulin Secretion
GK.....	Glucokinase
HEPES.....	(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)
IDF.....	International Diabetes Federation
KCl.....	Potassium Chloride
KRB.....	Krebs-Ringer Bicarbonate
LC-CoA.....	Long-chain-fatty-acyl—CoA
mRNA.....	Messenger Ribonucleic Acid
NEFA.....	Non-esterified Fatty Acid

NFDM.....	Non-Fat Dried Milk
NNT.....	Nicotinamide Nucleotide Transhydrogenase
pACC.....	Phospho-Acetyl-CoA Carboxylase
pAMPK.....	Phospho-AMP-Activated Protein Kinase
PC.....	Pyruvate Carboxylase
PFK.....	Phosphofructokinase
pMARCKS.....	Phospho-Myristoylated Alanine-Rich C Kinase
ROS.....	Reactive Oxygen Species
RPMI.....	Roswell Park Memorial Institute
SDS.....	Sodium Dodecyl Sulfate
T1DM.....	Type 1 Diabetes Mellitus
T2DM.....	Type 2 Diabetes Mellitus
TBST.....	Tris-Buffered Saline + Tween
TCA.....	Citric Acid Cycle

## INTRODUCTION

### Diabetes Mellitus

Diabetes is defined by the presence of chronic hyperglycemia (Schmidt 2018, Petersmann et al. 2019). This might be a result of the destruction of pancreatic beta cells as it appears in type 1 diabetes mellitus (T1DM), or a defect in insulin secretion in relation to genetics, inflammation and metabolic stress as is the case with T2DM. Patients with T1DM need insulin treatment to survive, whereas T2DM, which is previously called non-insulin-dependent diabetes doesn't need insulin treatment for an individual to survive. Most patients with T2DM are obese or have an increased percentage of abdominal fat, and with age and low physical activity the risk of developing the disease increases, and even dyslipidemia (EISayed et al. 2022). Their insulin levels may appear normal or even elevated (Association 2013).

Diabetes mellitus (DM) affects 1 out of 11 adults worldwide (International Diabetes Federation (IDF) 2015), and over 90% of them are type 2 diabetes mellitus (T2DM) (Zheng, Ley and Hu 2018). This number is estimated to be 415 million people and is four times higher than the number of diabetic people thirty years ago. By the year 2040, it's expected to that this number will reach 642 million. It has long been known that genes could contribute to an individual's risk in developing the disease (Zheng et al. 2018). In addition to the genetic component, other factors might contribute to developing diabetes, such as obesity, a sedentary lifestyle, energy-dense diets, and population aging (Zheng et al. 2018, Schmidt

2018). Fortunately, some of these factors can be avoided by adopting a healthy lifestyle (Zheng et al. 2018).

## **Insulin**

Insulin was first isolated by Fredrick Banting and Charles Best in 1921, and since then it's been known to be the hormone that controls glucose levels in the blood. It's the most important anabolic hormone in the body (Tokarz, MacDonald and Klip 2018). Its main function is to regulate the storage of nutrients (storage for use as a fuel) including glucose, lipids, and amino acids (Corkey, Deeney and Merrins 2021). The synthesis of insulin in the body occurs inside the  $\beta$ -cell located in the pancreas to be ready for secretion (Tokarz et al. 2018). In the last few decades, studies on diabetes primarily focused on the outcome, which is controlling the glucose level, while ignoring other related problems such as basal hyperinsulinemia. It has been suggested that a change in focus to include these related problems might provide insight into new drug development to reverse the disease. In the early stages of hyperglycemia, elevated blood glucose can lead to ectopic lipid deposition, elevated basal insulin secretion, insulin resistance, and chronic low-grade inflammation (Corkey et al. 2021).

## **Insulin secretion in T2DM**

Insulin secretion in the bloodstream in vivo is oscillatory (Polonsky, Given and Van Cauter 1988). In a comparison between normal and obese subjects, which mimics the normal and excess nutrients, it has been found that they have a similar number of pulses of insulin after a meal, but with hypersecretion of insulin

in the obese group. This pattern similarity isn't shown in the diabetic subjects. Diabetics were shown to have disrupted insulin oscillation with damped pulses and higher pulses number post-meal (Polonsky et al. 1988).

It's important to know the difference in the pathway regulating secretion of insulin in the two states, GSIS and basal secretion. GSIS is the insulin secretion in response to fuel excess, while basal secretion in the fasting state. As we mentioned before that lipids and ROS play an important role in GSIS, and it seems that these may have a major effect on insulin secretion in the basal state (Corkey et al. 2021). A study was done on T2DM subjects, where they measured their fasting FFA and divided them into tertiles. They found that the tertile with the highest FFA levels had the lowest insulin secretion in response to glucose (Salgin et al. 2012). Another major difference we should mention is that during excess fuel with a high demand for insulin to be secreted, insulin stores could reach depletion (Corkey et al. 2021).

### **Mechanisms of Glucose-Stimulated Insulin Secretion (Triggering)**

Glucose is the major metabolic signal for insulin secretion. Secretion occurs after the production of a series of glucose-induced metabolic and electrical signals. The canonical pathway of insulin secretion starts with glucose entering the pancreatic  $\beta$ -cell through a glucose transporter GLUT1/GLUT2 (Tokarz et al. 2018). Glycolysis of glucose takes place in the cytoplasm starting with phosphorylation of glucose into glucose-6-phosphate by glucokinase (GK) followed by a series of reactions resulting in the production of 2 adenosine

triphosphates (ATP) and 2 pyruvate molecules (Dashty 2013, Tokarz et al. 2018). Pyruvate then enters the citric acid cycle (TCA) in the mitochondria (Tokarz et al. 2018). Further metabolism of pyruvate through pyruvate dehydrogenase, TCA cycle, and oxidative phosphorylation leads to the production of more than 30 ATP generated from one glucose molecule (Yetkin-Arik et al. 2019). It has been shown that the ATP required to close the  $K_{ATP}$  channel is initially produced not in the mitochondria but rather at the plasma membrane close to the channel. It has been demonstrated that Pyruvate kinase (PK) tethered to the plasma membrane converts phosphoenolpyruvate (PEP) produced in the mitochondria to pyruvate in the cytosol generating high levels of ATP in close proximity to the  $K_{ATP}$  channel. The ATP in the microenvironment of the channel then acts to alter the ATP/ADP ratio to initially close the channel and depolarize the membrane, while ATP generated in the mitochondria is then utilized to maintain the  $K_{ATP}$  channel closed (Foster et al. 2022). The inhibition of the  $K_{ATP}$  channel depolarizes the plasma membrane and opens voltage gated  $Ca^{2+}$  channels, allowing for  $Ca^{2+}$  to enter the cell (Fridlyand, Ma and Philipson 2005). A fall in the ATP/ADP ratio then allows for the cell to return to a resting membrane potential.  $Ca^{2+}$  channel activity follows the membrane potential to allow  $Ca^{2+}$  influx and stimulate exocytosis. It was long believed that glucose-glucose induced insulin secretion was mediated by signals to close the  $K_{ATP}$  channel leading to  $Ca^{2+}$  influx (Gembal et al. 1993). The question of whether  $Ca^{2+}$  is the sole signal to stimulate insulin secretion was addressed by Gembal. Diazoxide (DZ) was used to activate the  $K_{ATP}$  channel and prevent  $Ca^{2+}$

influx until the cell was depolarized with the addition of KCl. Increasing concentration of glucose was then added to examine insulin secretion. The results showed an increase in insulin secretion as the glucose concentration was increased suggesting there were other signals in addition to an increase in intracellular  $\text{Ca}^{2+}$  responsible for regulating exocytosis. Such signals were thought to amplify insulin secretion. Thus, insulin secretion was regulated by two distinct pathways, triggering and amplification (Gembal et al. 1993).

### **Amplification Of Insulin Secretion**

Fatty acids (FA) are a major fuel of the pancreatic  $\beta$ -cell oxidized after transport into the mitochondria. In addition FA are known to acutely stimulate glucose-induced insulin secretion without stimulating basal insulin release (Stein et al. 1996). The metabolic effects of FA in cells are mediated by the intracellular FA-CoA ester, long-chain acyl-CoA (LC-CoA). Our lab has shown a number of effects of LC-CoA in the  $\beta$ -cell including activating endoplasmic reticulum  $\text{Ca}^{2+}$ -ATPases (Deeney et al. 1992) and the  $K_{\text{ATP}}$  channel (Larsson et al. 1996) as well as stimulating exocytosis (Deeney et al. 2000).

The LC-CoA hypothesis proposes a coordinated regulation of glucose and FA oxidation controlled by the level of cytosolic malonyl-CoA (Prentki et al. 1992). Glucose metabolism leads to mitochondrial anapleurosis leading to citrate transport to cytosol. Citrate is converted to malonyl-CoA by the actions of Citrate lyase (CL) and acetyl-CoA carboxylase (ACC). Malonyl-CoA inhibits carnitine palmitoyl transferase 1, the rate limiting step for transport of LC-CoA into the

mitochondria. Thus, FA oxidation is inhibited when glucose is available as a fuel and cytosolic LC-CoA is proposed to increase and be available for incorporation into lipid signals to support insulin release. GSIS is thought to be regulated by numerous amplification pathways. Among all of the pathways, these are thought to be crucial: anaplerosis/cataplerosis, the ATP citrate lyase (ACL)/acetyl-CoA carboxylase (ACC)/malonyl-CoA/carnitine palmitoyltransferase-1 (CPT-1) axis, the glycerolipid/NEFA cycle, post-translational attachment of small ubiquitin-like modifier to target lysine residues (SUMOylation), NADPH, reactive oxygen species, and the redox control of exocytosis proteins (Prentki, Corkey and Madiraju 2020).

As it has been suggested that when pyruvate resulting from glycolysis enters the mitochondria, it ends up providing citrate, which flows out into the cytosol, and it's converted into acetyl-CoA before it's converted again to malonyl-CoA by acetyl-CoA carboxylase (ACC) (Corkey et al. 1989). In the end, malonyl-CoA ends up being an important component for FFA synthesis in the cytosol, and it has another role to inhibit carnitine palmitoyl transferase I (CPT1) thus inhibiting long chain fatty acid oxidation (Corkey et al. 1989, Mills, Foster and McGarry 1983, Prentki et al. 1992). With these effects, lipids will accumulate in the cytosol impacting insulin secretion (Corkey et al. 2021).

