MÄLARDALEN UNIVERSITY
SWEDEN

School of Sustainable Development
of Society and Technology

A study of TRPV1 and TRPV4 ion channels in the beta cells by using fura-2 based microfluorometry

Banina Hadrovic

Degree project ECTS 30.0

Study performed 2009 in the department of Clinical Science and Education. Karolinska Institute

Supervisor

Md. Shahidul Islam, M.D, Ph.D.

Examiner at Mälardalen University

Magnus Neumüller, Ph.D.

Abstract

The calcium ion (Ca^{2+}) is an important ion that regulates many cellular functions including exocytosis, contraction of muscles, neural functions, fertilization and cell division. In the plasma membrane of cells there are different Ca^{2+} channels, including the transient receptor potential (TRP) family of cation channels. The TRP channels are activated by physical stimuli like temperature, stretch, osmolality, and also various ligands. These channels are divided into seven subfamilies, namely TRPC, TRPV, TRPM, TRPML, TRPA, TRPP, and TRPN.

TRP channels can regulate the cytoplasmic free Ca^{2+} concentration ($[Ca^{2+}]_i$) and are therefore important for research of insulin secretion from beta (β) cells. With TRP research new and more effective treatment methods for people with diabetes can be developed. People with type 2 diabetes have a decreased insulin secretion from beta (β) cells, in response to glucose. Cytoplasmic free Ca^{2+} concentration ($[Ca^{2+}]_i$) is important for insulin secretion. It is therefore desirable to find compounds that can increase $[Ca^{2+}]_i$ in pancreatic β cells and thereby increase insulin secretion.

The aim of this project was to investigate whether pancreatic β cells express TRPV1 and TRPV4 ion channels. If the channels are expressed in β cells the $[Ca^{2+}]_i$ can be increased by identifying substances that stimulate TRPV1 and TRPV4 channels. The results can then be used for providing better treatment for patients with diabetes type 2. Insulinoma cells from rat (S5 cells) were used as a model for β cells. $[Ca^{2+}]_i$ was measured from single fura-2 loaded S5 cells by ratiometric microfluorometry. To test whether TRPV1 is expressed, N-(4-hydroxyphenyl)-Arachidonoylamide (AM404) and [5-hydroxyl-1-(4-hydroxy-3-methoxyphenyl)decan-3-one] ([6]-gingerol) were used. To test whether TRPV4 was expressed, a TRPV4-selective agonist 4alpha-Phorbol 12,13-Didecanoate namely 4α–PDD was used.

The two agonist of TRPV1, AM404 and [6]-gingerol increased $[Ca^{2+}]_i$. Capsaicin a classical activator of TRPV1 used as a control also increased $[Ca^{2+}]_i$. These increases were inhibited by capsazepine, a specific blocker of TRPV1. 4α–PDD, a specific agonist of TRPV4 also increased $[Ca^{2+}]_i$. These results suggest that S5 cells express both TRPV1 and TRPV4 channels and that AM404, [6]-gingerol and 4α–PDD are potential substances for increasing the insulin secretion from β cells.

Key words:
Calcium signalling, TRPV1, TRPV4, AM404, [6]-gingerol, 4α–PDD, insulin secretion, pancreatic β cells, fluorescence techniques, fura-2.

Sammanfattning

Kalciumjonen (Ca^{2+}) är en viktig jon och förmedlar signaler i processer som cellutsöndring, muskelkontraktion, nervfunktion, fertilisering och celldelning. I cellers plasmamembran finns det olika sorters Ca^{2+} -kanaler, inklusive transient receptor potential (TRP) jonkanalerna. TRP kanalerna aktiveras av fysisk stimulans, så som temperatur, utsträckning, osmolalitet men också av olika ligander. TRP kanalerna är indelade i sju underfamiljer, TRPC, TRPV, TRPM, TRPML, TRPA, TRPP,och TRPN.

TRP kanalerna reglerar den fria Ca^{2+} koncentrationen ($[Ca^{2+}]_i$) i cytoplasman och är därmed viktiga för forskning inom insulinutsöndringen från beta (β) celler. Med denna forskning kan nya och effektivare behandlingsmetoder för personer med diabetes utvecklas. Personer med typ 2 diabetes har bl.a. en minskad insulinfrisättning i beta (β) celler som orsakar en glukosökning i blodet. Den fria Ca^{2+} -koncentrationen ($[Ca^{2+}]_i$) i cytoplasman är viktig för insulinfrisättningen. Det är därför önskvärt att hitta kemiska föreningar som kan bidra till en ökning av $[Ca^{2+}]_i$ i bukspottkörtelns β celler och därmed också ge en ökad insulinfrisättning.

