Studies on molecular mechanisms in calcium signaling and cellular energy consumption

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A special dedication to my dear Mother, Father and Sister
To discern the truth in everything, by whom so ever spoken, is wisdom.

- Thiruvalluvar
Abstract

Ion signaling plays fundamental role in cell survival. Na\(^+\) and Ca\(^{2+}\) are critical players in ion signaling. Cells spend the major amount of energy to maintain and regulate Na\(^+\) and Ca\(^{2+}\) gradients across the cell membrane. Any disruption in cellular energy consumption by plasma membrane ATPases affects ion signaling and vice versa. In this thesis, I first aimed to quantify ATP consumption dynamics of the Na\(^+\)/K\(^+\)-ATPase (NKA) at a single cell level using live cell imaging techniques. Secondly, I extended my research using similar technique to describe mechanisms that are involved in Ca\(^{2+}\) signaling pathway that contribute to the pathobiology of hypertension. In the third and fourth part, I used ratiometric fluorometry technique to understand the role of Ca\(^{2+}\) signaling in insulin secretion dynamics in the pancreatic β-cells.

Adenosine triphosphate (ATP) is the major energy source in cells and NKA, among plasma membrane ATPases represents the main energy consumer. In the first part of the thesis, we demonstrate a method to quantify ATP consumption dynamics of NKA at a single cell level in human embryonic kidney cells (HEK293a) using PercevalHR, a genetically encoded fluorescent biosensor that reports changes in the ATP:ADP during live cell imaging. The results showed that inhibition of mitochondrial ATP production caused a reversible reduction in basal ATP:ADP in rat primary proximal tubule cells (PTC) but not in HEK293a cells. Upon inhibiting glycolysis, a rapid decline in ATP:ADP in HEK293a but not in PTC was observed, suggesting a Warburg effect in cell lines where metabolism has been adapted to a high glucose and low oxygen environment. It was also observed that the rate of ATP hydrolysis was higher at optimal working temperature of ATPases i.e. 37°C, compared to room temperature. The rate of ATP consumption was significantly reduced when the sarco-endoplasmic reticulum Ca\(^{2+}\) ATPase and NKA were inhibited and ATP consumption increased when the NKA was forced to function at maximal velocity. The above findings allow us to conclude that PercevalHR is a robust and sensitive fluorescent probe to measure ATP consumption dynamics of NKA in HEK293a and primary PTC cells.
Hypertension treatment strategy includes the use of a wide range of blockers of angiotensin II type 1 receptor (AT1R) and the voltage gated calcium channel 1.2 (Cav1.2). The effect of physiological concentrations of angiotensin II (AngII) on AT,R signaling remains elusive and it is also unclear if there is a reciprocal regulation of AT,R signaling by Cav1.2. The main aim of the second part of my thesis was to examine the Ca^{2+} dynamics upon exposure to physiological and pharmacological AngII doses in HEK293a cells, cardiomyocytes in primary culture and aortic smooth muscle cells using live cell imaging technique. Repeated exposure to physiological (1 nM) AngII concentrations led to upregulation of Ca^{2+} response in contrast to pharmacological (100 nM) AngII exposure. Upon blocking Cav1.2, amplified Ca^{2+} response to 1 nM AngII in both HEK 293a cells and cardiomyocytes was observed. The inhibition of the Ca^{2+} permeable transient receptor potential (TRP) channels amplified the AT,R response suggests that the activity of AT,R is sensitive to Ca^{2+} influx. These findings provide new insights into AngII signaling and open opportunities for future research into understanding of hyperactivity of the angiotensin system and for use of Ca^{2+} channel blockers as mono-therapy in hypertension.

In pancreatic β-cells, an increase in the cytoplasmic free Ca^{2+} concentration ([Ca^{2+}]_i) triggers insulin secretion. Several mechanisms have been proposed that regulate membrane depolarization upon Ca^{2+} influx and closure of K_ATP channels. Cation channels belonging to TRP family have been shown to play important role in depolarization. In the third part of my thesis, I aimed to study the role of the TRPM5 channel in regulating insulin secretion, and [Ca^{2+}]_i in rat β-cells. The results showed that upon inhibition of TRPM5 channels in the presence of KCl or fructose, an insulin inhibitory response was not seen, but reduced insulin secretion was seen in response to glucose, L-arginine, and GLP-1. It also inhibited the K_ATP channel-independent insulin secretion by glucose. Inhibition of TRPM5, reduced the [Ca^{2+}]_i increase in response to L-arginine. It also inhibited the [Ca^{2+}]_i increase triggered by glucose in a K_ATP channel independent mechanism. However, inhibition of TRPM5 did not alter the [Ca^{2+}]_i response triggered by KCl, fructose, glucose and GLP-1. From the above, it is evident that TRPM5 plays an important role in mediating insulin secretion
by glucose, and L-arginine, and in potentiating the glucose-induced insulin secretion by GLP-1. This study implies that TRPM5 could be a potential therapeutic target for treating insulin secretion dysfunction.

The findings of TRPM5 mediated alteration in $\text{Ca}^{2+}$ signaling and insulin secretion in the rat $\beta$-cells prompted to fourth part of this thesis, which aimed at studying $\text{Ca}^{2+}$ signaling in the recently developed genetically engineered human insulinoma cell line (EndoC-BH1) thus promoting a newer alternative platform to researches on $\text{Ca}^{2+}$ signaling in the context of type II diabetes in humans. We stimulated the EndoC-BH1 cells with substances such as glucose, GLP-1, KCl, carbachol, L-arginine and tolbutamide which are generally known to increase $[\text{Ca}^{2+}]_i$ in the primary $\beta$-cells. A similar response was observed in the human insulinoma cells. Another additional finding was that GLP-1 was essential for eliciting a $\text{Ca}^{2+}$ response in the EndoC-BH1 cells upon stimulation by tolbutamide and glucose. We conclude that the EndoC-BH1 cells respond with a $[\text{Ca}^{2+}]_i$ increase upon stimulation by several well-known agonists and thus can serve as a novel model to study $\text{Ca}^{2+}$ signaling.

In conclusion, we demonstrate a robust cell imaging technique that can be used to quantify ATP consumption dynamics of NKA, to study several pathological conditions that results because of NKA dysfunction. My thesis additionally provides new insights into understanding $\text{Ca}^{2+}$ signaling and its role in hypertension and type II diabetes.
Sammanfattning


Adenosintrifosfat (ATP) är den största energikällan i celler och NKA utgör den största konsumenten av energi bland plasmamembran-ATPaser. I det första delarbetet demonstrerar vi en metod för att kvantifiera den NKA-beroende konsumtionen av ATP i enskilda humana embryonala njurceller (HEK293a) med hjälp av PercevalHR, en genetisk fluorescerande biosensor som rapporterar förändringar i förhållandet mellan ATP:ADP i levande celler. Resultaten påvisade att en hämning av mitokondriens ATP-produktion gav upphov till en reversibel minskning av basalt ATP:ADP i primära proximala tubuli celler (PTC) men inte i HEK293a-celler. Under inhibering av glykolys observerades en snabb minskning i ATP:ADP i HEK293a-celler men inte i PTC, vilket tyder på en Warburg-effekt i cellinjer där metabolismen har anpassats till en hög nivå av glukos och låg nivå av syre. Vi observerade också att ATP-hydrolysen var snabbare vid optimal arbetstemperatur för ATPaser dvs 37°C, jämfört med rumstemperatur. ATP-konsumtionen över tid minskade signifikant när det sarco-endoplasmatiska nätverkets Ca²⁺-ATPas (SERCA) och NKA inhiberades, och ATP-konsumtionen ökade när NKA pressades till maximal aktivitet. Dessa observationer visar att PercevalHR är en robust och känslig fluorescerande sensor som kan användas för att mäta dynamiken i ATP-konsumtionen av NKA i HEK293a-celler och primära PTC-celler.
Strategier för att behandla hypertoni innefattar användandet av ett brett spektrum av blockerare av angiotensin II typ 1-receptorn (AT1R) och den spänningsberoende kalciumkanalen 1,2 (CaV1.2). Lite är dock känt om effekten av fysiologiska koncentrationer av angiotensin II (AngII) på AT1R-signalering och om det finns en ömbesidig reglering av AT1R-signalering genom CaV1.2. Syftet med det andra delarbetet var att undersöka Ca²⁺-dynamiken under fysiologiska och farmakologiska AngII-doser i HEK293a-celler, hjärtmuskeler i primärkultur och glatt muskulatureller från aorta med mikroskopiavbildning på levande celler. Upprepad behandling med fysiologiska (1 nM) AngII-koncentrationer ökade Ca²⁺-responsen i motsats till farmakologisk (100 nM) AngII-exponering. En hämning av CaV1,2 förstärkte Ca²⁺-responsen efter behandling med 1 nM AngII i både HEK293a-celler och kardiomyocyter. Förstärkningen av AT1R-responsen observerades också efter hämning av kalciumpermeabla jonkanaler tillhörande TRPC-familjen, vilket tyder på att aktiviteten av AT1R är känslig för kalciuminflöde. De här observationerna ger ny kunskap om AngII-signalering och skapar möjligheter för framtida forskning att studera hyperaktivitet av angiotensin-systemet och användandet av Ca²⁺-kanalblockerare som monoterapi vid hypertoni.

En ökning av den fria Ca²⁺-koncentrationen i cytoplasman ([Ca²⁺]ᵢ) orsakar insulinutsöndring i β-celler från pankreas. En membran depolarisering följd av Ca²⁺-inflöde och en stängning av Kₐ₅₆-kanaler regleras av ett flertal mekanismer. Katjonkanaler tillhörande TRP-familjen tros spela en viktig roll för depolariseringen. I det tredje delarbetet studerade jag den roll som TRPM5-kanalen har i att reglera insulinutsöndring och [Ca²⁺], i β-celler från rätta. Inhibering av TRPM5-kanaler hämmade inte insulinutsöndringen utlöst av KCl eller fruktos, men minskade insulinutsöndringen efter behandling med glukos, L-arginin och GLP-1. TRPM5-inhibering hämmade också den Kₐ₅₆-oberoende insulinutsöndringen efter behandling med glukos. Inhibering av TRPM5-kanalen minskade även [Ca²⁺]-ökningen vid behandling med L-arginin och hämmade [Ca²⁺]-ökningen efter behandling med glukos genom en Kₐ₅₆-oberoende mekanism. Däremot hade TRPM5-inhiberingen ingen effekt på [Ca²⁺]-responsen efter
behandling med KCl, fruktos, glukos eller GLP-1. Våra observationer tyder på att TRPM5 spelar en viktig roll för att mediera insulinutsöndringen genom glukos och L-arginin och i att förstärka glukosinducerad insulinsekretion med GLP-1. Den här studien implicerar att en riktad reglering av TRPM5 skulle kunna användas för att behandla störningar i insulinutsöndringen.

Baserat på våra observationer att TRPM5 reglerar Ca\textsuperscript{2+}-signaleringen och insulinutsöndringen i β-celler från råtta, ville jag i det fjärde delarbete studera Ca\textsuperscript{2+}-signaleringen i en nyligen utvecklad genetiskt skapad human insulinom celllinje (Endoc-BH1). Detta för att undersöka en alternativ modell för Ca\textsuperscript{2+}-baserade studier med frågeställningar relevanta för typ 2-diabetes i människa. Vi stimulerade Endoc-BH1-celler med glukos, GLP-1, KCl, karbakol, L-arginin, och tolbutamid. Dessa substanter, kända för att öka [Ca\textsuperscript{2+}]\textsubscript{i} i primära β-celler, ökade också [Ca\textsuperscript{2+}]\textsubscript{i} i de humana insulinomcellerna. Vi fann också att GLP-1 var nödvändig för att framkalla en Ca\textsuperscript{2+}-respons i Endoc-BH1-celler vid behandling med tolbutamid och glukos. Endoc-BH1-celler svarar alltså på ett likartat sätt som β-celler med en [Ca\textsuperscript{2+}]\textsubscript{i}-ökning vid behandling med flera välkända agonister.