Reactive oxygen species (ROS) is produced in the mitochondria at complex I and III of the electron transport chain (ETC). It results from the response to excess fuel, high mitochondrial NADH, and low ADP concentrations, and can leak to the

cytosol. In addition to that,  $\beta$ -cells are characterized by low expression of antioxidant enzymes (Corkey et al. 2021).

ROS is part of a major communication system in  $\beta$ -cells influencing hundreds of proteins including phosphatases and kinases, pyruvate kinase is one of them, which regulates  $K_{ATP}$  channels. Lower levels of ROS are needed to save important signaling functions, such as the proliferation of  $\beta$ -cell. ROS interacts with reactive cysteines by oxidizing proteins thus inhibiting cysteine-threonine and tyrosine phosphatases, which leads to activating kinases involved in vesicle trafficking. And at the state of basal respiration, when there is low energy demand plus adequate or excessive fuel, resulting in a sufficient amount of ATP, ROS production will increase significantly (Corkey et al. 2021).

### **Insulin Secretion Oscillates**

Insulin secretion is pulsatile. Cunningham et al. demonstrated  $\beta$ -cell oscillatory behavior in isolated rat islets, HIT T-15 cells and INS-1 832/13 cells. Oscillations were seen at 10 mM glucose in perfused islets with the oscillation amplitude increasing as the glucose concentration was increased to 12.5 and then 20 mM glucose. The oscillation amplitude changed with glucose concentration but there was no change in the period (Cunningham et al. 1996).

Phosphofructokinase (PFK) is a glycolytic enzyme that's activated by its product fructose-1,6-bisphosphate (F16BP), fructose-2,6-bisphosphate and AMP, while inhibited by ATP and citrate. PFK has been found in most tissues including pancreatic  $\beta$ -cells (INS-1), where all three isoforms of PFK have been found (M,

C, and L-type) (Yaney et al. 1995, Liu, Tornheim and Leahy 1998). When PFK(M) is activated by F16BP, it results in a high production of ATP from glycolysis which causes oscillations in the ATP/ADP ratio (Tornheim 1997). Oscillations are found not only in insulin secretion, but also with glycolytic intermediates and oxygen consumption, ADP and the ATP/ADP ratio, the  $K_{ATP}$  channel activity and membrane potential resulting in oscillatory cytosolic  $Ca^{2+}$  (Hopkins et al. 1992, Longo et al. 1991). Oscillations of insulin secretion are suggested to be due to alterations between on/off signals of insulin secretion. Deeney et al. demonstrated that these on/off signals of insulin secretion resulted in oscillations in insulin accumulation over time from both HIT and INS-1 cells cultured in a multi-well plate. These oscillations were correlated to the ATP/ADP ratio and  $Ca^{2+}$  influx. Coordinated oscillations between cells incubated in separate wells indicated that synchronization was due to glucose metabolism (Deeney et al. 2001).

### **The effect of Excess Nutrients (Glucolipotoxicity)**

Chronic incubation of insulin secreting cells with high glucose and FA results in Glucolipotoxicity (GLT), which is characterized by increased basal and decreased GSIS (Saadeh et al. 2012). GLT also reduces insulin content as excess nutrients stimulate insulin secretion in culture without an increase in compensatory insulin synthesis (Bollheimer et al. 1998).  $\beta$ -cells are often cultured in RPMI media containing 11 mM glucose and to study GLT investigators added higher glucose concentration with elevated FA (Saadeh et al. 2012).

More recently it has been argued that  $\beta$ -cell should be cultured at a glucose concentration more closely representing fasting serum glucose concentration. Erion et al compared INS-1 cells cultured at 4 mM and 11 mM glucose and found that cells exposed to chronic elevated glucose were already exhibiting characteristics of GLT including excess lipid accumulation, increased basal, decreased maximal GSIS and an increased glucose sensitivity leading to a left-shift in the glucose dose response for insulin secretion. Cells cultured at low glucose had increased insulin content, increased maximal GSIS and a right shifted glucose dose response to insulin secretion resulting in basal insulin secretion at a more physiological glucose concentration. The level of basal insulin secretion is often hard to compare between differently treated cells due to differences in insulin content. When the content was preserved under GLT conditions by adding DZ to inhibit insulin secretion during culture it was determined that basal insulin secretion was indeed increased suggesting that basal hypersecretion measured from cells cultured at high glucose is not an artifact of their reduced content (Erion et al. 2015).

### **ACC's Role In Fat Oxidation**

ACC1 has been noted to be the predominant ACC isoform expressed in INS-1 832/13 cells and pancreatic islets (Prentki et al. 2020). It has been shown to play a major role in insulin secretion in response to glucose and its activity is increased when INS-1 cells are incubated in high glucose. Reduced levels of ACC

expression in INS-1 cells results in a reduction in insulin secretion (Winder and Hardie 1999).

Malonyl-CoA is considered to be a metabolic switch and a regulatory metabolic coupling factor (MCF) for insulin secretion. This has been shown in vitro in INS-1 832/13 cells (Lorenz et al. 2013). The formation of malonyl-CoA is reduced when ACC expression declines (Prentki et al. 2020). When ACC was inhibited in INS-1 832/13 cells using CP-640186 and 5-tetradecyloxy-2-furoic acid (TOFA), GSIS was inhibited (MacDonald et al. 2008). This also has been shown in vivo, using the ACC inhibitor ND-630 resulting in low levels of malonyl-CoA and reduced GSIS (Harriman et al. 2016).

It is important to note that the ACC content in INS-1 cells is sensitive to the glucose condition. INS-1 cells incubated in increasing glucose concentrations (2, 5, 8, 11, 15, 20, 25, and 30 mM) for 24 hours, showed increased ACC expression (ACC mRNA) relative to the glucose concentration. ACC protein levels reflected similar changes after 72 hours. The effect was specific to the ACC protein and was not found in all of the carboxylating enzymes since PC levels were not changed (Brun et al. 1993)..

### **AMPK As A Metabolic Switch For Fat Oxidation**

AMP-activated protein kinase (AMPK) is considered as a metabolic master switch alternating between carbohydrate and fat metabolism. AMPK increases or decreases metabolic pathways by phosphorylation of the target proteins such as

ACC. It's activated by AMP and antagonized by a high concentration of ATP and therefore is responsive to the AMP/ATP ratio. Phosphorylation of ACC inhibits its activity. Also, in the liver cell, when AMPK deactivates ACC, there is a decrease in ACC's product malonyl-CoA, thus increasing fat oxidation. It's important to note that AMPK isn't the only regulator of ACC. Both citrate and LCA-CoA regulate too. A study done with INS-1 cells incubated at low glucose concentration shows both increased AMPK activity and decreased ACC activity (Winder and Hardie 1999).

### **AIMS**

Polonsky et al. reported similar number of insulin oscillations between normal and obese subjects but with hypersecretion in the latter, and in diabetic subjects, these oscillations were disrupted (Polonsky et al. 1988). In her MS thesis Narmuratova presented initial results indicating that oscillations in GSIS from INS-1 cultured at low and high glucose were similar in period with different amplitudes reflecting the insulin content of cells grown in different glucose conditions. pACC/ACC ratio also oscillated with the peak of pACC occurring between peaks of insulin secretion in 4G cells measured over a 15 min timecourse. The Aim of this thesis is to extend Narmuratova's study and compare oscillations in insulin with pACC/ACC and pAMPK/AMPK ratios (Narmuratova 2022).

AIMs.

1. Replicate the comparison of insulin oscillations between 4G and 11G cells with a longer time course and increased sample frequency.

2. Confirm protein oscillations of pACC/ACC ratio and its relationship to oscillations in insulin secretion,
3. Extend measure of protein oscillations to include pAMPK/AMPK ratio comparing the oscillatory pattern to oscillations in both pACC/ACC ratio and insulin secretion.

Our hypothesis was oscillations in glucose-stimulated insulin secretion and protein phosphorylation are coordinated to provide lipid signals to amplify insulin secretion, and dysregulation in 11G oscillations may reflect disrupted oscillations in T2DM patients.

## Methods

### Cell culture

Clonal pancreatic  $\beta$ -cells (INS-1) were cultured in RPMI media containing 4 or 11 mM glucose, 10 mM of HEPES buffer, 10 mM Sodium Pyruvate, 2 mM L-Glutamine, 50 IU/ml Penicillin, 50  $\mu$ g/ml Streptomycin, 50  $\mu$ M  $\beta$ -Mercaptoethanol (BME) and 10% of Fetal Bovine Serum (FBS). Cells were used from passage 58 to 83. Cells were seeded into 48 well plates to achieve an approximate density of 250-350k cells/well at the time of the experiment. Plating density was determined by the average doubling time of the cells (32 hrs cultured in 11 mM and 56 hrs cultured in 4 mM glucose) and the time they spent in culture prior to the experiment (4-6 days). Cells were plated noting all these considerations, in alternating 4 and 11 mM glucose wells. Cells were initially plated in 11 mM glucose and media was changed to the appropriate glucose concentration the next day. Thus each condition was plated for every other well.