Målet med detta projekt har varit att undersöka om β celler från bukspottkörtel uttrycker jonkanalerna TRPV1 och TRPV4. Om β celler uttrycker dessa kanaler kan $[Ca^{2+}]_i$ i cytoplasman ökas genom att identifiera substanser som stimulerar just TRPV1 och TRPV4 kanaler. Resultaten kan användas för att bidra med bättre behandling till diabetespatienter med typ 2 diabetes. Tumoriserade celler från råtta (S5) användes som modell för β celler. $[Ca^{2+}]_i$ mättes från enskilda fura-2 laddade S5 celler med hjälp av ett ratiometriskt mikrofluorometriskt system. För att undersöka om TRPV1 finns testades ämnena N-(4-hydroxyphenyl)-Arachidonoylamide (AM404) och [5-hydroxyl-1-(4-hydroxy-3-methoxyphenyl)decan-3-one] ([6]-gingerol). För att undersöka om TRPV4 finns användes det TRPV4-specifika ämnet (4alpha-Phorbol 12,13-Didecanoate) 4α–PDD.

De båda TRPV1 agonisterna AM404 och [6]-gingerol inducerade en ökning i $[Ca^{2+}]_i$. Capsaicin som är en klassisk TRPV1 agonist ökade också $[Ca^{2+}]_i$ och användes som kontroll. Alla dessa koncentrationsökningar inhiberades av capsazepine, som är en TRPV1- antagonist. 4α–PDD som är en specifik TRPV4 agonist ökade också $[Ca^{2+}]_i$.

Resultaten tyder på att S5 cellerna uttrycker både TRPV1 och TRPV4 kanaler samt att AM404, [6]-gingerol och 4α–PDD är alla substanser med potential att öka insulinfrisättningen från bukspottkörtelns β celler.

Nyckelord:
Kalciumsignalering, TRPV1, TRPV4, AM404, [6]-gingerol, 4α–PDD, insulinfrisättning, β cells, fluoroscensteknik, fura-2.

List of abbreviations

ADP	Adenosine diphosphate
AM	Acetoxymethyl ester
AM404	N-(4-hydroxyphenyl)-Arachidonoylamide
BSA	Bovine Serum Albumin
DMSO	Dimethyl sulfoxide
EGTA	Ethylene Glycol Tetraacetic Acid
ER	Endoplasmic Reticulum
FAAH	Fatty Acid Amide Hydrolase
HBSS	Hank's Buffered Salt Solution
KRBH	Krebs-Ringer bicarbonate/Hepes buffer
RR	Ruthenium Red
RPM	Revolutions per minute
RPMI	Roswell Park Memorial Institute medium
TRP	Transient Receptor Potential
TRPV	Transient Receptor Potential Vanilloid
SERCA	Sarcoplasmic/Endoplasmic Reticulum Ca^{2+}-ATPase
SOC	Store Operated Channels
4α-PDD	4alpha-Phorbol 12,13-Didecanoate
[6]-gingerol	[5-hydroxyl-1-(4-hydroxy-3-methoxyphenyl)decan-3-one]

TABLE OF CONTENTS

Introduction

The Calcium ion

The calcium ion (Ca^{2+}) is the most important signalling ion in the cell. Ca^{2+} is responsible for mediating processes such as, fertilization, cell division, exocytosis and apoptosis. Ca^{2+} is suitable as a second messenger due to the fact that it has specific and flexible capability of binding to different proteins. Ca^{2+} needs to be in an ionized form for intracellular signalling. In resting conditions the cytoplasmic free Ca^{2+} concentration ($[Ca^{2+}]_i$) is 10 000 times lower than the extracellular $[Ca^{2+}]$. The endoplasmic reticulum (ER) is a major site for Ca^{2+} store in the cell. To maintain low $[Ca^{2+}]_i$ and to avoid toxicity, the cell uses Ca^{2+}-ATPases and Na^{+}/Ca^{2+} -exchangers to move Ca^{2+} into ER. When Ca^{2+}-signalling takes place the $[Ca^{2+}]$ of the cytoplasm increases but it returns rapidly to its resting level. The plasma membrane of cells contains various types of Ca^{2+} channels. One group of such channels belong to the transient receptor potential (TRP)-family. The TRP channels are activated by various stimuli. TRP channels are divided into seven subfamilies and one or other of them is present in almost all kind of cells (Gustafsson and Islam, 2005).