Sammanfattningsvis så demonstrerar vi en avbildningsmetod som kan användas på levande celler för att kvantifiera dynamiken av NKAs ATP-konsumtion och för studera ett flertal patologiska tillstånd orsakade av en dysfunktion hos NKA. Min avhandling ger också ny kunskap om Ca\textsuperscript{2+}-signaleringen och dess roll vid hypertoni och typ 2-diabetes.
List of publications and manuscripts

This thesis is written based on the following three publications and one manuscript:

**Paper I**

**Krishnan K,** Fritz N, Huličiak M and Brismar H. Study of energy consumption by Na⁺/K⁺ ATPase using PercevalHR. (Manuscript)

**Paper II**

Bernhem K, **Krishnan K,** Bondar A, Brismar H, Aperia A, Scott L. AT₁-receptor Response to non-Saturating Ang-II concentrations is amplified by calcium channel blockers. (Provisionally accepted in the journal BMC cardiovascular disorder)

**Paper III**


**Paper IV**

**Contribution to the publications**

**Paper I**

I was involved in project design, planned and executed all experiments, analysed results, made figures and wrote most of the manuscript.

**Paper II**

I designed and performed experiments on HEK293a cells that addressed the role of VGCC and TRPC channels in AT1R signaling, analyzed the results and contributed to manuscript writing.

**Paper III**

I designed and performed most of the experiments, analysed results, made figures and wrote the manuscript.

**Paper IV**

I designed the study, performed all the experiments, analysed results, made figures and contributed in manuscript writing.
List of abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>ACE</td>
<td>Angiotensin Converting Enzyme</td>
</tr>
<tr>
<td>ADP</td>
<td>Adenosine diphosphate</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>ATP:ADP</td>
<td>ATP: ADP ratio</td>
</tr>
<tr>
<td>AngII</td>
<td>Angiotensin II</td>
</tr>
<tr>
<td>AT1R</td>
<td>Angiotensin II type 1 receptor</td>
</tr>
<tr>
<td>ARG</td>
<td>L-arginine</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BSA</td>
<td>Albumin from bovine serum fraction V</td>
</tr>
<tr>
<td>BTP2</td>
<td>3,5-bis(trifluoromethyl)pyrazole</td>
</tr>
<tr>
<td>CaV1.2</td>
<td>Voltage gated calcium channel subtype 1.2</td>
</tr>
<tr>
<td>[Ca^{2+}]_i</td>
<td>Cytoplasm Ca^{2+} concentration</td>
</tr>
<tr>
<td>CCH</td>
<td>Carbachol</td>
</tr>
<tr>
<td>CICR</td>
<td>Ca^{2+} induced Ca^{2+} increase</td>
</tr>
<tr>
<td>CPA</td>
<td>Cyclopiazonic acid</td>
</tr>
<tr>
<td>CTP</td>
<td>Cytidine triphosphate</td>
</tr>
<tr>
<td>DAG</td>
<td>Diacylglycerol</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's Modified Eagle Medium</td>
</tr>
<tr>
<td>DZ</td>
<td>Diazoxide</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>FADH$_2$</td>
<td>Flavin adenine dinucleotide</td>
</tr>
<tr>
<td>FRAP</td>
<td>Fluorescence recovery after photo bleaching</td>
</tr>
<tr>
<td>Term</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>---------------------------------------------------------------------------</td>
</tr>
<tr>
<td>FRET</td>
<td>Förster resonance energy transfer</td>
</tr>
<tr>
<td>Fura-2 AM</td>
<td>Fura-2 Acetoxymentyl ester</td>
</tr>
<tr>
<td>Gαq</td>
<td>G-protein subunit αq</td>
</tr>
<tr>
<td>GK</td>
<td>Glucokinase</td>
</tr>
<tr>
<td>GLP-1</td>
<td>Glucagon like peptide-1</td>
</tr>
<tr>
<td>GLUT</td>
<td>Glucose transporter</td>
</tr>
<tr>
<td>GSIS</td>
<td>Glucose stimulated insulin secretion</td>
</tr>
<tr>
<td>GTP</td>
<td>Guanosine triphosphate</td>
</tr>
<tr>
<td>HEK 293a</td>
<td>Human embryonic kidney cells</td>
</tr>
<tr>
<td>hTERT</td>
<td>Human telomerase reverse transcriptase</td>
</tr>
<tr>
<td>IAA</td>
<td>Iodo acetic acid</td>
</tr>
<tr>
<td>IP₃</td>
<td>Inositol 1,4,5-trisphosphate</td>
</tr>
<tr>
<td>IP₃R</td>
<td>Inositol 1,4,5-trisphosphate receptor</td>
</tr>
<tr>
<td>KₐATP</td>
<td>ATP sensitive K⁺ channel</td>
</tr>
<tr>
<td>KCa</td>
<td>Calcium activated K⁺ channels</td>
</tr>
<tr>
<td>KCl</td>
<td>Potassium chloride</td>
</tr>
<tr>
<td>KRBH</td>
<td>Krebs Ringer bicarbonate HEPES buffer</td>
</tr>
<tr>
<td>NADH</td>
<td>Nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>NCX</td>
<td>Na⁺-Ca²⁺ exchanger</td>
</tr>
<tr>
<td>NKA</td>
<td>Na⁺/K⁺ ATPase</td>
</tr>
<tr>
<td>OUA</td>
<td>Ouabain</td>
</tr>
<tr>
<td>PIP₂</td>
<td>Phosphatidylinositol 4,5-bisphosphate</td>
</tr>
<tr>
<td>PI-PLC</td>
<td>Phosphatidyl inositol specific phospholipase C</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>PMCA</td>
<td>Plasma membrane Ca²⁺ ATPase</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>PP</td>
<td>Pancreatic polypeptide</td>
</tr>
<tr>
<td>PTC</td>
<td>Proximal tubule cells</td>
</tr>
<tr>
<td>RPMI</td>
<td>Roswell Park Memorial Institute 1640 medium</td>
</tr>
<tr>
<td>RVCM</td>
<td>primary rat ventricular cardiomyocytes</td>
</tr>
<tr>
<td>RyR</td>
<td>Ryanodine receptor</td>
</tr>
<tr>
<td>SCID</td>
<td>Severe combined immunodeficiency</td>
</tr>
<tr>
<td>SERCA</td>
<td>Sarco-endoplasmic reticulum Ca(^{2+}) ATPase</td>
</tr>
<tr>
<td>STED</td>
<td>Stimulated emission depletion fluorescence microscopy</td>
</tr>
<tr>
<td>STIM</td>
<td>Stromal interaction molecule</td>
</tr>
<tr>
<td>SUR1</td>
<td>Sulphonylurea receptor 1</td>
</tr>
<tr>
<td>SU</td>
<td>Sulphonylurea</td>
</tr>
<tr>
<td>SV(_{40})LT</td>
<td>Simian Virus 40 large T antigen</td>
</tr>
<tr>
<td>TCA</td>
<td>Tricarboxylic acid cycle</td>
</tr>
<tr>
<td>TIRF</td>
<td>Total Internal Reflection Microscopy</td>
</tr>
<tr>
<td>TOL</td>
<td>Tolbutamide</td>
</tr>
<tr>
<td>TPP</td>
<td>Trphenylphosphine</td>
</tr>
<tr>
<td>TPPO</td>
<td>Trphenylphosphine oxide</td>
</tr>
<tr>
<td>TRPM(_{3})</td>
<td>Transient Receptor Potential Melastatin-like subtype 3</td>
</tr>
<tr>
<td>TRPM(_{4})</td>
<td>Transient Receptor Potential Melastatin-like subtype 4</td>
</tr>
<tr>
<td>TRPM(_{5})</td>
<td>Transient Receptor Potential Melastatin-like subtype 5</td>
</tr>
<tr>
<td>TRPC</td>
<td>transient receptor potential cation channels</td>
</tr>
<tr>
<td>VGCC</td>
<td>Voltage gated Ca(^{2+}) channel</td>
</tr>
<tr>
<td>(V_{\text{max}})</td>
<td>Maximal velocity</td>
</tr>
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</table>
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Thesis outline

This thesis is a combination of four separate research studies.

In the first study, I measured ATP consumption dynamics of Na+/K+-ATPase using a genetically encoded fluorescent indicator called Perceval HR. We demonstrate that Perceval HR is an excellent tool to visualize ATP:ADP in mammalian cells.

In the second study, I studied the role of calcium signaling and TRP channels in angiotensin II type 1 receptor (AT1R) signaling cascade. We prove that low inhibition of CaV1.2 with physiological and therapeutically relevant concentration of Angiotensin II upregulate AT1R signaling.

In the third study, I studied the role of the TRPM5 channel in regulating insulin secretion, and cytoplasmic free calcium concentration in the rat β-cells by using triphenyl phosphine oxide, a selective inhibitor of the channel.

In the fourth study, I tested whether, the genetically engineered human β-cell line (EndoC-BH1) could be used as models for studying Ca²⁺ signaling in the context of Type II Diabetes. We found that the EndoC-BH1 cells could be a relevant model to study stimulus-secretion coupling and Ca²⁺ signaling in the human β-cells.
1. Introduction

1.1 ATP - The Fuel in living cells

Adenosine triphosphate (ATP) is the important form of energy in all living cells. ATP contains adenosine that comprises of the purine adenine and the sugar ribose that are linked with three phosphates by a phosphate ester bond and two phosphate anhydride bonds. All foods are broken down to produce ATP that drives as well as regulates majority of the metabolic processes (1) (fig 1.). ATP is an energy rich compound that is widely distributed in all the cells in a human body (2). This ubiquitous compound provides energy for vital biological process ranging from cell fertilization, muscle movement, signal transduction in neurons, to apoptosis. Nucleosides like Guanosine triphosphate (GTP), Cytidine triphosphate (CTP), Uridine triphosphate (UTP) have similar chemical properties like that of ATP, but they serve relatively fewer roles (3).

Fig 1: Illustration explaining the components of ATP, ADP and ATP hydrolysis
1.1.1 ATP production mechanisms

ATP is an unstable molecule because of its high energy bonds and is readily hydrolyzed to release its energy in the presence of appropriate enzymes in cellular reactions. During food digestion polysaccharides are converted into sugars, proteins into aminoacids and fats into fatty acids and glycerol. In glycolysis (4), each glucose molecule is transported in cytosol and broken down into two pyruvate molecules. The net energy produced during this conversion is 2 ATP and 2 NADH. The pyruvate is transported into the mitochondria from cytosol where it undergoes oxidative breakdown. Fatty acids are broken down to acetyl CoA by oxidation. The Acetyl CoA enters Kreb’s cycle (5) undergoes several steps to produce 2 ATP, 8 NADH, 2 FADH$_2$, and 6 CO$_2$. Further NADH and FADH$_2$ enters into electron transport chain gets broken to transfer electrons, that they gained when oxidizing other molecules, to the electron-transport chain. The energy produced during electron transfers along the mitochondrial complexes is used to pump H$^+$ to outer compartment of mitochondria. The generation of H$^+$ gradient drives ATP production at the end of electron transport chain. Electron transport chain produces $\sim$32 ATP per glucose molecule whereas glycolysis yields 2 ATP per glucose molecule (fig 2.).
Fig 2: Schematic block diagram illustrating ATP synthesis from glucose.

1.1.2 Na⁺/K⁺-ATPase (NKA)

NKA is an omnipresent enzyme. Jens Christian Skou discovered NKA in 1957 (6); he was awarded Nobel Prize for his discovery of the first ion transporting enzyme, NKA. This transmembrane enzyme contains α, β and γ subunits. Their crucial functions are to maintain Na⁺ and K⁺ gradients across the cell membrane. NKA achieve this by transporting three Na⁺ ions outside the cell and promoting influx of two K⁺ ions at the same time (fig 3.). This exchange is an energetically unfavorable event, due to high Na⁺ in extracellular space and high K⁺ in the intracellular space, so ion transport is done against the electrochemical gradient at the expense of ATP (7). This process is believed to account for approximately 30% of the total ATP consumption in humans (8). Maintenance of Na⁺ and K⁺ are essential for maintaining membrane potential (9). NKA, in addition, is also responsible for regulation of cell volume and transepithelial movement of salt (10).
Fig 3: Left to right: schematic diagram illustrating function of NKA step by step. Blue circles: Na⁺, purple diamonds: K⁺.