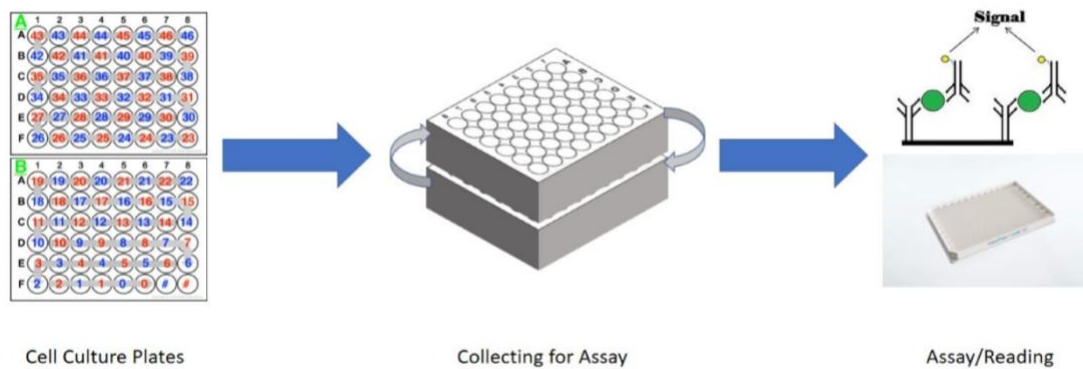
### Insulin secretion-Static incubation

On the day of the experiment culture media is replaced with RPMI media with 1 mM glucose (1G RPMI) without serum and penicillin/streptomycin and incubated at 37° C (CO<sub>2</sub> incubator) for 2 hrs. Media of the wells is replaced with 1G RPMI using a 10 ml culture pipette after dumping the contents all at once. After that cells were incubated for 30 min at 37° C (water bath) in a modified Krebs-Ringer buffer (KRB) containing (in mM) 109 NaCl, 4.6 KCl, 5 NaHCO<sub>3</sub>, 2 CaCl<sub>2</sub>, 1 MgSO<sub>4</sub>, 0.15 Na<sub>2</sub>HPO<sub>4</sub>, 0.4 KH<sub>2</sub>PO<sub>4</sub>, 20 HEPES, 1 glucose and 0.05% Bovine



30 sec intervals over a single 48 well plate. On the day of the experiment, the plate is placed in a warm water bath (37 °C) and the old medium is suctioned with a gel loading tip and replaced with 500  $\mu$ L 1G RPMI medium. Starting from well number 46 until well number 0. Starting from 46 with 15 seconds intervals (30 seconds for each condition). The plate was then moved to the CO<sub>2</sub> incubator (37 °C) for 1 hour. One well for each condition is replaced with Trypsin in order to count cells and extract for insulin content.

After 1 hour, the plates were moved back to the water bath to change the 1G RPMI to 300  $\mu$ L 1G KRB (0.05% BSA) in a similar time course fashion. The plates were kept in the water bath for an additional 7 minutes, for a total of 30 mins. After that, wells were changed to 300  $\mu$ L of 12 mM glucose KRB with 0.5% BSA.



**Figure 2. The samples of interest are collected by flipping the cell culture plates onto a new plates, before diluting and assaying.**

Collecting the secretion samples was done by flipping the plates (A) into a similar new plates (B). Plate B was stored frozen at -20 °C until assayed for insulin. The A plate containing the cells was treated immediately with SDS-PAGE sample buffer (50-300  $\mu$ L). The plate was quickly vortexed and heated to 80 °C for 2-4 min.

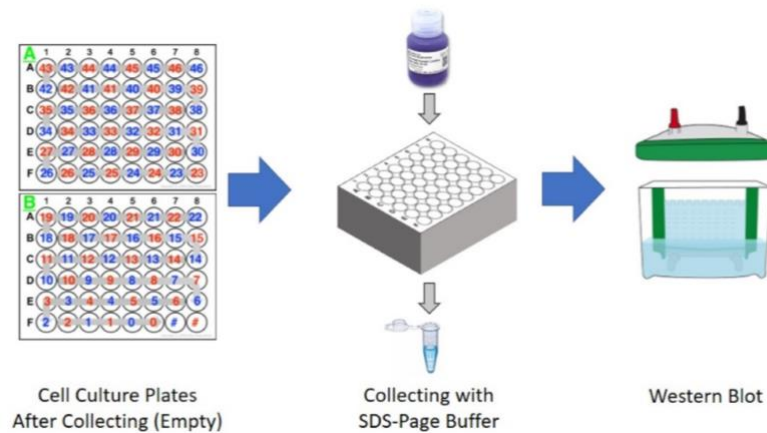
This was repeated three times. Protein samples were collected and transferred into microcentrifuge tubes to be boiled for 5 minutes and then stored at  $-20^{\circ}\text{C}$  for Western analysis.

### **Insulin assay**

Insulin was measured using a Homogeneous Time-Resolved Fluorescence immunoassay (CisBio). 2  $\mu\text{L}$  of insulin sample is added to 2  $\mu\text{L}$  of the antibody mix in a 1536 well microwell plate and incubated overnight. The plate is read at two distinct fluorescent wavelengths as per the manufacturer's instructions using a TECAN M1000 Pro Multimode plate reader. Insulin in oscillation experiment samples were diluted and compared to insulin standards similarly treated. Data was analyzed using Excel. Insulin content was measured after extracting the cells in PBS with 25 mM NaOH and 0.1% triton-x 100.

### **Western Blot**

The workflow of the western blot is shown in Figure 3. Criterion gradient gels (4-15%, 15  $\mu\text{L}$ , and 26 wells) (BioRad) were used to separate proteins for identification. The running buffer consisted of 25 mM of Tris, 192 mM of glycine, and 0.1% sodium dodecyl sulfate (SDS). The gel was run at constant 15 milliamperes for the stacking gel and then moved to the cold room ( $5^{\circ}\text{C}$ ) and the separation was continued at a constant voltage of 60 volts for 5 hours. The gel was removed and put in Towbin Transfer Buffer (25 mM Tris, 192 mM glycine, 20% methanol) for 20 minutes. Proteins in the gel were transferred to nitrocellulose membrane overnight in the cold room at a constant voltage of 7 volts.



**Figure 3. Steps by which the cells in the 48-well plates are frozen in liquid nitrogen, then they're treated with SDS-PAGE Buffer and collected in eppendorfs, and finally running a western blot.**

### Primary Antibody Incubations

After transfer, ponceau was added to the membrane and washed several times with water to visualize the transferred proteins. The membrane was blocked for one hour using 5% non-fat dried milk (NFDM) in Tris-buffered saline (TBST) (20 mM Tris, 150 mM NaCl, and 0.1% Tween). After blocking, the membrane was cut into two pieces through the 100 kDa standard that was added to border the gel samples. The two pieces were washed again several times with TBST, before treating with the primary antibodies rocking overnight in the fridge (5 °C). All antibodies were diluted (1:1000) in TBST with 3% BSA and 0.05% Sodium Azide.. The top membrane strip, which was above 100 kDa was treated with ACC (Acetyl CoA Carboxylase) (total), or P-ACC (Phospho-Acetyl CoA Carboxylase). The lower membrane strip, which is below 100 kDa was treated with

AMPK (Adenosine Monophosphate-Activated Protein Kinase (total)), or P-AMPK (Phospho-Adenosine Monophosphate-Activated Protein Kinase).

### Secondary Antibody Incubations and Imaging The Blot

The next day, the primary antibody solution was collected and the membranes were washed three times with TBST for 10 minutes each, before incubating with the secondary antibody (goat anti-rabbit) in TBST with 5% NFDM. Blots were incubated for 45 minutes on a rocker at room temperature. The membranes were washed 6 times with TBST for 10 minutes each. The blots were imaged with enhanced chemiluminescence (ECL) (SuperSignal West Femto Maximum Sensitivity Substrate) with one image collected every 30 seconds. After

	<b>Antibody type</b>	<b>Name of The Antibody</b>
<b>1</b>	<b>Primary antibody</b>	Anti-Phospho-AMPK
		Anti-AMPK- $\alpha$
		Anti-Pan Actin
		Anti-Acetyl-CoA Carboxylase
		Anti-Phospho-Acetyl CoA Carboxylase
<b>2</b>	<b>Secondary antibody</b>	Anti-rabbit goat antibody

**Table 1. The list of the antibodies used for the western blot.**

imaging ACC and AMPK phosphorylation, the blots were stripped of antibodies by incubating blots in stripping buffer (62 mM Tris (pH of 6.8), 2% SDS, and 350  $\mu$ M 2- $\beta$ ME) and rocking in a water bath (65 °C) for 30 minutes. After that, the membranes were washed thoroughly several times with TBST before blocking

again with 5% NFDM for 15-30 minutes and reincubating with antibodies for total ACC and AMPK.

## Results

### Insulin Secretion

GSIS was measured from INS-1 cells cultured in 4 mM RPMI. Figure 4 shows the insulin secretory response from cells acutely incubated with 1, 2, 3, 4, 6, 8, 10, and 12 mM glucose. Insulin secretion in response to 2 to 4 mM glucose was low and not different suggesting this represented basal insulin secretion at levels of glucose below the threshold required to stimulate insulin secretion in these cells. A small insignificant increase was observed at 6 mM glucose, which was followed by greater incremental increases in insulin secretion when glucose was increased from 8-12 mM. The percent increases in secretion compared to basal were 23% for 6 mM, 104% for 8 mM, 288% for 10 mM and 428% for 12 mM glucose.

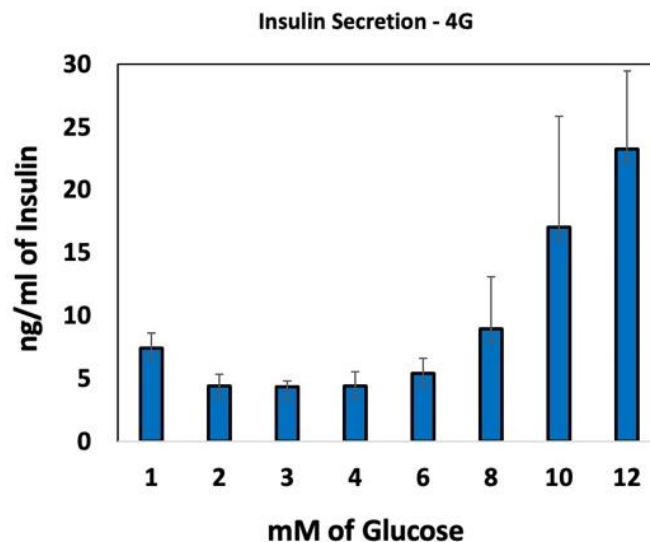
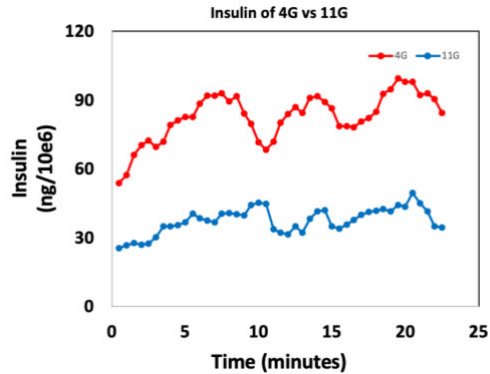


Figure 4. INS-1 cells cultured in a 96-well plate to examine glucose-stimulated insulin secretion in chronic 4G cells.