Diabetes

Type 1 diabetes is a disease that occurs when beta (β) cells are destroyed by immune mechanisms, while type 2 diabetes results from insulin resistance. Type 2 diabetes is often caused by excessive body weight and physical inactivity. About 90 % of people with diabetes have type 2 diabetes. In 2005 approximately 1.1 million people died from diabetes and according to the world health organisation (WHO), this number is likely to more than double by the year 2030.

Studies of Ca^{2+} signalling in β cells of people suffering from type 2 diabetes show that a decreased number of β cells are activated by glucose and that $[Ca^{2+}]_i$ increase takes place more slowly. Also the maximum $[Ca^{2+}]_i$ increase is lower than that under normal conditions. Ca^{2+} can become toxic at very high concentrations. It is thought that β cells in both type 1 and type 2 diabetes may undergo apoptosis or necrosis due to toxicity caused by Ca^{2+} (Gustafsson and Islam, 2005).

Aim of the work

The aim of this thesis was to identify whether TRPV1 and TRPV4 channels are expressed in S5 cells. The intended was to test effects of AM404, [6]-gingerol, and 4α–PDD on intracellular $[Ca^{2+}]_i$ in S5 cells. If TRPV1 and TRPV4 are expressed in S5 cells and the substances able to increase $[Ca^{2+}]_i$ the same research can be performed on β cells from human where the substances can be used for a more effective insulin secretion. In the future the results can useful for developing better therapies for treating diabetes.

More specifically the following questions were asked:

- Does AM404 increase $[Ca^{2+}]_i$?
- Does [6]-gingerol increase $[Ca^{2+}]_i$?
- Does capsazepine inhibit the Ca^{2+} response to AM404 and [6]-gingerol?
- Does 4α–PDD increase $[Ca^{2+}]_i$?

The substances AM404 and [6]-gingerol are both TRPV1 agonist. An increase in $[Ca^{2+}]_i$ caused by AM404 and [6]-gingerol will indicate that TRPV1 channels are expressed in S5 cells. If TRPV1 is expressed a TRPV1 agonist, capasazepine will be used for blocking the $[Ca^{2+}]_i$ increase caused by AM404 and [6]-gingerol and thereby provide a even more convincing indication for the presence of TRPV1 channels. An increase in $[Ca^{2+}]_i$ caused by 4α–PDD will similarly indicate that TRPV4 channels are expressed in S5 cells.

Background

Pancreatic Beta Cell

Islets of Langerhans are responsible for regulating blood glucose and body energy metabolism. The islets possess their own microvasculature to carry insulin and other secreted factors rapidly out of the islets, as well as to receive nutrient and regulatory factors into the islets. β cells in islets account for over 70% of the total islet mass. When the glucose concentration rises in the blood, β cells secrete insulin to stimulate the conversion of glucose to glycogen in the liver and the uptake of glucose into insulin target tissues. As a result of insulin action the blood glucose level will drop and the insulin secretion will be inhibited. The β cell is electrically silent at low glucose concentrations (<3 mM). Under such condition the cell secrete insulin at a basal rate. Figure 1 shows how Ca^{2+} regulates insulin secretion from β cells when they are stimulated by glucose (Nunemaker and Satin, 2005).

When blood glucose increases, β cells take up glucose through the glucose transporter GLUT2 (a, figure 1). Glucose is then metabolized through a series of processes. An increase in cellular energy occurs as a result of mitochondrial production of adenosine triphosphate (ATP) (b). The increase in the ratio of ATP to ADP (ATP/ADP) closes the ATP-sensitive potassium (K_{ATP}) channels (c). This initiates the repetitive firing of Ca^{2+} dependent action potentials and the influx of Ca^{2+} into the β cell (d). The resulting increase in $[Ca^{2+}]_i$ causes insulin secretion by triggering the exocytosis of insulin (e). When blood glucose finally returns to its basal level through insulin action, ATP/ADP ratio drops in the β cell, leading to the re-opening of K_{ATP}-channels that in turn shuts off the glucose-induced electrical activity, (Nunemaker and Satin, 2005).