1.1.3 Quantification of NKA's ATP consumption

There have been studies done to measure the NKA’s total ATP consumption. The ratio of Na⁺ transported across NKA per molecule of glucose is approximately 6:1 in human erythrocytes (11). Oxygen consumption is used as a parameter for determining NKA activity, In vitro study show that excess of 20 % of energy is consumed by NKA in skeletal muscle, duodenal epithelium and liver of domestic ruminants (12). In vivo measurement of Rb⁺ ion flux is used to determine NKA activity in sea urchins (13). ATP hydrolysis provides energy for NKA pumps. Enzymatic ATP hydrolysis rate can be measured by means of colorimetric reactions (14, 15) and spectroscopic method (16) is used to quantify the NKA activity. Despite availability of wide range of tools to quantify NKA activity, there is a lack of clear evidence on quantification of energetic costs of transport of Na⁺ and K⁺ across the NKA in live and intact cells.

1.2 Hypertension and cardiovascular disease

Cardiovascular diseases are one of the most common factors of human mortality despite the advancement in medical facilities
and cardiovascular research throughout the world. Hypertension is the most common risk factor for cardiovascular related disorders and renal diseases. The renin angiotensin system is a well-studied regulator of blood pressure and plays an important role in pathophysiology of cardiovascular disorders (17). A central component in this system is the angiotensin II type 1 receptor (AT1R). AT1R is a G Protein couple receptor. It functions as a primary regulator for blood pressure homeostasis. The AT1 receptor contains an extracellular NH₂ terminus followed by seven helical transmembrane domains connected by three intracellular and three extracellular loops, and an intracellular carboxyl terminus. The carboxyl terminus contains three phosphorylation sites for protein kinase C (PKC). AT1R is capable of exhibiting several active conformations to trigger different signal pathways with different physiological outcomes, including vasoconstriction, aldosterone release, renal sodium reabsorption, adrenergic facilitation, vascular smooth muscle cells hypertrophy and cardiac myocyte hyperplasia (18).

1.2.2 Angiotensin and AT1R signaling

The protein precursor angiotensinogen is cleaved by renin to release inactive angiotensin I, which is further cleaved by angiotensin converting enzyme to generate AngII. AngII is an important modulator of the sympathetic nervous system. The main receptor of AngII in the cardiovascular system is the AT1R. Binding of AngII to AT1R results in activation of phospholipase C (PLC) and protein kinase C (PKC), release of inositol 1,4,5-triphosphate (IP3) and Ca²⁺ mobilization from intracellular stores. It is well documented that AngII activation of AT1R can be followed by desensitization and internalization of the
activated receptor (19, 20). Although blockers of AT_1R and voltage gated calcium channel 1.2 (Cav1.2) are commonly used for treatment of hypertension, there is a need for clarification about the effect of physiological concentrations of AngII on AT_1R signaling as well as feedback regulation by Cav1.2 on AT_1R signaling.

1.3 Ca^{2+} signaling and its primary regulators

Intracellular Ca^{2+} signaling is one of the most basic but at the same time most complex and multi-faceted cellular signaling systems. Cytoplasmic Ca^{2+} concentration ([Ca^{2+}]_i) plays a pivotal role in many cellular processes that include triggering insulin exocytosis (21), fertilization of eggs (22), and enhancing ATP synthesis in mitochondria (23). Measurements in resting cells have established that the concentration of Ca^{2+} in the cytoplasm is ~20-100 nM while the extracellular concentration is ~1-2 mM. The strong concentration gradient over the plasma membrane make Ca^{2+} as a highly specific and sensitive signal system, where a local increase in [Ca^{2+}]_i rapidly can trigger a global [Ca^{2+}]_i increase (24). The entry, duration of presence and exit of the Ca^{2+} in the cytoplasm is regulated by many channels, pumps, ion exchangers, Ca^{2+}-binding proteins, and also other organelles (25).

Channels present on the plasma membrane like receptor operated Ca^{2+} channels, store operated Ca^{2+} channels, and voltage gated Ca^{2+} channels (VGCC) regulate the Ca^{2+} entry from extracellular region in to the cells. The Ca^{2+} influx, through the L-type VGCCs in response to extracellular glucose is a predominant and a well-established mechanism (26).
[Ca\(^{2+}\)]_i is also regulated by store operated Ca\(^{2+}\) entry, in which the reduction in the Ca\(^{2+}\) in the ER (Ca\(^{2+}\) store) sensed by Stromal interaction molecule (STIM), that translocate itself to plasma membrane to signal the Orai1 channel to activate Ca\(^{2+}\) entry (27).

In the G protein coupled signaling, 1,4,5-inositol trisphosphate (IP\(_3\)), produced by PLC, binds to the 1,4,5-inositol trisphosphate receptor (IP\(_3\)R) activating the channel to mobilize Ca\(^{2+}\) from the ER Ca\(^{2+}\) stores into the cytoplasm (28). IP\(_3\)R and ryanodine receptors (RyR) are activated or inhibited by Ca\(^{2+}\) depending on the concentration of [Ca\(^{2+}\)]_i (29). The Ca\(^{2+}\) entry through VGCCs activates the RyRs to mobilize Ca\(^{2+}\) from endoplasmic reticulum to further amplify the Ca\(^{2+}\) signals; this process is called Ca\(^{2+}\) induced Ca\(^{2+}\) release (CICR) (30).

Sarco-endoplasmic reticulum Ca\(^{2+}\) ATPase (SERCA) replenishes the Ca\(^{2+}\) stores in the endoplasmic reticulum (31). The Mitochondrial Ca\(^{2+}\) buffering (32, 33) and efflux of Ca\(^{2+}\) across the plasma membrane through plasma membrane Ca\(^{2+}\) ATPase, sodium-calcium exchangers also regulates the [Ca\(^{2+}\)]_i (34), thereby preventing the Ca\(^{2+}\) toxicity in the \(\beta\)-cells. The activation of several TRP channels also regulates the [Ca\(^{2+}\)]_i, either by providing background depolarizing current or by permeatingCa\(^{2+}\) ions through them (25).

1.3.1 TRP channels

Transient receptor potential (TRP) channels were first discovered in *Drosophila*. A mutation of an ion channel in the photoreceptor cells elicited a transient change in membrane potential in response to continuous light and thus the name transient receptor potential channels (35). So far 28 TRP (27 in
human) channels have been found and they are classified into two groups and seven subfamilies. Group A contains five subfamilies TRPC (canonical), TRPV (vanilloid), TRPM (melastatin), TRPA (ankyrin), and TRPN. Group B contains two subfamilies TRPP (polycystic) and TRPML (mucolipin) (36). The transmembrane topology of TRP channels show that TRP channels have six transmembrane sections with a pore forming unit between the 5th and 6th transmembrane domain. TRP channels are cation channels that are permeable to K⁺, Na⁺, or Ca²⁺. They are involved in perceiving various types of sensory reception that includes mechanoreception, thermoreception, chemoreception, and photoreception. TRPC1, TRPC3, TRPC4 and TRPC6 channels are endogenously expressed in human embryonic kidney (HEK 293a) cells (37-39). β-cells are found to possess at least 13 TRP channels and among them TRPs like TRPM2, TRPM3, TRPM4 and TRPM5 are thought to play important role in insulin secretion in the β-cells in rodents (40).

1.4 Islets of Langerhans

Paul Langerhans, a German pathologist, was the first person to describe these microstructures in the pancreas in 1869, but he failed to identify their function. Later in 1893, Edouard Laguesse, a French histologist, coined the name ‘islets of Langerhans’ and suggested that could produce hormones that lowers hyperglycemia.

Pancreas houses two different types of tissues, namely the exocrine and the endocrine tissues. Islets of Langerhans are the endocrine tissues, and they are more in the body and tail region of the pancreas. Islets contain at least five types of cells including the α-cells that secrete glucagon, the β-cells that secrete insulin, the δ-cells that secrete the growth hormone inhibiting
hormone, somatostatin, pancreatic polypeptide producing (PP) cells, and ghrelin producing epsilon cells (41). β-cells sense the change plasma nutrient concentration and in responses stimulate insulin exocytosis to maintain glucose homeostasis in the blood.

The islet failure results in diabetes, a heterogeneous metabolic disorder associated with high blood glucose. Diabetes is classified mainly into two types namely the type-1 diabetes mellitus and the type-2 diabetes mellitus. Type-1 diabetes is an autoimmune disorder in which β-cells are killed by the immune system. Type-2 diabetes is associated with progressive β-cell dysfunction in secreting insulin due to aging or developing insulin resistance. Studying the physiology of β-cells may give a clearer picture about the pathogenesis of diabetes and may also useful in identifying therapeutic targets for treatment of the disorder.

1.4.1 Stimulus secretion coupling in the β-cells

The adult human islets comprise 70-80% of β-cells that play critical role in the maintenance of blood glucose level. The β-cells also act as sensors for fatty acids, amino acids, neurotransmitters, growth factors, incretins and other hormones. Insulin exocytosis in β-cells requires increase in [Ca^{2+}]. Stimulus secretion coupling in the pancreatic β-cells includes a combination of metabolic and electrical signaling cascades. After consumption of a meal blood glucose concentration increase. Glucose is carried in to the β-cells through specific glucose transporters (GLUT 2 in rodents, and GLUT 1 and 3 in humans). Glucose is metabolized via glycolysis in the cytoplasm to produce adenosine triphosphate (ATP) and pyruvic acid. This pyruvic acid in turn is metabolized via
tricarboxylic acid cycle in the mitochondria to produce ATP. The combined ATP production from glycolysis and TCA cycle increases the ATP:ADP in the cytoplasm (42, 43).

ATP sensitive K⁺ channels (K_{ATP}) that are present on the plasma membrane is a primary sensor that couples the metabolic changes exerted by glucose to electrical activity and finally to secrete insulin in the β-cells. K_{ATP} channel comprises of sulphonylurea receptor (SUR1) and four pore forming subunits K{IR}6.2, member of inward rectifier superfamily (26).

The increase in ATP:ADP in cytoplasm causes closure of K_{ATP} channels that prompts cell membrane depolarization; the change in membrane potential activates the voltage gated Ca^{2+} channels (VGCCs) that allows entry of Ca^{2+} into the cytoplasm. The increase in [Ca^{2+}]_i triggers insulin exocytosis (Fig. 4).

Upon reduction in plasma glucose levels, the decrease in ATP:ADP increases the opening probability of K_{ATP} channels causing repolarization that acts as a feedback mechanism to stop insulin secretion. Diazoxide, opens the K_{ATP} channels to prevent depolarization of plasma membrane, which makes it a therapeutic tool for treating hyper insulinism (44). Closure of K_{ATP} channels is an undisputed signaling event in plasma membrane depolarization. However, the K_{ATP} channel closure alone is not adequate to depolarize the membrane. An additional inward depolarizing currents caused by other ion channels present on the membrane is essential to depolarize the membrane. It is thought that some members of TRP family of channels may account for these inward depolarizing currents.
Fig. 4 Stimulus secretion coupling in the β-cell. The figure shows the cell organelles, ion channels, pumps, exchangers, and transporters involved in the regulation of stimulus secretion coupling. ADP-Adenosine diphosphate; ATP- Adenosine triphosphate; CICR-Ca\(^{2+}\) induced Ca\(^{2+}\) increase; DAG- diacylglycerol; ER-Endoplasmic reticulum; GK- Glucokinase; GLUT- Glucose transporter; IP\(_3\)- inositol 1,4,5-trisphosphate; IP\(_3\)R- inositol 1,4,5-trisphosphate receptor; K\(_{ATP}\)-ATP sensitive K\(^+\) channel; KCa-Calcium activated K\(^+\) channel; MC- Mithochondria; NCX- Na\(^+\)-Ca\(^{2+}\) exchanger; NKA- Na\(^+\)/K\(^+\)-ATPase, PIP\(_2\)-Phosphatidylinositol 4,5-bisphosphate; PI-PLC- phosphatidylyl inositol specific phospholipase C; PKC- protein kinase C; PMCA- Plasma membrane Ca\(^{2+}\) ATPase; RyR- Ryanodine receptor; SERCA- Sarco-endoplasmic reticulum Ca\(^{2+}\) ATPase; SU- Sulphonylurea; SUR1- Sulphonylurea receptor 1; TCA cycle-tricarboxylic acid cycle; TRPM5- Transient Receptor Potential Melastatin-like subtype 5; VGCC- Voltage gated Ca\(^{2+}\) channel.
1.4.2 \(K_{ATP}\) channel independent insulin secretion

The closure of \(K_{ATP}\) channel and depolarization of plasma membrane are important events in stimulus secretion coupling. In glucose stimulated insulin secretion (GSIS), there are other coupling factors apart from \(K_{ATP}\) channels in bringing about plasma membrane depolarization and insulin secretion. In the presence of diazoxide, which is known to activate the \(K_{ATP}\) channels and hyperpolarizes the plasma membrane (45), glucose fails to, depolarize the plasma membrane, and secrete insulin in the islets isolated from mice. However, in the presence of high concentration of \(K^+\), glucose potentiates insulin secretion even in the presence of diazoxide (46) proving that glucose can amplify insulin secretion without involving the \(K_{ATP}\) channel activity. This validates that glucose initiates insulin secretion via \(K_{ATP}\) channel dependent manner and further amplifies insulin secretion in \(K_{ATP}\) independent manner.