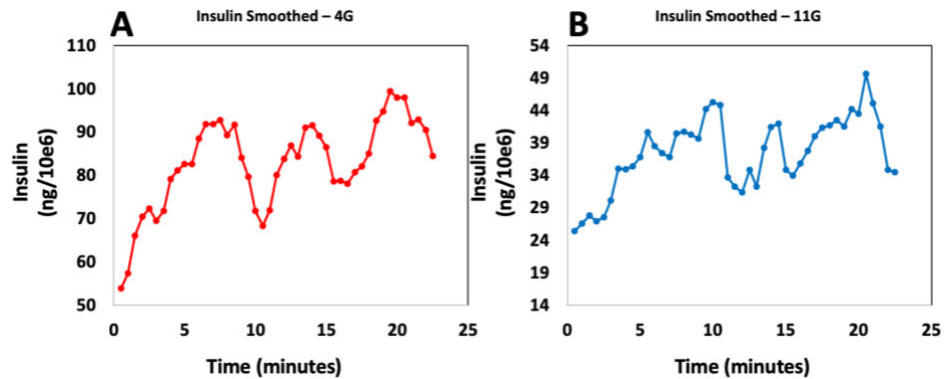
### Glucose-Stimulated Insulin Oscillations-High Resolution

In order to determine the kinetics of secretion and establish a relationship to metabolic changes within the cell we stimulated cells with glucose at timed intervals over 24 min. By culturing the cells in every other well with either 4 or 11 mM glucose we were able to do a full 24 min time course by stimulating one well at a time with 12 mM glucose every 15 sec over two 48 well plates.

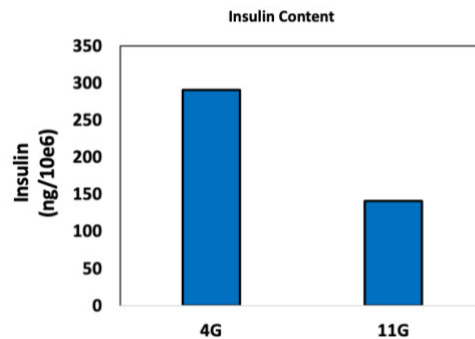
Insulin secreted from cells cultured in 11 mM glucose (11G cells) was less in concentration than that secreted from cells cultured in 4 mM glucose (4G cells) (Figure 5 and Table 2). The average insulin secretion from 4G cells was  $81.9 \pm 9.6$  ng/ $10^6$  cells, and the average of 11G was  $37.1 \pm 7.7$  ng/ $10^6$  cells. Insulin release from chronic 4G cells was 2.2 times higher than that from chronic 11G cells. Insulin content was 2-fold higher in 4G cells compared to 11G cells (290 vs 141 ng/ $10^6$  cells). Oscillations from 4 and 11 mM G cells were plotted individually as shown in Figure 6 to better visualize the oscillatory behavior in both conditions. With chronic incubation in 4G (Figure 6A) we can see clearly 3 big oscillations on the 23 min time scale with a resolution of 30 seconds. The peaks of the oscillations occur at 7.5, 14 and 19.5 min with amplitudes of 39, 23 and 21 ng/ $10^6$  cells respectively. With cells incubated in 11G (Figure 6B), similar patterns appear with 3 oscillations. The peaks of the oscillations occur at 10.5, 14.5 and 20.5 min with amplitudes of 20, 10.5 and 10 ng/ $10^6$  cells respectively. The average amplitude is 29% higher in 4G cells compared to 11G cells but was not statistically different (27.9 vs 21.6).



**Figure 5. INS-1 cells cultured in two 48-well plates to examine glucose-stimulated oscillatory insulin secretion. A 30-second interval of each condition (4 mM glucose on the top [red] and 11 mM glucose on the bottom [blue])**

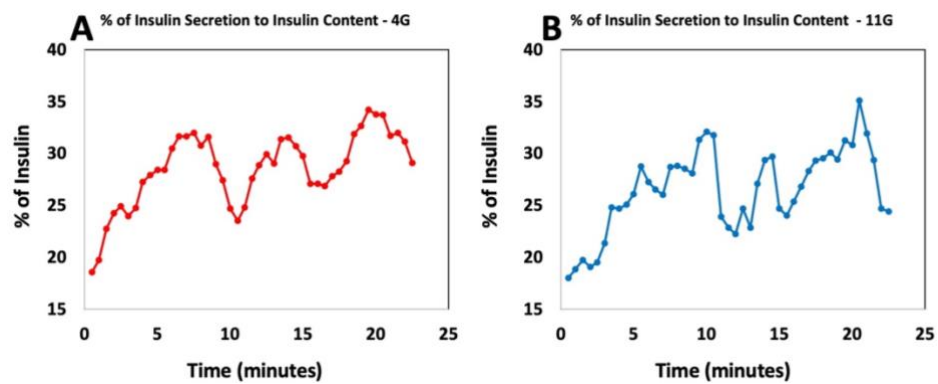


**Figure 6. INS-1 cells cultured in two 48-well plates to examine glucose-stimulated oscillatory insulin secretion. A 30-second interval of each condition (4 mM glucose on red [A] and 11 mM glucose on blue [B])**

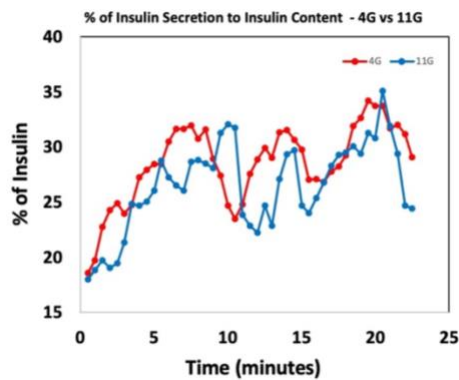


**Figure 7. Insulin content of INS-1 cells cultured in 4 mM glucose on the left and 11 mM glucose on the right.**

After correcting the insulin secretion with the insulin content of each condition as shown in Figure 7, they both have a similar percentage of insulin secreted and when we put them in one figure they show similar oscillatory behavior (Figure 9). On average, 4G cells secreted 28.2% of insulin compared to its insulin content, and 11G cells secreted 26.3%. Differences in basal insulin secretion typically found between cells cultured in low and high glucose were not observed, since the KRB was changed in the time zero well just prior to collecting the sample for insulin release.



**Figure 8. INS-1 cells cultured in two 48-well plates to examine glucose-stimulated oscillatory insulin secretion as a percentage of insulin content. A 30-second interval of each condition (4 mM glucose on the top [red] and 11 mM glucose on the bottom [blue])**



**Figure 9. INS-1 cells cultured in two 48-well plates to examine glucose-stimulated oscillatory insulin secretion as a percentage of insulin content. A 30-second interval of each condition (4 mM glucose on the top [red] and 11 mM glucose on the bottom [blue])**

<b>Average of</b>	<b>4G</b>	<b>11G</b>
<b>Secretion ng/10e6 <math>\pm</math>SD</b>	81.9 $\pm$ 13	37.1 $\pm$ 7.7
<b>Content ng/10e6 <math>\pm</math>SD</b>	290 $\pm$ 13.4	141.2 $\pm$ 35.1
<b>% of content % <math>\pm</math>SD</b>	28.2% $\pm$ 4.5	26.3% $\pm$ 5.5
<b>Amplitude ng/10e6 <math>\pm</math>SD</b>	27.9 $\pm$ 9.6	21.6 $\pm$ 5.5
<b>Time minutes <math>\pm</math>SD</b>	7.3 $\pm$ 2.3	7.3 $\pm$ 4

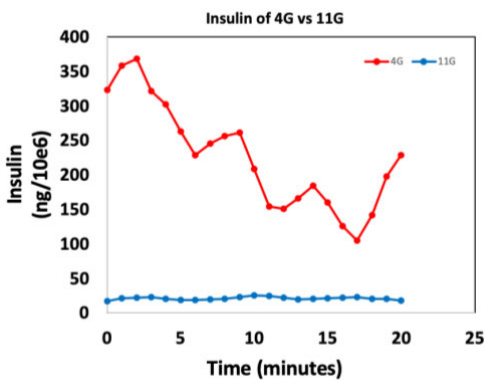
**Table 2. Shows the average secretion, content, percentage of insulin secretion to the content, oscillation amplitude, and oscillation time of both 4G and 11G cells (30-seconds experiment).**

The experiment shown above was done with glucose stimulation at 15 sec intervals, which resulted in a high time resolution (30 sec) image of oscillatory insulin secretion from both 4G and 11 G cells. In order to compare changes of insulin secretion with those of protein phosphorylation we replicated the experiment with a longer interval of glucose stimulation to achieve a 1 min resolution in both 4G and 11G cells in a single 48 well plate. The oscillations achieved at 30 sec resolution (Figure 8) would still be very well resolved if only every other point was plotted.