1.4.3 TRPM5 channel associated functions

The human TRPM5 gene was first described in 2000 by Enklaar and his co-workers (47, 48). The human TRPM5 gene is located on chromosome 11 and it contains 24 exons composed of 1165 amino acids whereas, the mouse TRPM5 gene is located on chromosome seven and composed of 1158 amino acids. TRPM5 is a monovalent cation channel that is permeable to \(Na^+\), \(K^+\), \(Cs^+\) and \(Li^+\). The channel is activated by voltage, \([Ca^{2+}]_i\), phosphoinositide-4,5-bisphosphate (PIP\(_2\)) and heat (15 and 35ºC) (49). TRPM5 has been best studied in taste cells where channel is associated with sensing sweet, umami and bitter tastes in the taste receptor cells (50, 51). The expression of TRPM5 in the stomach and small intestine are thought to be
associated with chemosensation in the post ingestion process (52). TRPM5 is also shown to be playing important role in sensing of odors in mice (53).

1.4.4 Role of TRPM5 in \([\text{Ca}^{2+}]_i\); increase and insulin secretion

TRPM5 channels provide inward depolarizing current that is believed to contribute to depolarization and thereby Ca\(^{2+}\) influx in to the cytoplasm. TRPM5 knockout mice exhibited the lack of glucose induced fast oscillation in membrane potential and \([\text{Ca}^{2+}]_i\). The islets from these mice secreted less insulin in response to glucose stimulation (54). In the same knockout mice model, TRPM5 mediated depolarization is proposed to be coupled with nutrient and L-arginine mediated insulin secretion in the β-cells (55, 56). Until now, understanding of the physiological role of TRPM5 channel in insulin secretion is dominated by data obtained from knockout mice from a single source (54-56). It should also be noted that drawing conclusion about the role of the TRPM5 channel, derived from a model devoid of TRPM5 gene from the embryonic stage can be deceiving. To further elucidate the role of TRPM5 in insulin secretion in response to different stimuli, more studies using different approaches and different animal models are needed. In our studies, using trprenylphosphine oxide (TPPO), a selective TRPM5 channel inhibitor, we have examined the role of TRPM5 channel in mediating insulin secretion and \([\text{Ca}^{2+}]_i\); levels in the rat islets and primary rat β-cells respectively.
1.4.5 Pharmacological tools for study of TRPM5 channel

A major challenge in determining the physiological role or TRPM5 channel is the lack of availability of potent and specific pharmacological tools. Flufenamic acid, a non-steroidal anti-inflammatory drug, inhibits TRPM5, however, it also inhibits a closely related TRPM4 channel with a 10 fold higher affinity (57). Quinine, a natural alkaloid and a bitter tastant, inhibits TRPM5 channel (58), along with inhibiting other ion channels (59, 60). Spermine, a polyamine, inhibits TRPM7 along with TRPM4 and TRPM5 in a micromolar range (61, 62).

Triphenyl phosphine oxide (TPPO) inhibited TRPM5 currents in a concentration dependent manner, in HEK 293a cells transfected with human TRPM5 (hTRPM5) or mouse TRPM5 (mTRPM5) gene. TPPO at 100 µM concentration did not inhibit the closely related hTRPM4b, hTRPV1, hTRPA1, and TRPM4, which shows TPPO’s high selectivity for the TRPM5 channel. Triphenyl phosphine (TPP) that has similar geometric shapes, volume and surface area to that of TPPO, did not inhibit the TRPM5 channels. TPPO features high negative charge above the oxygen atom (fig. 5) that is thought to be the prime factor involved in the inhibition of TRPM5 channel(63). The first selective and potent pharmacological antagonist for TRPM5 channel reported namely, TPPO, has made it convenient to probe the roles of TRPM5 in stimulus secretion coupling.
Fig 5: The figure shows the chemical structure of TPP (left) and TPPO (right).

1.5 Genetically engineered human β-cells

Insulinoma cell lines offer many research possibilities to study the pathophysiology of diabetes. For decades, the availability of wide range of insulinoma cell lines from rodents (64) plays important role in the β-cell research. These cell lines are used to overcome the limited availability of primary human β-cells. However, it should be noted that these cell lines are not from human origin. Conclusion from the data obtained from rodent cell lines can be redundant in understanding the physiological processes of β-cells in humans. Recently, a new functional human β-cell model has been established (65). These cells are obtained by transfecting the oncogene SV40LT in human fetal pancreatic buds followed by grafting the transfected buds into severe combined immunodeficiency (SCID) mice. The transfected buds formed insulinomas that were expanded to generate EndoC-BH1 cell lines (65). It is appropriate to use such human insulinoma cells for Ca\textsuperscript{2+} studies to better understand the Ca\textsuperscript{2+} signaling and regulation of insulin secretion in the human β-cells. Until now, Ca\textsuperscript{2+} signaling has not been reported in these cells. In this thesis, we have studied whether the EndoC-BH1 can used as a model to study Ca\textsuperscript{2+} signaling and stimulus secretion coupling in β-cells.
2. Materials and Methods

2.1 Brief history of microscopy

Zacharias Janssen, a Dutch spectacle-maker created the first microscope in 1590. This microscope could magnify images up to 10 times. In 1665, Robert Hooke, made a compound microscope (fig. 6), which he used to study different samples from nature and described in the book “Micrographia”. One of the samples was thin slices of cork. What he then observed he described as “cells” since it resembled him of the cells in a monastery. With this description he coined the term cell.

Later, in 1676, Anton van Leeuwenhoek used a single-lens microscope and observed for the first time living cells, bacteria in tooth scrapings. Those two events can be said to define the start point for the evolution of microscopy as an essential tool in biology and medicine.

Microscopes have today a wide range of applications and provide resolution from micrometers down to a few nanometers. On one end of the resolution range is optical coherence tomography that can be used to obtain real time read outs from live biological organisms with resolution down to ~10 µm. On the other extreme end is electron microscopy that can be used to discern details with spatial resolution on the molecular level. Electron microscopy is however invasive and require extensive preparation of the samples, thereby preventing observations of living cells. Fluorescence microscopy provides a wide range of spatial and temporal resolutions between these two resolution extremes and has found many applications in cell biology, much
due to the possibility to label biological material with fluorescent markers, providing both sensitivity and specificity (66).

Fig 6: Robert Hooke’s early compound microscope (67)

The evolution of fluorescence microscopy has been rapid during the last 50 years, a development that has accelerated even more during the last ten years. Today we have many advanced methods to choose between, including confocal (68) (69) and multi-photon microscopy (70) (71), TIRF (72), FRAP (73), FRET (74) microscopy and the recent development of super-resolution microscopy such as STED (75-77). The development has been possible thanks to technological advances in other fields, such as; the laser, the computer, immunofluorescence, and GFP.

In this thesis I have used fluorescence to study selected intracellular parameters. The techniques I have used were primarily fluorescence spectroscopy, scanning laser microspectroscopy and confocal microscopy.


2.2 Fluorescence

Fluorescence is a molecular energy conversion process where electrons in fluorescent molecules absorb light of short wavelength; get excited, and then re-emit light of a longer wavelength when they return back to the ground state. This can be described with a Jablonski diagram (fig. 7). In a molecule at room temperature the electrons are in their energy ground state (S0). The molecules absorb an energy quantum from a photon in the incident light, which excites an electron to a higher energy state (S2). Some of the energy is quickly lost due to internal conformational changes (vibrations) before the excited state (S1) is reached. From the state S1 the electrons can return to the ground state S0 and the energy difference S1-S0 is then emitted as a fluorescence photon. The energy of emitted photons is always lower than the energy of excitation photons, i.e the fluorescence is red shifted compared to the excitation light (78).

![Jablonski diagram](image)

**Fig 7**: simplified Jablonski diagram depicting the different states of electrons during excitation by light.

2.3 Properties of a good fluorophore

Fluorescent molecules (fluorophore) have several distinguishing
properties, the most important are: the excitation and emission spectra, the stokes shift, the efficiency to generate fluorescence photons, the sensitivity to environment factors and the photostability.

Stokes shift is defined as the distance between the wavelength of excitation maxima and emission maxima. For use in many fluorescence detection methods it is an important property of a good fluorophore to have a large stokes shift in order to efficiently be able to separate the incident light from the emitted fluorescence.

Quantum yield is the ratio of photons emitted by a fluorophore to the number of photons absorbed, i.e. the probability to generate a fluorescence photon. A fluorophore with high absorption, (also called extinction coefficient), and high quantum yield is bright and easily detectable even when used at low concentrations. (79).

Photo bleaching occurs when fluorophores get damaged irreversibly due to photochemical reactions (80), it is an important quality of fluorophores to be photo-stable in order to permit observation over longer times, such as in time lapse experiments of living cells.

Photo toxicity is a major concern in live cell fluorescence microscopy as the photo excited fluorophores can release reactive oxygen species which will damage living cells (81, 82). Reducing the intensity of excitation light as well as using sensitive detectors that allows for fast detection even of low light fluorescence can help in reducing photo toxicity.

When analyzing results based on fluorescence one must take potential artifacts into consideration. Artifacts can arise from
many sources, in particular when the fluorescence signal is used to measure an environment parameter, such as an ion concentration. The fluorescence intensity can be affected by non-specific interactions with cellular contents and other parameters, i.e. intracellular pH (83). One interesting development to alleviate some of those potential artifacts is nanosensors, where the reporter fluorophore is encapsulated within a protective matrix permeable only to the ions of interest and not to other cellular contents (84-86).

2.4 Cells

In this thesis I have used both cell lines and primary cells from rat. Cell lines are immortalized cells that preserve some but not all properties of their origin. A cell line can often be a good model for basic studies, but in order to study more complex functions and specific features closer to the In vitro situation primary cells are preferred. Here I briefly describe the procedure used to obtain and culture the different cell types used in this thesis.

In study 1 and 2, the cell line Human Embryonic Kidney 293a, (HEK239a), (Qbiogene Inc.) were cultured in DMEM medium (41966-029, Life technologies) supplemented with fetal bovine serum (10% v/v), penicillin (50 IU/ml), streptomycin (50 μg/ml). The cells were cultured and maintained in humidified incubator in 5% CO2 at 37°C. Cultures were trypsinised and passaged once a week. On the day of passaging, cells were plated on 18 mm diameter coverslips. The coverslips were incubated for 48 hours in 12 well plates with 1 ml of media for each coverslip. For introduction of fluorescent reporters, plasmids were transfected into the cells with the use of Lipofectamine LTX (Thermofisher scientific). Experiments were performed 24 hours
after transfection. The total concentration of plasmid DNA transfected was 2 µg per coverslip. HEK293a cells between passage 3 and 25 was used for the experiments.

In study 1 primary culture of rat proximal tubular cells (PTC) were obtained from Sprague Dawley rats. Animals were anesthetized by intraperitoneal injection of pentobarbital and the aorta cut, kidneys were excised and placed in 0.9 % NaCl (Sigma-Aldrich) at room temperature. The cortical layers were collected by using a microtome for 250µm slices. Slices were placed in 37°C basal solution (containing Hank’s balanced salt (HBSS), 0.2% Bovine Serum Albumine (BSA, Sigma-Aldrich), 10mM HEPES (Sigma-Aldrich), penicillin (10 µg/mL) and streptomycin (10 µg/mL) supplemented with 0.035% collagenase I (Sigma-Aldrich), and incubated for 20 min with gentle mixing using a fire-polished Pasteur pipette. The reaction was stopped by washing the cells twice in basal solution with 0.01% trypsin inhibitor. After washing, cells were plated on 18 mm glass coverslips in 12 well Petri dishes and allowed to attach for 30 min before growth media was carefully added. Cells were cultured for 2 or 3 days in supplemented Dulbecco’s modified Eagle's medium, HEPES (20 mM), NaHCO₃ (24 mM) (Merck KGaA, Darmstat, Germany), penicillin (10 µg/mL), streptomycin (10 µg/mL), and 10 % Fetal Bovine Serum (FBS) in 37°C at an approximate humidity of 95 – 98 % with 5 % CO₂. Growth media was changed after 24 h.

In study 2, primary rat ventricular cardiomyocytes (RVCM) were obtained from 3-5 day old Sprague Dawley and cultured on 18 mm diameter coverslips for 5 days following a previously described protocol (87). Rats were decapitated followed by removal of heart for generation of cardiomyocyte cultures. Cardiomyocytes were cultured five days before the experiments
were performed, contracting clusters, seen with transmission light were selected for experiments. Expression of cardiomyocyte markers were confirmed in this study using Troponin I (Chemicon) and Desmin (Chemicon) antibody staining according to manufacturer’s protocol. Rat aortic smooth muscle cells (ASMC) were purchased from (3H Biomedical, ScienCell) and cultured according to manufacturer’s instruction. Briefly, cells were thawed and plated on poly-L-lysine coated coverslips in complete smooth muscle cell medium (SMCM, ScienCell) including 2% FBS and supplemented with AngII (0.05 pM). Cells were cultured for three to five days before experiments.

In study 3, Langerhan islets were obtained from Sprague Dawley rats aged 2-3 months and weighed 250-350 g. Rats were sacrificed and their pancreas were dissociated. The Islets were dispersed using trypsin EDTA and similar sized Islets were picked under microscope for insulin secretion experiments. Furthermore, the Islets were dissociated into primary cells and plated on 18 mm coverslips for microfluorometry experiments.

In study 4, EndoC-BH1cells were obtained as a generous gift from Philippe Ravassard and Raphael Scharffmann, Institut du cerveau et de la moelle, Biotechnology & biotherapy, France. EndoC-BH1 cells is a genetically engineered human β-cell line generated by transducing human fetal pancreatic buds by a lentiviral vector system followed by grafting into severe combined immuno-deficient (SCID) mice. The oncogene Simian Virus 40 large T antigen (SV40LT) expressed in the resulted cells were transduced with human telomerase reverse transcriptase followed by grafting into other SCID mice. The resulting β-cells were expanded in cultures to form cell lines. EndoC-BH1 cells were cultured in DMEM with glucose (5.5
mM), albumin from bovine serum fraction V (BSA) (2%w/v), 2-mercaptoethanol (50 µM), nicotinamide (10 mM), transferrin (5.5 µg/ml), sodium selenite (6.7 ng/ml), penicillin (100 IU/ml) and streptomycin (100 µg/ml).

All experiments were performed according to Karolinska Institutet regulations concerning care and use of laboratory animals, and were approved by the Stockholm North ethical evaluation board for animal research.

2.5 Reagents and constructs

Bradykinin was purchased from Abcam. 3,5-bis(trifluoromethyl)pyrazole (BTP2) a TRPC channel blocker was obtained from Santa Cruz. Rat angiotensin II type 1 receptor, AT₁R, with Venus fused at C-terminus was used for Ca²⁺ experiments. AT₁R-Venus with addition of extracellular hemaglutinin tag (YPYDVPDYA) inserted on the first extracellular loop between Pro331 and Phe332 (HA-tagged AT₁R) was used for membrane recruitment experiments. pGβ-2A-YFP-Gγ2-IRES-GαqmTq (88) was kindly provided by the lab of Th. W. J. Gadella and used for Gαq-Gβγ FRET (Förster Resonance Energy Transfer) measurements.

2.6 Observing ATP:ADP changes using PercevalHR

HEK 293a cells were imaged using Zeiss LSM 510 Meta with 40X/0.75 N.A. PercevalHR (Addgene), a genetically encoded sensor was used to measure relative changes in ATP and ADP levels in the cell. ATP and ADP levels in a cell are a driving force for several biochemical and signaling pathways. A small stimulation of ATP hydrolysis triggers an amplified response as a decrease in ATP:ADP because of the simultaneous increase in
ADP levels. This characteristic feature of ATP:ADP makes it a robust indicator for observing rapid changes in cellular energy status (89). PercevalHR was excited at 405 nm and 488 nm, emission was collected at 530 nm. Increase in fluorescence emission by excitation at 488 nm corresponds to increased ATP levels and an increase in fluorescence by excitation at 405 nm corresponds to an increase in ADP levels. The occupancy of the sensor by ATP and ADP elicits primary response. The ratio between fluorescence at the two excitation wavelengths gives the ATP:ADP. pHRed (Addgene), was co-transfected in the cells along with percevalHR to monitor the pH changes during the experiments. pHRed was excited using 405 nm and 546 nm and emission was collected at 630 nm band pass filter.

2.7 Removal of pH artifacts

Inhibition of ATP production might have an effect on intracellular pH. Since, PercevalHR, as all sensors based on GFP, is sensitive to intracellular pH, we monitored pH along with ATP:ADP in all experiments. At the end of experiments we perifused the cells with NH4Cl (5 and 15 mM) for 1 min to intentionally alter intracellular pH for calibration (fig 8, left). We found, as expected, a linear correlation between pHRed and PercevalHR signals (fig 8, right). We used this pH calibrating measurement to correct for the potential influence of pH on the ATP:ADP.
Fig 8: pH compensation using NH₄Cl  (Left) Cells were perifused with NH₄Cl (5 mM or 15 mM) for the calibration, green trace represents PercevalHR signal, purple trace represents pH corrected PercevalHR signal, red trace represents pHRed signal and blue trace represents pH compensation factor. (Right) The plot between PercevalHR signal and the pHRed signal shows a linear correlation describing the pH sensitivity of PercevalHR, which is used to approximately correct for pH bias.

2.8 Rate of ATP consumption

HEK293a cells were perifused with Iodacetic acid (IAA) 1 mM to stop ATP production by irreversibly blocking the primary glycolysis. At this stage the cells have a finite amount of ATP. ATP is consumed by several ATPases leading to constant conversion of ATP into ADP. The rate of conversion follows a first order chemical reaction, which is used to calculate the time constant (α) for the reaction. This time constant indicate the rate of ATP consumption.

\[ A e^{-\alpha t} + B \]

2.9 Experimental setup for ATP analysis

HEK 293a cells grown on coverslips were washed with KREBS buffer containing NaCl (110 mM), KCl (4 mM), NaH₂PO₄·H₂O
(1mM), NaHCO₃ (25 mM), CaCl₂·2H₂O (1.5 mM), MgCl₂·6H₂O (1.25 mM), Glucose (5-10 mM) and HEPES (20 mM), pH 7.4, sterile filtered. Zero potassium (0K⁺) KREBS buffer contains NaCl (114 mM), NaH₂PO₄·H₂O (1mM), NaHCO₃ (25 mM), CaCl₂·2H₂O (1.5 mM), MgCl₂·6H₂O (1.25 mM), Glucose (5-10 mM) and HEPES (20 mM), pH 7.4. In case of glucose free buffer solutions, glucose was replaced by mannitol. The coverslips were mounted on a camlidge perfusion chamber where live cell imaging was performed. During the experiments, temperatures of buffer, stage and perfusion chamber were maintained between 34˚C to 37˚C with the help of heated optical stage, in-line perfusion solution heater. KREBS buffer was perfused at the rate of 2.8 ml/min using peristaltic pump. IAA 1mM was used to stop ATP production through glycolysis, Oligomycin (2.5 µM) and Rotenone (1µM) to arrest ATP production in the mitochondria, cyclopiazonic Acid (5 µM) to inhibit SERCA pumps and Ouabain 2mM to inhibit the NKA pumps. NH₄Cl (5 and 15 mM) were used as control to alter the extracellular pH.

2.10 Measurement of insulin secretion from islets

Islets of approximately similar sizes handpicked under microscope were chosen for all the experiments. In separate experiments we measured the insulin contents of the islets, and expressed the released insulin as percentage of the total insulin content. We found that insulin content of the islets were not highly variable. Islets of similar sizes were incubated in groups of three in 300 µl KREB buffer containing glucose (3.3 or 16.7 mM) with or without other pharmacological agents, for one hour at 37 °C on a shaking water bath. After incubation of the islets, the supernatant was removed, and stored at -20 °C. Total insulin content was measured after sonication of the islets for
10–15 s, followed by extraction of insulin overnight at 4 °C in acid-ethanol (70 % v/v). Immunoreactive insulin was measured by radioimmunoassay using polyclonal insulin antibodies (Fitzgerald Inc, USA), and rat insulin as standard (90).

### 2.11 Measurement of insulin secretion from the EndoC-BH1 cells

The EndoC-BH1 cells were seeded in a 24 well plate that were first coated with the cell culture support. The cells were seeded at a density of 226 000 cells/cm². After seeding, the cells were grown for 48 h in DMEM medium with glucose (5.5 mM), BSA (2%w/v), 2-mercaptoethanol (50 μM), nicotinamide (10mM), transferrin (5.5 μg/ml), sodium selenite (6.7 ng/ml), penicillin (100 IU/ml) and streptomycin (100 μg/ml). After that, the cells were incubated overnight in the glucose-starving medium containing DMEM with low glucose (2.8 mM), BSA (2%w/v), 2-mercaptoethanol (50 μM), nicotinamide (10mM), transferrin (5.5 μg/ml), sodium selenite (6.7 ng/ml), penicillin (100 IU/ml) and streptomycin (100 μg/ml). On the day of the experiments, the cells were incubated in KRBH buffer supplemented with glucose (0.5 mM) for one hour, and then with an increasing concentrations of glucose 0.5, 2.8, 5.6, 11, 16.7 and 25 mM glucose. The cells were incubated in 37°C at 5% CO₂ for one hour. After the incubation, the supernatant was removed, and stored at -20 °C. Total insulin content was measured after extraction of insulin overnight at 4 °C in acid-ethanol (70 % v/v). Immunoreactive insulin was measured by radioimmunoassay using polyclonal insulin antibodies (Fitzgerald Inc, USA), and human insulin as standard.
2.12 Measurement of $[\text{Ca}^{2+}]_i$ using Fura-2 AM

Cells grown on glass coverslips were incubated for 40 min in 2 ml of the 'loading buffer' consisting of RPMI-1640 medium, and BSA (2 %) along with fura-2-acetoxymentyl ester (fura-2 AM) (1 µM) (life technologies, Stockholm, Sweden). The glass coverslips were then incubated for another 15 min in modified KRBH buffer containing NaCl (140 mM), KCl (3.6 mM), NaH$_2$PO$_4$ (0.5 mM), MgSO$_4$.7H$_2$O (0.5 mM), CaCl$_2$ (1.5 mM), HEPES (10 mM), glucose (3 mM) and BSA (0.1%) (pH 7.4) to let the endogenous esterases hydrolyze the AM ester. The coverslip was then mounted at the base of an open perifusion chamber, which was then placed on the stage of an inverted microscope (CK 40, Olympus Inc, Germany). The modified KRBH buffer containing different pharmacological agents was perifused by a peristaltic pump. A water bath and a thermistor connected to the perifusion chamber were used to control the temperature of the solution in the perifusion chamber. It takes about 58 seconds for the new solution to reach the perfusion chamber.