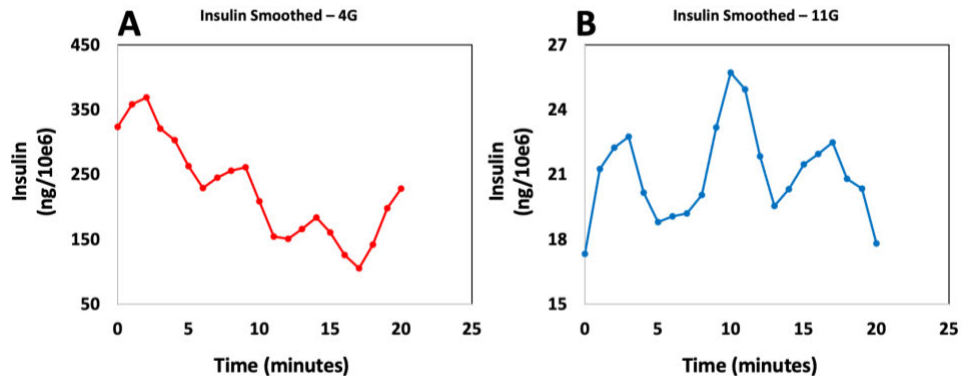
### **Glucose-Stimulated Insulin Oscillations-Low Resolution**

When secretion was measured at 1 min interval from 4G and 11G cells we obtained more disparate results from 4G and 11G cells. We measured a huge difference in the average secretion between 4G and 11G cells as shown in Figure 10 with an average secretion of 221.7 ng/10<sup>6</sup> cells in 4G cells and 20.7 ng/10<sup>6</sup> cells in 11G cells. When plotted together on the same graph the oscillations in 11G cells appear minimized by the scale of the oscillations in 4G cells. When plotted on optimal scales as in Figure 11 (A and B) at least three oscillations can clearly be seen at both glucose conditions. The peaks of the oscillations in 4G cells occur at 2, 9, and 14 with a partial peak at 20 min with amplitudes of 140, 107 and 79 ng/10<sup>6</sup> cells respectively. The peaks of the oscillations in 11G cells occur at 3, 10, and 17 with amplitudes of 5.4, 6.5 and 2.9 ng/10<sup>6</sup> cells respectively. The average amplitude in 4G cells is 93.8 and 5 in 11G cells, which is 17.8 times higher in 4G cells compared to 11G cells (Table 3). The average time from trough to trough is 5.3 minutes in 4G cells and 6 minutes in 11G cells. Insulin content was 6-fold higher in 4G cells compared to 11G cells (2784 compared to 398 ng/10<sup>6</sup> cells) (Figure 12). When normalized to insulin content 4G cells secreted 8.1% of insulin compared to its insulin content, and 11G cells secreted 5.3%. Insulin content affected the amplitude of insulin oscillations without appreciably affecting the oscillatory period. Normalizing to insulin content brought a 10-fold difference in average secretion to a less than a 2-fold difference.

The similarity between oscillations in insulin secretion with 30 sec and 1 min sampling gave us confidence to examine phosphorylation of proteins that may influence oscillatory insulin secretion in the 1 min samples.



**Figure 10. INS-1 cells cultured in one 48-well plate to examine glucose-stimulated oscillatory insulin secretion. A 1-minute interval of each condition (4 mM glucose on the top [red] and 11 mM glucose on the bottom [blue])**



**Figure 11. INS-1 cells cultured in one 48-well plate to examine glucose-stimulated oscillatory insulin secretion. A 1-minute interval of each condition (4 mM glucose on the left [red] and 11 mM glucose on the right [blue])**

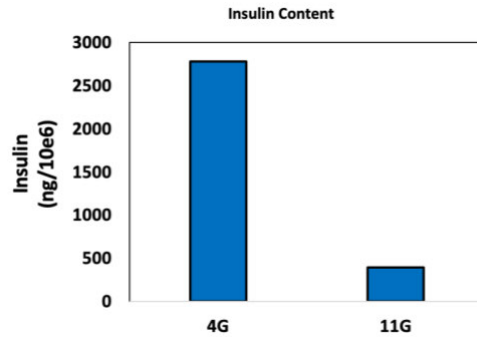


Figure 12. Insulin content of INS-1 cells cultured in 4 mM glucose on the left and 11 mM glucose on the right.

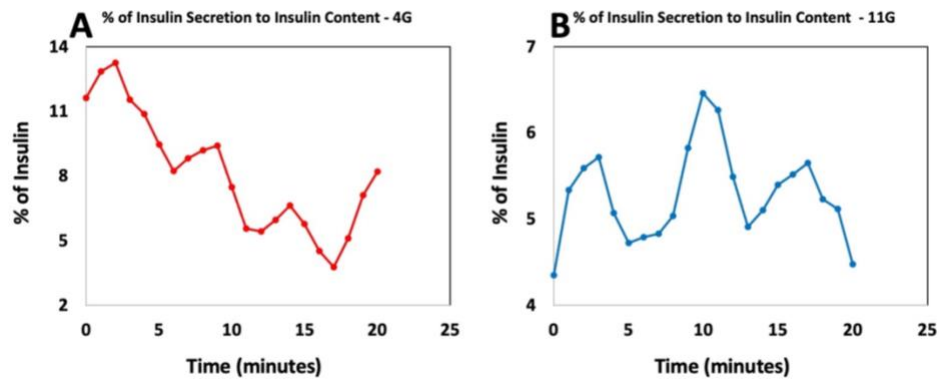


Figure 13. INS-1 cells cultured in one 48-well plate to examine glucose-stimulated oscillatory insulin secretion as a percentage of insulin content. A 1-minute interval of each condition (4 mM glucose on the top [red] and 11 mM glucose on the bottom [blue])

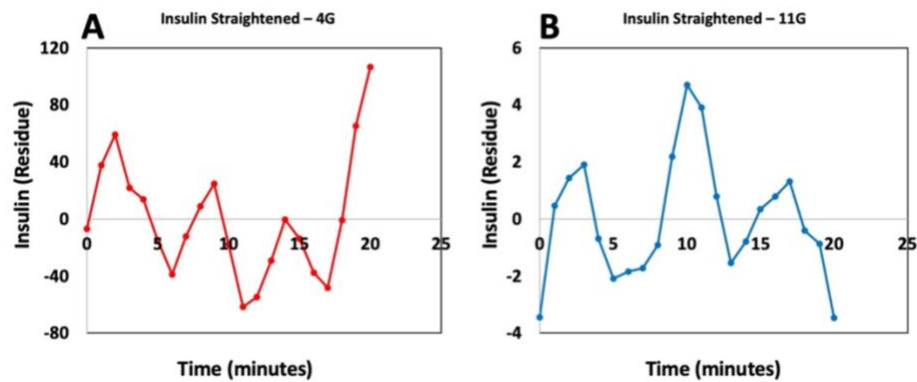
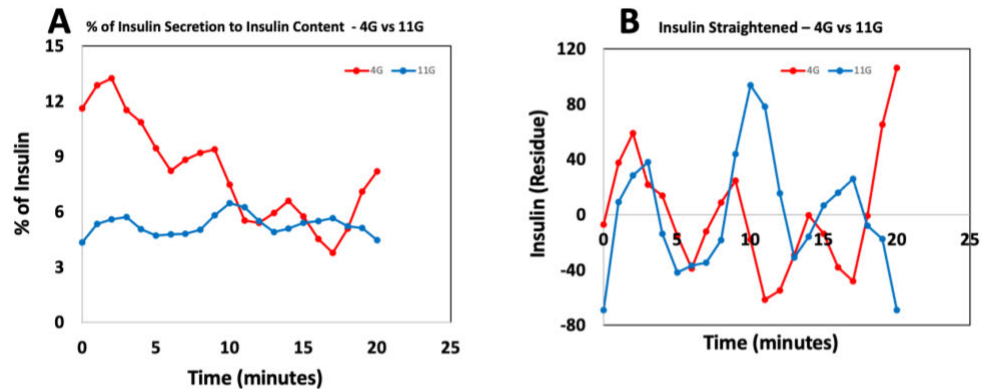


Figure 14. INS-1 cells cultured in one 48-well plate to examine glucose-stimulated oscillatory insulin secretion. A 1-minute interval of each condition (4 mM glucose on the left [red] and 11 mM glucose on the right [blue]). Ranges units are displayed in residue, which represents the distance of each point from a straight line.



**Figure 15.** INS-1 cells cultured in one 48-well plate to examine glucose-stimulated oscillatory insulin secretion as a percentage of insulin content (A) and insulin residue (B). A 1-minute interval of each condition (4 mM glucose on the top [red] and 11 mM glucose on the bottom [blue])

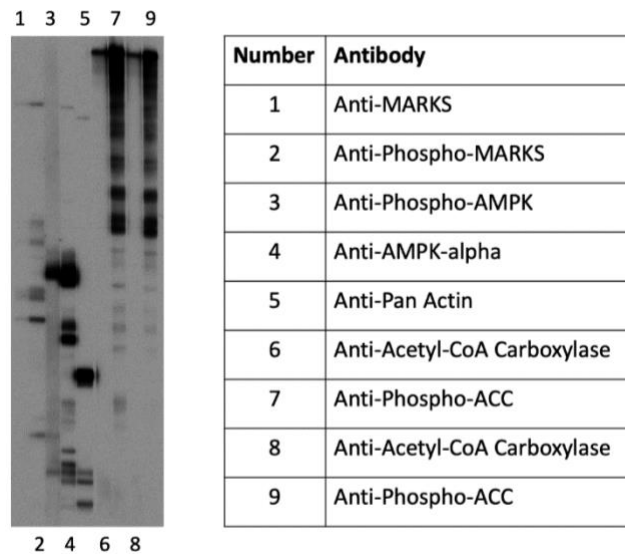
Average of	4G	11G
Secretion ng/10e6 $\pm$ SD	221.7 $\pm$ 80.7	20.7 $\pm$ 3.5
Content ng/10e6 $\pm$ SD	2784 $\pm$ 801	398 $\pm$ 79.3
% of content % $\pm$ SD	8.1% $\pm$ 2.8	5.3% $\pm$ 0.5
Amplitude ng/10e6 $\pm$ SD	108.6 $\pm$ 30.3	5 $\pm$ 1.8
Time minutes $\pm$ SD	5.3 $\pm$ 0.6	6 $\pm$ 1

**Table 3.** Shows the average secretion, content, percentage of insulin secretion to the content, oscillation amplitude, and oscillation time of both 4G and 11G cells (1-minute experiment).