For measurement of $[\text{Ca}^{2+}]_i$ from single β-cells, we chose only the large cells to exclude the non-β-cells as much as possible. The fluorescence was measured by dual wavelength excitation fluorometry. The monochromator (PhotoMedDeltaRam) has a diffraction grating that spatially separates the colors of white light emitted from xenon lamp. The generated lights of wavelengths 340 nm and 380 nm were directed onto the cells by a dichroic mirror. The emitted light chosen by a 510 nm filter was detected by the photomultiplier tube detector, and monitored by the Felix32 software (Photon Technology International, Inc).
The cells loaded with fura-2 were excited at 340 nm and 380 nm alternately. The ratios between the emitted fluorescence intensities upon excitations at 340 nm and 380nm (F340/F380) were calculated by the Felix32 software. One F340/F380 data point per second was recorded. The background fluorescence was measured by removing the cell from the area of the study. The background was subtracted from the traces before calculation of \([\text{Ca}^{2+}]_i\). For calibration, Rmin and Rmax were determined from external standards containing fura-2 free acid, and sucrose (2 M) (91). The \(K_d\) for \(\text{Ca}^{2+}\)-fura-2 was taken as 225 nM. \([\text{Ca}^{2+}]_i\) was calculated from F340/F380, Rmin and Rmax as described before (92).

2.13. Measurement of \([\text{Ca}^{2+}]_i\) using Fura Red

Cytosolic \(\text{Ca}^{2+}\) concentration (\([\text{Ca}^{2+}]_i\)) measurements were carried out in HEK293a cells using the fluorescent \(\text{Ca}^{2+}\) indicator Fura Red (Invitrogen). Cells were loaded with Fura Red using a buffer consisting of 5 µM Fura Red and 1 µl of 20 % Pluronic F-127 (Invitrogen) diluted in KREBS (NaCl 110 mM, KCl 4 mM, NaH₂PO₄·H₂O 1mM, NaHCO₃ 25 mM, CaCl₂·2H₂O 1.5 mM, MgCl₂·6H₂O 1.25 mM, Glucose 10 mM and HEPES 20 mM, pH 7.4, sterile filtered). Cells grown on 18 mm diameter coverslips (Warner Instruments) were rinsed with KREBs buffer and loaded for 45 min with the loading buffer at 37 °C with 5 % CO₂. After 45 min cells were washed again with KREBs prior to being mounted in a perfusion chamber (Chamlide). Through the use of heated optical table and in-line perfusion feed heater the cells were kept at 37 °C throughout experiments. The minimal dye concentration usable was identified through \(\text{Ca}^{2+}\) calibration of serial dilution of dye.
Cav1.2 inhibitors, nifedipine and verapamil, were both mixed fresh for each experimental day. Solutions were kept in fridge and protected from light. We chose to use a high concentration of nifedipine, 100 µM, to compensate for loss of active drugs during the longer experiments. Degradation was unavoidable within each experiment with cells and solutions kept at 37 °C. UV and 488 nm laser excitation also contributed to some drug degradation throughout experiments.

[Ca$^{2+}$] calibration was done by the following protocol, first a 3 min long washout of the KREBS buffer using a zero [Ca$^{2+}$] containing buffer (NaCl 110 mM, KCl 4 mM, NaH$_2$PO$_4$.H$_2$O 1mM, NaHCO$_3$ 25 mM, MgCl$_2$.6H$_2$O 1.25 mM, EGTA 250 µM, Glucose 10 mM and HEPES 20 mM, pH 7.4, sterile filtered) followed by 1 µM ionomycin in zero [Ca$^{2+}$] buffer for 4 min. Calibration ended with 7 min of 1.5 mM [Ca$^{2+}$] KREBs buffer perfusion.

The strongest AT$_1$R-expressing cells were excluded from the analysis. Cells not responding to the first stimuli were also excluded. Cells were individually selected and the mean Ca$^{2+}$ dye intensity was calculated and calibrated using the data from the end of each experiment. The mean intensity ratio, R, between the 488 nm excitation image and the 405 nm excitation image for each cell was calculated for each time point. $R_{\text{min}}$ and $R_{\text{max}}$ are the minimum and maximum intensity ratios obtained during calibration. Calibrated [Ca$^{2+}$]$_i$, was calculated with $k_d$ provided by Invitrogen as:

$$[\text{Ca}^{2+}]_i = k_d \frac{R - R_{\text{min}}}{R_{\text{max}} - R}$$

Ca$^{2+}$ measurements of RVCM were performed using the fluorescent Ca$^{2+}$ indicator Oregon Green BAPTA-1 (Invitrogen)
with the same protocol as described for Fura Red. Oregon Green BAPTA1 was chosen as its weaker binding affinity compared to Fura Red made it insensitive to background Ca\(^{2+}\) sparklets in RVCM. Calibration was not possible for the RVCM Ca\(^{2+}\) experiments, thus the non-calibrated Ca\(^{2+}\) response for each cell was obtained by the mean intensity within each selected cell.

Nifedipine effects on bradykinin activation of bradykinin B2 receptors were evaluated through comparing signaling strength with or without pretreatment of 100 µM nifedipine on 100 nM bradykinin (Abcam) treatment. Experiments were performed on Oregon Green Bapta 1 loaded HEK293a cells. Cells were loaded in the same manner as for Fura Red but with Oregon Green BAPTA-1 (5 µM). All other parameters were kept identical as for the 1 nM AngII experiments described above. Each experiment was calibrated as per the protocol described above.

Peak amplitudes were in all experiments calculated as peak above baseline. Baseline was defined as the mean intensity of a stable part of the trace prior to the peak. This calculation reflects the cytosolic increase in concentration rather than the absolute concentration.
3. Summary of studies 1-4

3.1 Study 1- Measurement of NKA activity using PercevalHR

A classical method to estimate ATPase activity is based on a colorimetric assay. Active transport of ions depends on energy generated from ATP hydrolysis. Initially we started with testing the feasibility of quantifying NKA pump activity by measuring the inorganic phosphate generated during the ATP hydrolysis using malachite green ATPase assay. To test the sensitivity of colorometric assay in our system we inhibited NKA and SERCA with Ouabain (2 mM) and CPA (5 µM) respectively, or in combination. We found that there were no differences observed in ATP hydrolysis in all three groups (fig 9). We ran this assay on microsomes obtained from HEK293a. Unlike the intact cells, ion gradient across the plasma membrane is non-existent in microsomes, lack of synergistic interplay between ion channels, transporters and ATPases affects the normal functionality of NKA thereby rendering them non-functional. It was evident that colorometric assay measures ATP turnover in membrane fractions, and is insensitive to measure NKA activity.
Fig 9: Malachite green ATPase assay showing no difference in ATP hydrolysis for different treatments in HEK 293a. The box plots shows no difference in ATPase activity when the microsomes from HEK 293a cells were treated with either Ouabain (2 mM) 27.8 ± 2.0 µM/min or Ouabain (2 mM) plus CPA (5 µM) 26.8 ± 1.7 µM/min compared to control 28.4 ± 2.1 µM/min (n = 10).

Then we decided to quantify NKA activity using a genetically encoded fluorescent indicator PercevalHR that measures ATP:ADP. It is reported to be sensitive to intracellular pH (89). Hence, calibration and compensation for pH changes are necessary to remove artifacts occurring due to pH influence and for obtaining correct estimates of ATP:ADP. We co-transfected HEK 293a cells with PercevalHR and pHRed to observe changes in the ATP:ADP and intracellular pH. The cells were perifused with IAA (1 mM) in order to block glycolysis. A representative trace shows that after correcting the Perceval signal for pH bias there were no detectable changes in Perceval signal during the initial half of the decrease in ATP:ADP after IAA treatment (fig 10). This demonstrate that change in the intracellular pH was negligible in our experiments and the pH
correction for PercevalHR signal was redundant. Therefore we based our analysis on first half of the curve immediate to pharmacological treatment.

**Fig 10:** HEK 293a Cells were perifused with IAA 1mM. Traces show that raw PercevalHR signal (green trace) does not vary from pH corrected PercevalHR signal (purple trace), illustrating that the pH calibration was not necessary.

K+ in the extracellular solution is essential for the proper functioning of the NKA pumps (93). The short K+ free KREBS pre-treatment for 2 min clamps the NKA pumps and forces them to function at maximal velocity ($V_{\text{max}}$) upon introduction normal KREBS buffer. With this background information we want to look at whether it is possible to use PercevalHR to examine the effect of K+ free KREBS pre-treatment on the rate of ATP consumption by NKA pumps. Control groups of HEK293a cells were pre-treated with K+ free KREBS buffer for 2 min followed by perifusion with IAA (1 mM) for 5 min. The other group was directly perifused with IAA 1mM without K+ free KREBS pre-treatment. We found that the rate of ATP hydrolysis was higher in the cells that were pretreated with K+ free KREBS proving
that the NKA consumed more ATP as they were forced to work at $V_{\text{max}}$ due to $K^+$ free KREBS pre-treatment (fig 11).

![Box plot showing increased rate of ATP consumption in NKA](image)

**Fig 11**: $K^+$ free KREBS pre-treatment increased rate of ATP consumption in NKA. Cells that were given $K^+$ free KREBS pretreatment followed by IAA (1 mM) showed higher mean rate constant $0.011$ s$^{-1}$, ($n=57$) compared to the cells that were given IAA (1 mM) without any pretreatment, mean rate constant = $0.0058$ s$^{-1}$, ($n=23$), ***: $P<0.0001$.

NKA is an enzyme and its rate of function is sensitive to change in temperature (94). Therefore we examined whether the changes in temperature can affect the rate of ATP consumption by NKA pumps. We pre-treated PercevalHR transfected HEK293a cells with $K^+$ free KREBS buffer for 2 min followed by treatment with IAA (1 mM) for 5 min at either 37 °C or room temperature (~23 °C). We found that the rate of ATP hydrolysis was higher for the cells at 37 °C compared to the cells at room temperature (fig 12). This is in line with the previous findings that demonstrates the optimal working temperature for NKA pump to be approximately 35-37°C (95).
ATP consumption rate of NKA is high at 37°C in relative to room temperature. Cells at 37°C showed higher mean rate constant $0.011 \text{ s}^{-1}$, $(n=57)$ compared to the cells treated at room temperature $0.0039 \text{ s}^{-1}$, $(n=37)$, ***: $P<0.0001$.

NKA (96) and SERCA (97) are the ATPases that are regarded as substantial consumers of ATP produced in a cell. Therefore, we wanted to see whether blocking these ATPases could affect the rate of ATP consumption. IAA (1 mM) was used to arrest ATP production through glycolysis, Ouabain (2 mM) was used to inhibit the NKA pumps and CPA (5 μM) to block SERCA. The PercevalHR transfected HEK293a cells were perifused with K+ free KREBS buffer for 2 min followed by treatment with pharmacological inhibitors for 5 min. We found that the rate of ATP consumption was higher in cells where NKA and SERCA were not inhibited, when compared to cells where these ATPases were inhibited (fig 13). This shows that the rate of ATP hydrolysis was significantly reduced in HEK 293a cells upon inhibition of SERCA and NKA.
Fig 13: Effect of inhibition of NKA and SERCA on ATP consumption rate. Cells that were given IAA (1 mM) alone showed higher mean rate constant 0.011 s⁻¹, (n=57) compared to the cells treated with IAA 1 mM, Ouabain (OUA) (2 mM) and CPA (5 µM), 0.0074 s⁻¹, (n=67), P< 0.0001.

The cells produce ATP via glycolysis and cellular respiration. Using PercevalHR, we were interested to see how the basal ATP:ADP levels get affected upon inhibition of ATP production via mitochondrial respiration or glycolysis in HEK 293a and PTC. We treated HEK 293a and PTC with Rotenone (1 µM) and Oligomycin (2.5 µM) for 5 min to inhibit mitochondrial ATP production. We observed a reduction in basal ATP:ADP in PTC but not in HEK 293a cells (fig 14a). We also tested blocking glycolysis using IAA (1 mM) for 5 min in HEK293a and PTC. Comparison of mean rate constant showed that decrease in ATP:ADP was faster in HEK 293a relative to PTC. This shows that IAA (1 mM), glycolysis inhibitor, the ATP hydrolysis was faster in HEK 293a cells when compared to PTC (fig 14b and 14c). These experiments illustrate the Warburg effect in cell lines where the metabolism of cell lines has been adapted to a higher glucose and lower oxygen environment.
**Fig 14**: Effect of inhibition of ATP production in HEK 293a and PTC. (A) Representative traces show that inhibition of mitochondrial ATP production by rotenone (rot) (1 µM) and oligomycin (olig) (2.5 µM) results in reduction of ATP:ADP in PTC (n=8) (red trace) but not in HEK 293a cells (n=30) (black trace). (B) The representative trace shows the rate of decrease of ATP:ADP is faster in HEK 293a cells compared to PTC during glycolysis inhibition using IAA (1 mM). (C) Boxplots show that mean rate constant for the decrease in the ATP:ADP was higher in the HEK 293a, 0.0068 s⁻¹ (n=7) compared to the PTC, 0.0039 s⁻¹ (n=6), **: P<0.01.

In summary, we demonstrated that PercevalHR can be used to measure ATP consumption dynamics of NKA in HEK 293a and primary PTC. We found that PercevalHR is a robust and sensitive tool in measuring ATP:ADP. During this project we
transfected PercevalHR in HEK 293a, PTC, and rat primary hippocampal neuronal cells. We observed that this tool is compatible with diverse cell models. It should also be noted that the transfection rate is lower in rat primary hippocampal neuronal cells ~ 20% when compared to HEK 293a and PTC, where the transfection rate was ~ 70-80%. We also observed that negligible bleaching of PercevalHR signal even after 40 min during our experiments shows the robustness of the fluorescent probe. The inherent feature of PercevalHR being a ratiometric probe is an added advantage to cancel out the effects of photo bleaching. Some consequences of photo toxicity includes free radicals rupturing cell membranes, and collapsing chemical and ionic compartmentalization (98). We did not observe any such abnormalities using PercevalHR in our long experiments that makes it a safe tool of choice to measure ATP:ADP dynamics.
3.2 Study 2 - AT1-receptor Response to Non-Saturating AngII Concentrations is amplified by Calcium channel blockers.

Hypertension is a chronic disease of multifactorial origin and it is a substantial risk factor for cardiac arrest and stroke. Antihypertensive medication includes drugs that block the Angiotensin receptors as well as selected calcium channels. These drugs are used alone or in combination. We were in this study interested in the potential for a feedback mechanism by calcium channels regulating the response of AT1R.

The majority of studies on AT1R signaling have used pharmacological (non-physiological) AngII concentrations. This has consistently been shown to result in desensitization of the Ca^{2+} signal response. We therefore started the study by analyzing how AT1R respond to repeated stimulation of both pharmacological (100 nM) and physiological (1 nM) doses of AngII on a single cell level. We transfected HEK 293a cells with Venus tagged AT1R in order to both express the receptor and to identify cells for analysis. We used the Ca^{2+} sensitive dye Fura Red in time lapse experiments to monitor the response to AngII stimulation. Cells were exposed to 3 pulses of AngII, each 2 min long. Between the first and second exposure we introduced a delay of 5 min and between second and third exposure 30 min. This is a common timescale for desensitization studies (99, 100).

As expected, we found that the high concentration of AngII gave a stronger response than the lower concentration of AngII, and also a significant desensitization of the Ca^{2+} signal in the repeated exposures. In contrast, no desensitization was observed during repeated stimulation at the lower dose of AngII. (fig 15a and 15b). Based on this data we conclude that the AT1R is not
desensitized by repeated stimulation at physiological concentrations of AngII.

**Fig 15**: (A) Representative $[\text{Ca}^{2+}]_{i}$ traces from Fura Red loaded HEK293a cells treated with repeated 1 nM (left) or 100 nM (right) AngII for 2 min at times indicated by the arrows with 5 min recovery after the first exposure and 30 min after second exposure. (B) Quantification of peak $[\text{Ca}^{2+}]_{i}$ response to repeated AngII treatment 1 nM (open bars) and 100 nM (grey bars) as shown in A. Responses are calculated as peak signal above baseline. *: $p \leq 0.05$ compared to 1st AngII exposure, **: $p \leq 0.01$ compared to 1st AngII exposure. n = 4 - 7 experiments.
There are reports suggesting a crosstalk between the Cav1.2 and AT1R (101-103). We therefore wanted to see if Nifedipine and Verapamil, which are inhibitors of Cav1.2, could have an effect on the AngII response. Cells were pretreated with nifedipine or verapamil and then exposed to AngII. In the case of either drug treatment, there was a significant increase of the calcium response to 1 nM AngII (fig 16).

Fig 16: Quantification of peak [Ca\textsuperscript{2+}]i response in Fura Red loaded HEK 293a cells that are exposed to repeated doses of AngII (1 nM) in the presence or absence of nifedipine (1 µM and 100 µM) (n = 4-6), **: p ≤ 0.01 compared to 1 nM AngII exposure alone (left graph). HEK293a cells exposed to repeated doses of AngII (1 nM) in the presence or absence of Verapamil (2 µM) (n= 10) *: p ≤ 0.05 compared to 1 nM AngII exposure alone (right graph). Peaks are calculated as height above baseline.

We then aimed to clarify if the stronger response from At1R after blocking Cav1.2 is due to a direct interaction between At1R and Cav1.2 or more general related to the flux of Ca\textsuperscript{2+}. We therefore tested a different pathway for Ca\textsuperscript{2+} entry by blocking TRPC channels. Cells were exposed to two pulses of AngII (1 nM), 2 min, the first with only AngII and the second after 5 min of
BTP2 treatment or vehicle. BTP2 is an inhibitor of TRPC3 and TRPC5. We found that the Ca\(^{2+}\) response to AngII was higher following the treatment of BTP2 compared to the vehicle (fig. 17). This suggests that the mechanism in the potentiation of AngII response is due to a reduced capacity for influx of Ca\(^{2+}\).

**Fig 17:** Representative [Ca\(^{2+}\)]\(_i\) traces from Fura Red loaded HEK 293a cells exposed to AngII (1 nM) (at arrows) followed by 5 min treatment with either vehicle (left trace) or BTP2 (10 \(\mu\)M) (right trace). Bar graph shows relative changes in response between first and second AngII stimulation for vehicle (open bar) and BTP2 (grey bar). (n = 4–5), **: \(p \leq 0.01\) for BTP2 compared to vehicle.

A cell line is relatively artificial compared to the cells in the living organism (as is demonstrated in study 1). In our experiments, we needed to transfet AT1R into the cells since this receptor is not normally expressed in a conventional cell lines. To establish the relevance of our findings we therefore concluded the study by using primary cells from tissues relevant in hypertension, cardiomyocytes and aortic smooth muscle cells. Cells were exposed to two pulses of AngII (1 nM, 2 min), the first with only AngII and the second after 15 min recovery followed by 15 min treatment with vehicle (fig 18a, 18c), nifedipine or verapamil. In both cardiomyocytes and aortic smooth muscle
cells, we verified the finding that nifedipine as well as verapamil significantly enhance the Ca$^{2+}$ response to AngII (fig 18b, 18d).

Fig 18: Representative [Ca$^{2+}$]$_i$ traces of Oregon Green Bapta 1 loaded aortic smooth muscle cells (A) and rat ventricular myocytes (C) treated for 2 min with AngII (1 nM) at times indicated by arrows. Both cells were co-treated from 15 min after first AngII exposure throughout the experiment with vehicle in top trace, nifedipine (0.2 µM) in middle trace and verapamil (0.9 µM) in bottom trace. (B) Bar graph shows the relative change between first and second response to AngII.
stimulation for vehicle (open bar), nifedipine (0.2 µM) (dark grey bar) and verapamil (0.9 µM) (light grey bar) in aortic smooth muscle cells. Peaks are calculated as signal above baseline. n = 6 - 7 individual experiments, *: p ≤ 0.05 compared to vehicle. (D) Bar graph shows the relative change between first and second response to AngII stimulation for vehicle (open bar), nifedipine (0.2 µM) (dark grey bar) and verapamil (0.9 µM) (light grey bar) in rat ventricular myocytes. Peaks are calculated as signal above baseline. (n = 6–10), *: p ≤ 0.05 compared to vehicle, *#: p ≤ 0.05 compared to nifedipine.

In summary, we demonstrate that inhibition of Ca\textsuperscript{2+} channels can upregulate AT\textsubscript{1}R signaling. These effects were observed with physiological and therapeutically relevant concentration of AngII, nifedipine, verapamil. Calcium channel blockers are used to treat hypertension, either in combination with other drugs such as inhibitors of AT\textsubscript{1}R or ACE, or as mono-therapy. When calcium channels blockers are used as mono-therapy, the possibility for stimulation of AT\textsubscript{1}R signaling should be taken into account.
3.3 Study 3 - Role of the TRPM5 channels in insulin secretion and Ca\textsuperscript{2+} signaling in the rat β-cells.

We studied the role of the TRPM5 channel in insulin secretion from rat islets and Ca\textsuperscript{2+} signaling from rat pancreatic β-cells. From the preliminary experiments done in our lab, we found that TPPO did not affect the tolbutamide induced [Ca\textsuperscript{2+}]\textsubscript{i} increase, and [Ca\textsuperscript{2+}]\textsubscript{i} increase in response to depolarization by KCl. This shows that TPPO does not affect the activity of the VGCC's. TPPO, did not increase [Ca\textsuperscript{2+}]\textsubscript{i} by itself in the primary rat β-cells making this compound a suitable pharmacological tool to study the TRPM5 channel.

TPPO inhibited the glucose induced insulin secretion from the rat islets. Our results were in line with previous reports that showed impaired insulin secretion in TRPM5 knockout mice (54, 56). However, in our study TPPO did not affect the glucose induced [Ca\textsuperscript{2+}]\textsubscript{i} increase. The difference in the experimental conditions could account for this apparent discrepancy. The insulin secretion was measured from whole islets over a period of one hour, whereas [Ca\textsuperscript{2+}]\textsubscript{i} was measured from single β-cells for only 500 s. Colson et al (2010) (54), showed that the absence of TRPM5 reduced the frequency of glucose induced Ca\textsuperscript{2+} oscillations in the intact mouse islets. In our Ca\textsuperscript{2+} experiments we used single β-cells which usually do not show Ca\textsuperscript{2+} oscillations as seen in the intact islets.

One of the major finding in our study was that TPPO reduced both the K\textsubscript{ATP} channel-independent insulin secretion and [Ca\textsuperscript{2+}]\textsubscript{i} increase in response to glucose (fig 19a, 19b, and 19c), as tested by the commonly used KCl plus diazoxide protocol (104). Thus, TRPM5 channels seem to play an important role in the insulin
secretion by glucose, by the $K_{ATP}$ channel independent mechanism.

**Fig 19:** TPPO inhibited the $K_{ATP}$ channel-independent insulin secretion and $[Ca^{2+}]_i$ increase by glucose. (A) Islets were stimulated by glucose (16.7 mM) plus KCl (25 mM) with diazoxide (Dz) (100 µM), in the presence of TPPO (100 µM) (white bar), or in the absence of TPPO (black bar). TPPO inhibited the $K_{ATP}$ channel independent insulin secretion by
glucose \((P < 0.005, n = 12)\). (B) The trace shows the changes in \([\text{Ca}^{2+}]_i\) in a single rat β-cell stimulated first with glucose (16.7 mM), plus KCl (25 mM) plus diazoxide (100 µM), in the presence of TPPO (100 µM), and after washout, with glucose (16.7 mM), KCl (25 mM), and diazoxide (100 µM). (C) The bars show the maximal \([\text{Ca}^{2+}]_i\) increase obtained by stimulation by glucose (16.7 mM) plus KCl (25 mM) with diazoxide (100 µM), in the presence of TPPO (100 µM) (white bar), or in the absence of TPPO (black bar). TPPO inhibited the \(K_{\text{ATP}}\) channel independent \([\text{Ca}^{2+}]_i\) increase by glucose \((P < 0.05, n = 8)\).

The presence of fructose together with a stimulatory concentration of glucose, further amplifies the insulin secretion (105). TRPM5 has been shown to be involved in this process in the mouse islets (55). In our experiments TPPO did not inhibit either fructose mediated insulin secretion or \([\text{Ca}^{2+}]_i\) increase suggesting that in the rat islets, TRPM5 was not involved in insulin secretion in response to this hexose. In this respect, our results contradict the results reported by Kyriazis et al (2012) (55), who demonstrated that TRPM5 is involved in insulin secretion in response to fructose in mouse β-cells. The reason for this discrepancy is unclear. These authors have studied insulin secretion using islets from TRPM5 knockout mice, while we have used a pharmacological tool to study insulin secretion form rat islets. Thus, species differences, and differences in the experimental approach, may partly explain the differences in the results obtained.