### Western Blots

We tested the efficacy of the antibodies to be used in this experiment on chronic 4G INS-1 cells. The antibodies including anti-MARKS, anti-pMARKS, ,

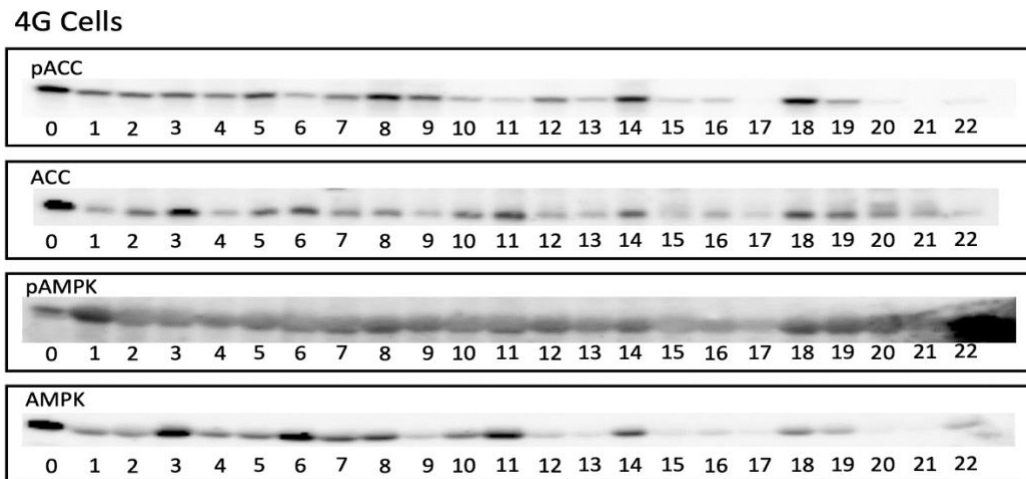
anti-AMPK, anti-pAMPK, anti-ACC, anti-pACC and anti-Actin were tested on individual strips cut from the same protein blot. The signals obtained are shown in Figure 16. Antibodies that were used in the following analysis are ACC, pACC, AMPK, and pAMPK.



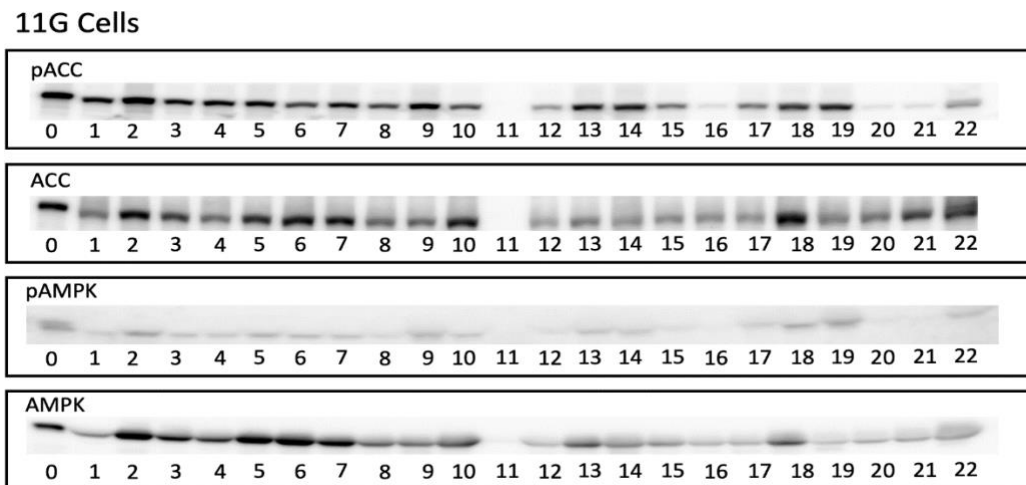
**Figure 16. Testing antibodies in INS-1 cells cultured in flask with 4 mM glucose.**

We next analyzed the samples extracted from the 48 well plate of INScells cultured in 4 mM and 11 mM glucose (in alternating wells) that were stimulated with 12 mM glucose at 1 min intervals. The time course starts with a zero time representing the last well of each condition that was changed to fresh basal (1 mM) glucose KRB and continued for 22 min. The protein signals obtained from the 4G cells are shown in Figure 17. Although it appeared that oscillations in pACC were clearly evident throughout the time course, similar variable results in total ACC were also evident after stripping and re-probing for total ACC. A similar pattern of total AMPK was seen in the same extracts indicating that the pattern was due to

variability in the protein extraction from individual wells. It was more difficult to determine clear oscillations in pAMPK in the blot that was obtained. The protein signals obtained from the 11G cells are shown in Figure 18. It can be seen that there was a similar problem in the total protein extraction from the wells with variability that could not be explained by differences in cell number assessed by visual inspection before the experiment.



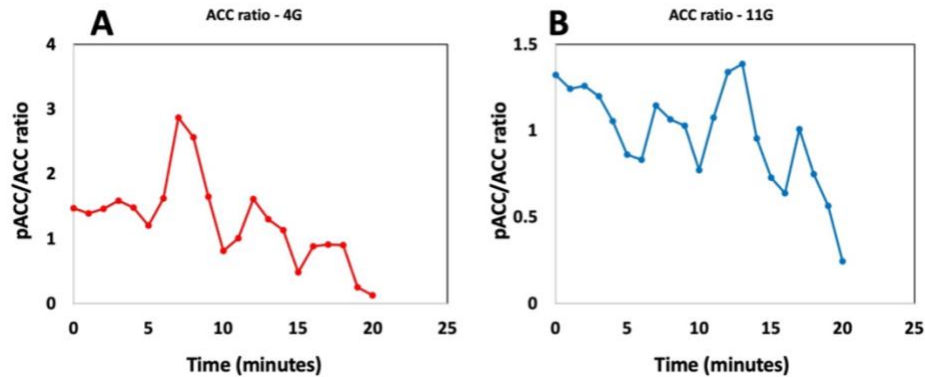
**Figure 17. INS-1 cells cultured in one 48 well plates to examine glucose-stimulated oscillatory pACC/ACC and pAMPK/AMPK ratio in chronic 4 mM glucose.**



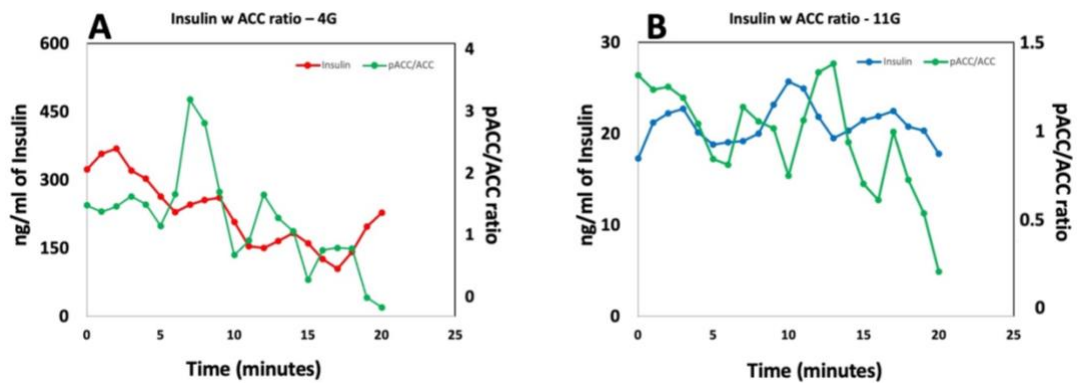
**Figure 18. INS-1 cells cultured in one 48 well plates to examine glucose-stimulated oscillatory pACC/ACC and pAMPK/AMPK ratio in chronic 11 mM glucose.**

After analyzing the blots with ImageJ software, pACC/ACC and pAMPK/AMPK ratios were calculated, plotted and compared to each other and insulin secretion. Figure 19 shows a plot of the calculated pACC/ACC from 4G (A) and 11G (B) cells. In Figure 19A, the pACC/ACC ratio of 4G cells appears to have 3 oscillations, with peaks occurring at 7, 12 and 17 min and having an average period of 5 min. 11G cells (19B) also show 3 oscillations with peaks occurring at 7, 13 and 17 min and having a similar average period of 5 min. When looking at the oscillation of insulin and pACC/ACC ratio plotted together (Figure 20), the peaks of pACC/ACC ratio are never coincident with peaks of insulin secretion in 4G cells (21 A). In the case of 11G cells (21 B) there are some peaks of pACC/ACC ratio (first and last) that occur simultaneously with peaks of insulin secretion. This is illustrated with colored bars at the peaks of each oscillation in both 4G and 11G cells. Interestingly there are small changes in the pACC/ACC ratio in both 4G and 11G cells that may or may not represent an oscillation, but do seem to line up with the first oscillation in insulin secretion.

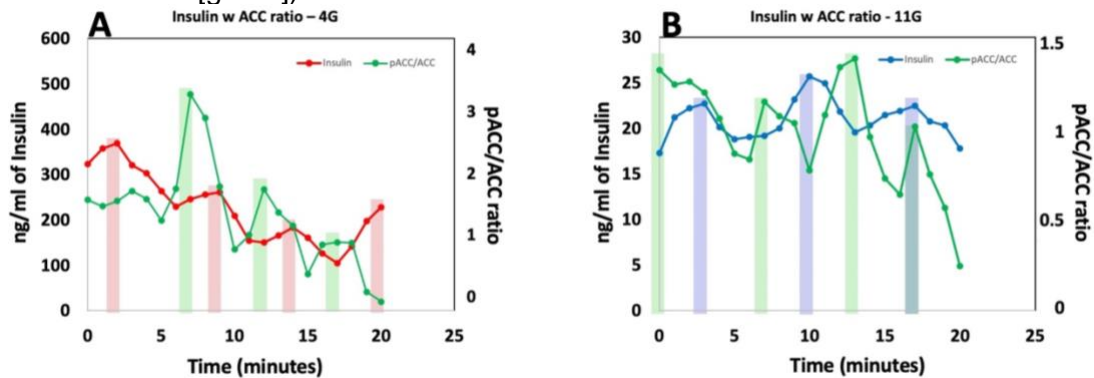
To demonstrate the relationship between pACC/ACC ratio and insulin secretion more clearly, the two were plotted as their residuals compared to the best fit line of their individual data in Figures 22-24. The residual plot of the pACC/ACC removed the negative slopes in the 4G and 11G data (compare to Figure 20). This allows a better visual of the pACC/ACC peak (green) preceding the insulin peak (red) in 4G cells while showing the contrast in the 11G cells with more disarray between the peaks of pACC/ACC (green) and insulin (blue).



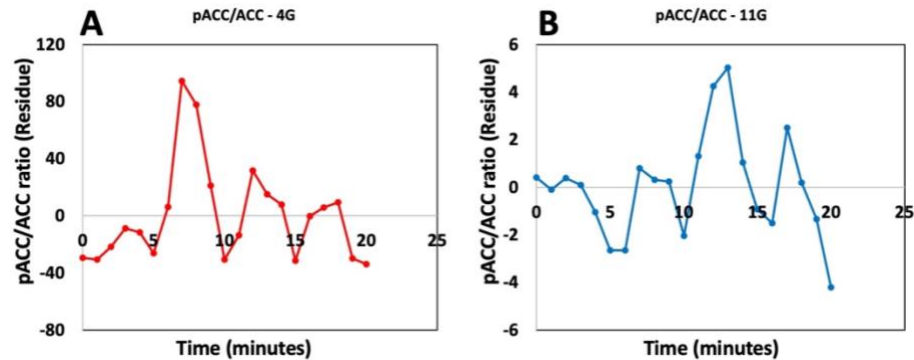
**Figure 19.** INS-1 cells cultured in one 48-well plate to examine glucose-stimulated oscillatory pACC/ACC ratio. A 1-minute interval of each condition (4 mM glucose on the left [red] and 11 mM glucose on the right [blue])



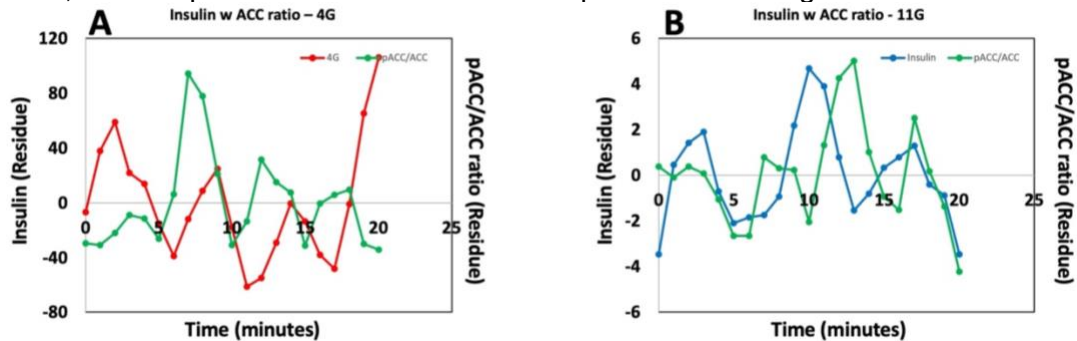
**Figure 20.** INS-1 cells cultured in one 48-well plate to examine glucose-stimulated oscillatory insulin secretion and pACC/ACC ratio. A 1-minute interval of each condition (4 mM glucose on the left [red], 11 mM glucose on the right [blue], and pACC/ACC ratio [green])



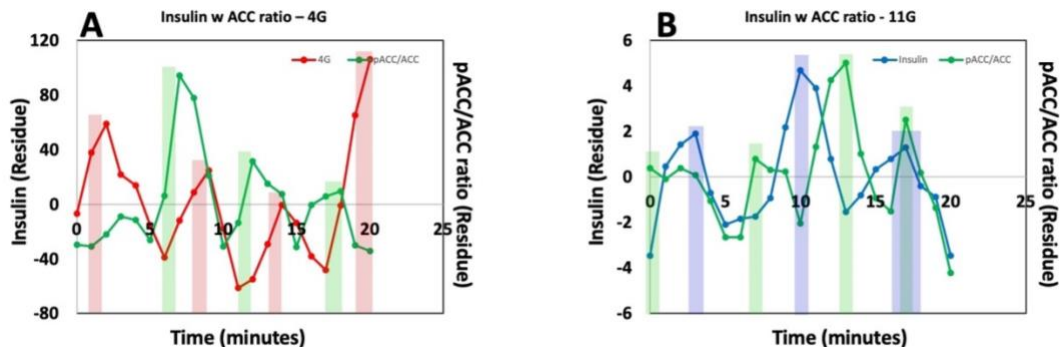
**Figure 21.** INS-1 cells cultured in one 48-well plate to examine glucose-stimulated oscillatory insulin secretion and pACC/ACC ratio with peak points. A 1-minute interval of each condition (4 mM glucose on the left [red], 11 mM glucose on the right [blue], and pACC/ACC ratio [green])



**Figure 22. INS-1 cells cultured in one 48-well plate to examine glucose-stimulated oscillatory pACC/ACC ratio.** A 1-minute interval of each condition (4 mM glucose on the left [red] and 11 mM glucose on the right [blue]). Ranges units are displayed in residue, which represents the distance of each point from a straight line.

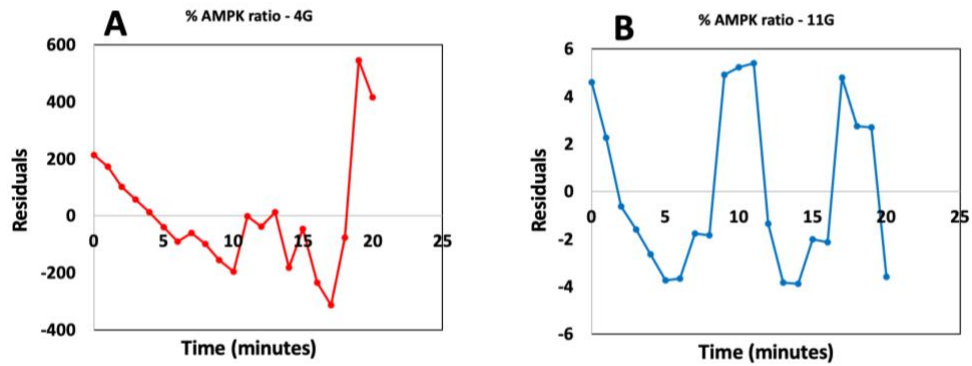


**Figure 23. INS-1 cells cultured in one 48-well plate to examine glucose-stimulated oscillatory insulin secretion and pACC/ACC ratio.** A 1-minute interval of each condition (4 mM glucose on the left [red], 11 mM glucose on the right [blue], and pACC/ACC ratio [green]). Ranges units are displayed in residue, which represents the distance of each point from a straight line.

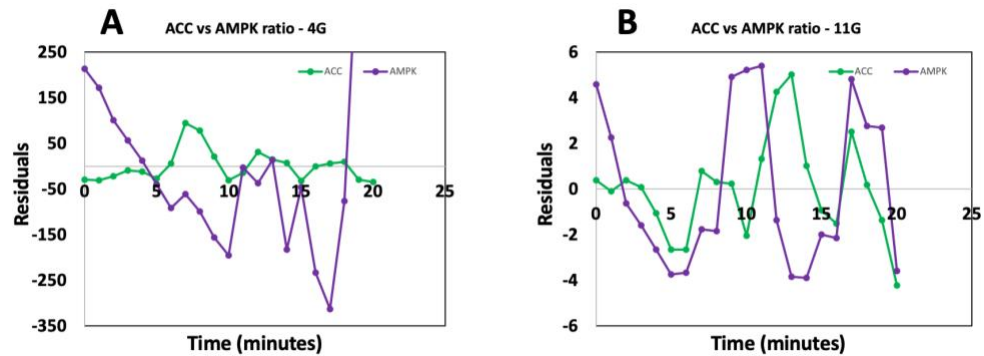


**Figure 24. INS-1 cells cultured in one 48-well plate to examine glucose-stimulated oscillatory insulin secretion and pACC/ACC ratio with peaks points.** A 1-minute interval of each condition (4 mM glucose on the left [red], 11 mM glucose on the right [blue], and pACC/ACC ratio [green]). Ranges units are displayed in residue, which represents the distance of each point from a straight line.

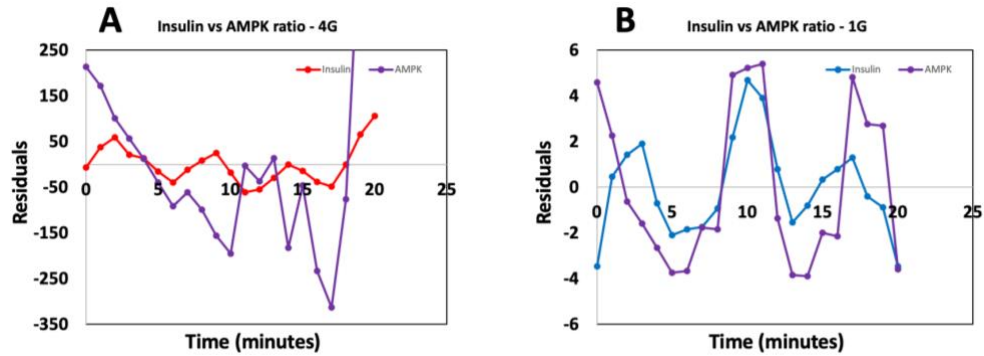
Oscillations in pAMPK/AMPK ratio are calculated and plotted for both 4G and 11G cells (Figure 22). Three peaks can be seen in 4G cells while two peaks are clearly resolved in 11G cells. When the pAMPK/AMPK ratio was plotted with insulin it can be seen that the two exhibit coincident peaks in both 4G and 11G cells.



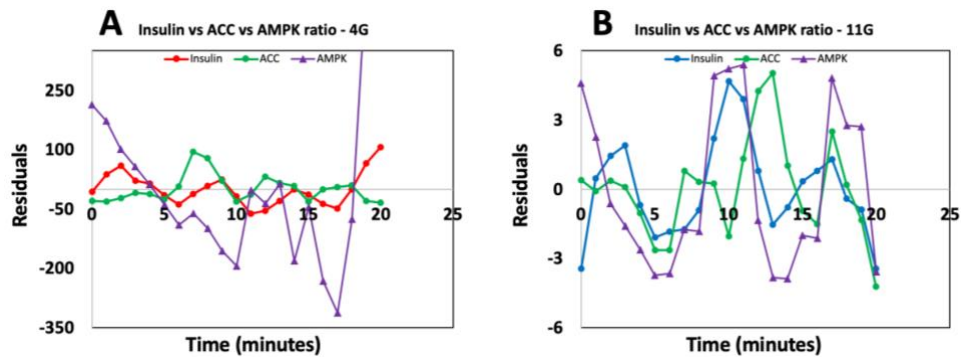
**Figure 25. INS-1 cells cultured in one 48-well plate to examine glucose-stimulated oscillatory pAMPK/AMPK ratio.** A 1-minute interval of each condition (4 mM glucose on the left [red] and 11 mM glucose on the right [blue]). Ranges units are displayed in residuals, which represent the distance of each point from a straight line.