Another important finding in our study was that TRPM5 was involved in L-arginine induced insulin secretion and \([\text{Ca}^{2+}]_i\) increase. L-arginine is a commonly used tool for \textit{In vitro} mechanistic studies of stimulus-secretion coupling and \([\text{Ca}^{2+}]_i\) signaling in the β-cells (106). It is postulated that L-arginine,
which is a cationic amino acid, depolarizes the plasma membrane upon entering into the β-cell, without requiring metabolism of the amino acid. However, this view may be over simplistic. We found that TPPO markedly reduced, but did not completely inhibit the L-arginine induced insulin secretion and \([\text{Ca}^{2+}]_i\) increase (fig 20a, 20b, and 20c). These results indicate that while L-arginine alone can increase \([\text{Ca}^{2+}]_i\) partially, maximal increase of \([\text{Ca}^{2+}]_i\) by the amino acid requires a second event, namely, the activation of the TRPM5 channel by the initial increase of \([\text{Ca}^{2+}]_i\) caused by L-arginine.
Fig 20: TPPO inhibited insulin secretion, and [Ca^{2+}]_i increase by L-arginine. (A) Rat islets were stimulated by L-arginine (Arg) (20 mM) without TPPO (black bar), and with TPPO (100 μM) (white bar) in the presence of glucose (7 mM). TPPO inhibited L-arginine-stimulated insulin secretion by ~40% (P < 0.05, n = 8). (B) The trace shows the changes in [Ca^{2+}]_i in a single rat β-cell stimulated by L-arginine (20 mM), first without TPPO, and then after washout, with TPPO (100 μM). Glucose (7 mM) was present throughout the experiment. TPPO inhibited
the L-arginine-induced \([\text{Ca}^{2+}]_i\) response in a reversible manner in all experiments. (C) The figure shows the maximal \([\text{Ca}^{2+}]_i\) increase obtained by L-arginine (20 mM) without TPPO (black bar), or with TPPO (100 µM) (white bar). TPPO inhibited L-Arginine-induced \([\text{Ca}^{2+}]_i\) increase by \(\sim 48\%\) \((P < 0.05, n = 5)\). The data were derived from experiments similar to one shown in Fig.12B.

GLP-1 makes the β-cells competent to stimulation by glucose, GLP-1 has been shown to exert its insulinotropic effect via various mechanisms, including depolarizing plasma membrane by closure of the \(K_{\text{ATP}}\) channels (107), \(\text{Ca}^{2+}\) influx through \(\text{Ca}^{2+}\) channels present on the endoplasmic reticulum (108), and activation of non-specific cation channels (109). In this context, another important finding in our study was that TPPO significantly inhibited the GLP-1 potentiated GSIS (fig. 21a). This suggests that TRPM5 may be involved in mediating the action of this incretin hormone. It is noteworthy that GLP-1 stimulates \(\text{Na}^+\) entry through non-selective cation channels in the plasma membrane (110), which could be TRPM5. We also found that while TPPO inhibited the insulin secretion, it did not inhibit the \([\text{Ca}^{2+}]_i\) response triggered by glucose plus GLP-1(fig 21b, and 21c). This is consistent with the fact that GLP-1 mediates its actions primarily by activating the cAMP dependent pathways, rather than the \(\text{Ca}^{2+}\) signaling pathways. It remains a possibility that GLP-1 elicited a local \(\text{Ca}^{2+}\) signaling which was not detectable by our method, which measured the global \([\text{Ca}^{2+}]_i\) changes.

In summary, our results suggest that in the rat islets TRPM5 is involved in mediating insulin secretion in response to glucose, GLP-1, and high concentration of L-arginine, but not in response to fructose. We speculate that TRPM5 is one of the many
putative channels that provide the so called inward depolarizing “leak current”. Thus, increased activity of the TRPM5 could make the β-cells more readily electrically excitable by the incretin hormones.

**Fig 21:** Effect of TPPO on GLP-1-induced insulin secretion and \([\text{Ca}^{2+}]_i\) changes. (A) Rat islets were stimulated by glucose (16.7 mM), and GLP-1 (50 nM) without TPPO (white bar), and with
TPPO (100 µM) (black bar). GLP-1 increased insulin secretion by ~3 fold compared to that by 16.7 mM glucose alone (checked bar). Stimulation of insulin secretion by GLP-1 (white bar) was inhibited by TPPO (black bar) \((P < 0.05, n = 8)\). (B) The trace shows \([\text{Ca}^{2+}]_i\) changes in a \(\beta\)-cell elicited by glucose (16.7 mM) plus GLP-1 (50 nM) without TPPO in the first stimulation, and then after washout, by the same agents in the presence of TPPO (100 µM). (C) The figure shows the maximal \([\text{Ca}^{2+}]_i\) increase obtained by glucose (16.7 mM) plus GLP-1 (50 nM) without TPPO (100 µM) (black bar), or with TPPO (100 µM) (white bar). TPPO did not inhibit the \([\text{Ca}^{2+}]_i\) increase by glucose plus GLP-1 \((P > 0.05, n = 7)\). The data were derived from experiments similar to one shown in Fig. 13B. It should be noted that we used only one pharmacological tool, namely TPPO, which is relatively new. It is possible that this pharmacological tool may have other effects which we still do not know. For instance, it may inhibit glucose metabolism. However this possibility is less likely, because glucose increased \([\text{Ca}^{2+}]_i\) in the presence of TPPO. It is however unclear why TPPO inhibited insulin secretion in spite of the fact that it did not inhibit \([\text{Ca}^{2+}]_i\). One possibility is that glucose induced \([\text{Ca}^{2+}]_i\) increase does not activate the TRPM5 in the presence of TPPO leading to sub-optimal depolarization, both \([\text{Ca}^{2+}]_i\) and depolarization are necessary for optimal insulin secretion.

In spite of the fact that our conclusion is based on the results obtained by using one pharmacological tool, our results are convincing given that previous studies also reported reduced insulin secretion in TRPM5 knockout mice.
3.4 Study 4 - Insulin secretion and Ca\textsuperscript{2+} signaling in a genetically engineered human pancreatic β-cell line

The development of genetically engineered human insulinoma cell lines that can be easily cultured and propagated has opened up possibilities for studying signal transduction in the β-cells. In this study, we demonstrate insulin secretion in EndoC-BH1 cells in response to glucose, GLP-1 and we show for the first time that [Ca\textsuperscript{2+}]\textsubscript{i} can be measured from these cells by using fura-2 based microfluorometry. We have shown that several physiological or pharmacological agonists that are known to increase [Ca\textsuperscript{2+}]\textsubscript{i} in the primary β-cells also increase [Ca\textsuperscript{2+}]\textsubscript{i} in the EndoC-BH1 cells.

Glucose stimulated the insulin secretion in a concentration dependent manner in the EndoC-BH1 cells (fig. 22a). These cells secreted more insulin in response to glucose (16.7 mM) and GLP-1 (50 nM) compared to glucose (16.7 mM) alone (fig. 22b), suggesting that the metabolic coupling pathways involved in glucose and GLP-1 stimulated insulin secretion were intact in these cells.

**Fig 22:** Glucose stimulated insulin secretion in the EndoC-BH1 cells. (A) The figure shows the magnitude of insulin secretion from EndoC-BH1 cells in response to various glucose (Glu)
concentrations. (B) Insulin secreted in response to glucose (16.7 mM) (white bar) and glucose (16.7 mM) plus GLP-1 (50 nM) (black bar).

KCl, which is known to depolarize the plasma membrane and activate VGCCs increased [Ca\textsuperscript{2+}]\textsubscript{i} in the fura-2 loaded EndoC-BH1 cells proving the presence of functional VGCCs in these cells. They also responded to the cholinergic agent carbachol indicating that the PI-PLC-IP\textsubscript{3} pathway for Ca\textsuperscript{2+} signaling was intact in these cells. The cationic amino acid L-arginine also increased [Ca\textsuperscript{2+}]\textsubscript{i} in the EndoC-BH1 cells.

Sulphonylureas are known to increase the [Ca\textsuperscript{2+}]\textsubscript{i} primarily by inhibiting the K\textsubscript{ATP} channels and thereby causing depolarization followed by activation of VGCC. We found that, Tolbutamide, a commonly used sulphonylurea, increased [Ca\textsuperscript{2+}]\textsubscript{i} in the presence of GLP-1 (fig. 23a) indicating that EndoC-BH1 cells possess functional K\textsubscript{ATP} channels and voltage-gated Ca\textsuperscript{2+} channels. GLP-1 was essential for eliciting Ca\textsuperscript{2+} response in the EndoC-BH1 cells upon stimulation by glucose (fig. 23b). These observations are consistent with the fact that GLP-1 makes β-cells competent to glucose (107). However, we found that even in the presence of GLP-1 the [Ca\textsuperscript{2+}]\textsubscript{i} response to glucose in the EndoC-BH1 cells was small compared to that reported in the primary human β-cell (111). It should be noted that, Insulin secretion in response to glucose is poor (or absent) in most of the rodent insulinoma cell lines available. These observations prove that the ion channels and the signaling molecules that are essential for the regulation of the membrane potential and Ca\textsuperscript{2+} signaling in the primary β-cells are present also in the EndoC-BH1 cells. We conclude that EndoC-BH1 cells could be used as a model to study stimulus-secretion coupling and Ca\textsuperscript{2+} signaling in the human β-cells.
Fig 23: GLP-1 makes EndoC-BH1 cells competent (A) The representative trace shows increase in $[Ca^{2+}]_i$ when stimulated with tolbutamide (100 µM) plus GLP-1 (50 nM) ($n = 5$). (B) The representative trace shows increase in $[Ca^{2+}]_i$ when stimulated with glucose (16.7 mM) plus GLP-1 (50 nM). KCl (25 mM) was used as a positive control. ($n = 17$).
Cell metabolism requires energy. ATP is major source of energy that drives most of the bio-chemical reactions. During my PhD I have studied bioenergetics in NKA and developed a method to quantify ATP turnover using the genetically encoded fluorescent sensor PercevalHR. This technique has a wide range of applications as a robust tool to study cellular energy dynamics in diverse cell models. For example, in brain, the neuronal and glial cells consume high energy and they are susceptible to sudden increase in energy demands. Quantifying the energy consumption using PercevalHR will prove to be a valuable method to study functional and physiological characteristics of these cells. Quantification of ATP hydrolysis using Perceval HR can be used in cancer studies to determine the metabolic state of the cells. Mitochondria are powerhouse of a cell, the density of mitochondria increases where the demand for energy is high. In neurons, the post and pre synaptic buttons which, are called as spines are in high demand for energy during synaptic transmissions. It is still unknown how mitochondria meet the energy needs in spines. The combined use of PercevalHR along with fluorescent mitochondria marker can be used in combination to study the mitochondrial dynamics in neurons i.e. whether the mitochondria moves to spines to produce ATP or the ATP diffuses into the spines from dendrites in order to prevent energy crisis in neurons. PercevalHR can also be coupled with super resolution microscopy such as STED to measure intracellular parameters at nanoscale level. It should also be noted that Perceval HR can measure the total ATP turnover as well as it is sensitive to intracellular pH changes. Considering the advancement in development of fluorescent
probes, it is promising that future development will yield ATP sensitive fluorescent probes that can measure absolute ATP concentration, which will be a better tool for measuring intracellular bioenergetics. One of the major sources of energy to cells comes from glucose. Cells use insulin to transport glucose into them. Ca\(^{2+}\) signaling is one the major stimulus for insulin secretion in \(\beta\)-cells in pancreas. Ca\(^{2+}\) signaling is omnipresent and it regulates cellular metabolism through various Ca\(^{2+}\) channels and pumps. This includes TRP channels, which are involved in several physiological processes ranging from cold sensation, hormone secretion, vision processing etc. Several of the TRP channels conduct calcium ions. Studying role of these Ca\(^{2+}\) conducting TRP channels in various types of cells may open new targets for treatment of several metabolic disorders. The EndoC-BH1 cells are of human origin, these cells have good insulin secreting properties in response to glucose. Future research could be intensified to find out whether these cells could be used for \(\beta\)-cell transplantation.
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