**Figure 26. INS-1 cells cultured in one 48-well plate to examine pACC/ACC ratio and pAMPK/AMPK ratio.** A 1-minute interval of each condition (pACC/ACC ratio [green] and pAMPK/AMPK ratio [purple]). Ranges units are displayed in residuals, which represent the distance of each point from a straight line.



**Figure 27. INS-1 cells cultured in one 48-well plate to examine glucose-stimulated oscillatory insulin secretion and pAMPK/AMPK ratio.** A 1-minute interval of each condition (4 mM glucose on the left [red], 11 mM glucose on the right [blue], and pAMPK/AMPK ratio [purple]). Ranges units are displayed in residuals, which represent the distance of each point from a straight line.



**Figure 28. INS-1 cells cultured in one 48-well plate to examine glucose-stimulated oscillatory insulin secretion, pACC/ACC and pAMPK/AMPK ratio.** A 1-minute interval of each condition (4 mM glucose on the left [red], 11 mM glucose on the right [blue], pACC/ACC ratio [green], and pAMPK/AMPK ratio). Ranges units are displayed in residuals, which represent the distance of each point from a straight line.

## Discussion

Oscillations in insulin secretion have been reported for a long time, and over the years other metabolic factors (ATP/ADP ratio,  $\text{Ca}^{2+}$ , etc.) involved in insulin exocytosis have also been shown to oscillate in a coordinated fashion to drive secretion. Insulin oscillations have been measured in both isolated pancreatic islets and clonal pancreatic  $\beta$ -cells. Although much attention has been given to examining the contribution of metabolites to insulin exocytosis, not as much is known about the time sensitive changes in protein phosphorylation regulating protein activity. Our results replicate previous results that insulin oscillations can be measured in a multiwell plate format (Narmuratova 2022, Deeney et al. 2001). We demonstrate that the period of oscillations in insulin secretion is similar in 4G and 11G cells measured in a longer time course, in agreement with previous studies (Narmuratova 2022). The amplitude of these oscillations is dependent on the insulin content of the cultured cells and when corrected for content the amplitude of the oscillation as a percent of total insulin content is similar at 4G and 11G. Both pACC/ACC and pAMPK /AMPK ratios oscillate over the same time course as insulin in 4G and 11G cells. Oscillation peaks in the pACC/ACC ratio consistently precede the peaks in insulin secretion from 4G cells. This is less so in 11G cells where the relationship between pACC/ACC and insulin is not as regular. The pAMPK/AMPK ratio oscillations seem to be coincident with the pACC/ACC ratio in 4G and 11G cells, although pAMPK/AMPK ratio oscillations in 4G cells are not as clear as the oscillations in 11G cells.

Curiously, the plotted oscillations in insulin secretion consist of peaks followed by troughs that often come back to baseline. In addition the time course sometimes follows a negative slope. In this multiwell system it would be expected that insulin continually accumulates and would show a positive slope with no deep troughs. To rule out that this could be attributed to the insulin assay itself we assayed a 1-min time course in 4G cells in triplicate by assaying the samples in sequence 3 times. The average value at each time plotted and smoothed (3 point moving average). The results are consistent with the oscillations being generated by the cells and not an artifact of the assay itself (Figures 29 and 30, appendix).

When insulin secretion from 4G cells is matched to oscillations in the pACC/ACC ratio (Figure 21A), we can see that the peaks of insulin secretion are found at the trough of the pACC/ACC ratio and vice versa. This result makes sense since the phosphorylation of ACC will inhibit its activity. The reduction in ACC activity will lead to decreased malonyl-CoA and reduced inhibition of CPT-1 allowing LC-CoA to enter the mitochondria for oxidation. Thus LC-CoA oxidation is increased and incorporation into lipid signals to support insulin exocytosis will be reduced when exocytosis is at a low point. The opposite is true when the insulin secretion is at its highest. In this case the pACC/ACC ratio is at its lowest allowing for malonyl-CoA production, inhibition of CPT-1 and decreased LC-CoA oxidation when lipid signals are needed for exocytosis.

In 11G cells (Figure 21B), the correlation of pACC/ACC to insulin release is not as clear as in 4G cells, whether the peaks of pACC/ACC ratio are consistently

opposing the insulin peak or not. It's hard to tell by this single experiment whether phosphorylation of the ACC in 11G cells is significantly altered, although there does appear to be more of a disconnection. It is interesting to think that this approach may lead to insight into altered lipid signaling in cells that may be altered by GLT at high glucose. In this regard it is important to remember that ACC expression in INS-1 cells has been shown to increase with culture at increasing glucose concentrations. Thus part of a putative disruption in lipid signaling may be that in 11G cells the ACC is elevated to the point where oscillations in its activity has less of a controlling effect as inhibition of the enzyme may never be so much as to alter LC-CoA oxidation and incorporation into lipid. This may mean that there would be a more constant lipid signaling input into cells that have been chronically exposed to high glucose leading to altered oscillations in insulin secretion. On the other hand, the secretion we show here in 4G and 11G cells matches remarkably well.

With regard to the correlation of insulin secretion to changes in phosphorylation, another possibility that we should mention is that insulin samples are collected before collecting the cell proteins with SDS-PAGE Buffer during the experiment, and this may shift the oscillation of pACC/ACC ratio toward the right. Experiments in the future may need to be done by quickly freezing the cells in liquid N<sub>2</sub> to fix the phosphorylation as close to insulin collection as possible.

For the pAMPK/AMPK ratio, we expect it to follow the pattern of pACC/ACC ratio since pAMPK is going to phosphorylate ACC. By looking at pACC/ACC with

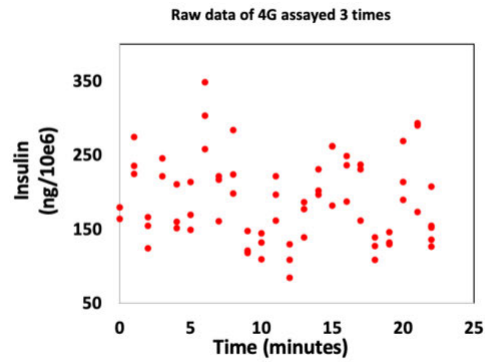
pAMPK/AMPK ratio (Figure 26), it seems that the pACC increases when pAMPK shows an increase. And it's hard to judge by looking at insulin oscillation with pAMPK ratio since it doesn't follow a consistent pattern.

It's important to mention the difficulty that we faced during our journey. We had multiple problems but the most important among them was the collection of the cells for western blot. This resulted in uneven signals measured for total proteins. This is a variable that should not exist, as the cell number is the same from between wells of the same glucose incubation (4G and 11G). If it turns out that this is due to extracting in PAGE sample buffer in the well, we will switch to extracting in a more traditional RIPA buffer and adding concentrated PAGE sample buffer prior to running the gel. The low extraction may have led to our problem in sensitivity of the measures as the antibodies had to be used at a higher concentration and with more sensitive chemiluminescence reagents to detect our signals.

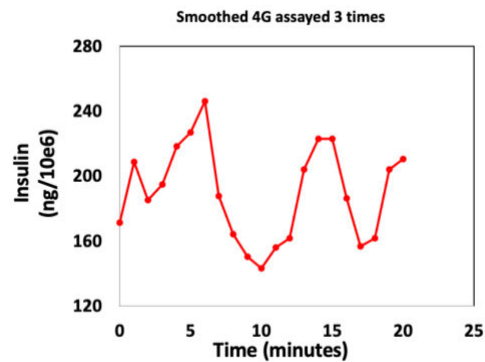
In light of the difficulties discussed it's interesting to see promising results, at least with pACC/ACC ratio, which encourages us to adjust the protocol to achieve clearer blots. Narmuratova et al. reported in 2021 an oscillation in INS-1 cells using a 48-well plate and has been able to measure proteins such as MARKS and ACC ratio for 4G and 11G cells (Narmuratova 2022). We wanted to take this to the next step and see if we're able to see a high resolution of the oscillation with a longer time course. It will also be interesting to extend our studies to measure other proteins or lipids involved in this pathway.

In conclusion, oscillations in insulin secretion are preceded by oscillations in ACC and AMPK phosphorylation to regulate lipid signals that amplify normal glucose-stimulated insulin secretion. These lipid signals that amplify insulin secretion may be disrupted in excess nutrient conditions. Chronic excess nutrients may alter the coordination of ACC and AMPK phosphorylation with insulin secretion resulting in impaired oscillations in insulin release. Regulation of lipid signals in the pancreatic  $\beta$ -cell may provide therapeutic benefit in the treatment of hyperinsulinemia, insulin resistance and Type 2 diabetes.

## APPENDIX



**Figure 29. INS-1 cells cultured in one 48-well plate to examine glucose-stimulated oscillatory insulin secretion.** A 1-minute interval of each condition (4 mM glucose on red and 11 mM glucose not shown) was assayed 3 times. Each point represents a one well assay.



**Figure 30. INS-1 cells cultured in one 48-well plate to examine glucose-stimulated oscillatory insulin secretion.** A 1-minute interval of each condition (4 mM glucose on red and 11 mM glucose not shown) was assayed 3 times, averaged and smoothed.

### List of Abbreviated Journal Titles

ArteriosclerThrombVascBiol.....	Arteriosclerosis, thrombosis, and vascular biology
Am J Physiol.....	American journal of physiology
Arch Biochem Biophys.....	Archives of biochemistry and biophysics
Association, A. D.....	American Diabetes Association
Biochem J.....	Biochemical Journal
Clin Biochem.....	Clinical biochemistry
ExpClinEndocrinolDiabetes...	Experimental and clinical endocrinology & diabetes
J Biol Chem.....	The Journal of biological chemistry
J Clin Invest.....	The Journal of clinical investigation
J Clin Endocrinol Metab.....	The Journal of clinical endocrinology and metabolism
N Engl J Med.....	The New England journal of medicine
Nat Rev Endocrinol.....	Nature reviews. Endocrinology
Physiol Endocrinol Metab...	American journal of physiology. Endocrinology and metabolism
Proc Natl Acad Sci U S A.....	Proceedings of the National Academy of Sciences of the United States of America
Sci Rep.....	Scientific reports

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**CURRICULUM VITAE**