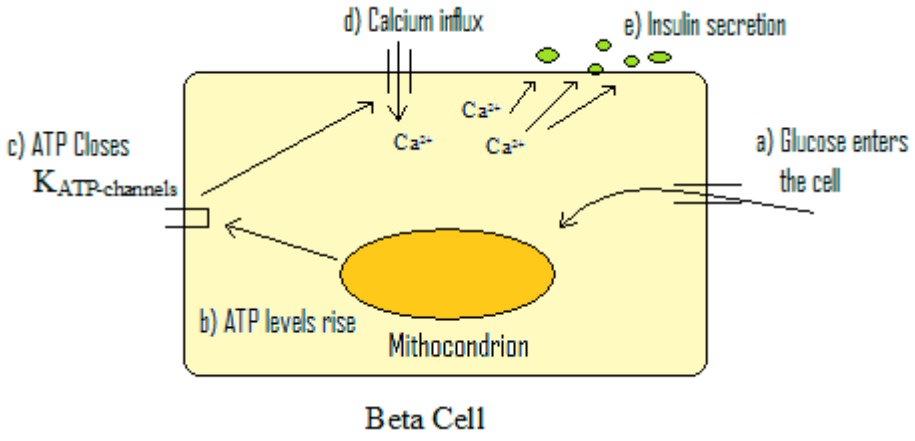


Figure 1: Molecular mechanisms involved in glucose-induced increase of insulin secretion from β cells. When blood glucose concentration rises glucose enters the beta-cell and the ATP/ADP ratio increases. This leads to closure of K_{ATP} channels, plasma membrane depolarization, Ca^{2+} influx, and exocytosis.

Transient Receptor Potential channels

Transient receptor potential (TRP) channel was first identified as ion channel in Drosophila, where it is involved in light perception. So far, 28 members of TRP channels have been identified and 27 of them have been found in human cells. The channels are divided into seven subfamilies, including TRPC (canonical), TRPV (vanilloid), TRPM (melastatin), TRPML (mucolipin), TRPA (ankyrin), TRPP (polycystin), and TRPN (no mechanopotential), (Montell and Rubin, 1989). When TRP channels are activated, conductance for cations such as, Na^+ , K^+ and Ca^{2+} is increased and the membrane potential changes. The TRP channels are activated by multiple stimuli and modes of activation like activation of G-protein coupled receptors, ligand activation, temperature-sensitive activation and mechanical activation (Nagata K et al., 2007).

TRP channels are attracting much attention from various research areas including physiology, pharmacology and toxicology because of their variety of biological functions as well as their existence in organisms from yeast to mammals. There is also mounting evidence to suggest that channels of the TRP family might be the next generation of ion-channel targets that are involved in inflammatory pain (Szallasi et al., 2007).

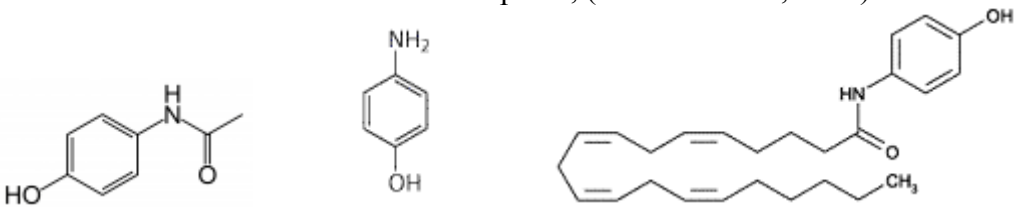
TRPV1

TRPV1 is the most studied and validated TRP channel. In 1997 the first cloning of TRPV1was reported. TRPV1 is a non-selective cation channel with permeability for divalent cations like Ca^{2+}. Vanilloid substances like capsaicin, which is the active component of chilli pepper, activates TRPV1 (Montell and Rubin, 1989). The TRPV1 channel also responds to temperatures over 42 ^oC, meaning that TRPV1 is the ion channel responding both to heat and the sense of heat when eating chilli pepper (Kornfeldt T., 2007). Capsaicin is a flexible natural compound and its biological use is covered by close to 1000 patents. It is used in products ranging from food flavouring to pepper spray for self defence and for ointments for relief of neuropathic pain. This also makes TRPV1 an important target for pain relief, and a number of small-molecule TRPV1 antagonists are already undergoing phase I and II clinical trials for the indications of chronic inflammatory pain and migraine. Animal models also show a therapeutic value for TRPV1 antagonists in the treatment for pain caused by cancer. The central fibres of capsaicin sensitive neurons enter the dorsal horn of the spinal cord where they form synapses with second order neurons. TRPV1 is also present in brain nuclei and non-neuronal tissues.

The list of agents that can activate TRPV1 is growing. One agent that has been reported to activate TRPV1 is N-(4-hydroxyphenyl)-Arachidonoylamide (AM404). The lack of effective drugs for treatment of pain also shows the need for investigation into TRPV1 agonists and antagonists. Data indicate that TRPV1 antagonists could be useful in treating disorders other than pain, for example chronic cough, and bowel syndrome (Szallasi et al., 2007).

AM404

Paracetamol is one of the most widely used drugs for treatment of pain and fever. Unlike non steroidal anti-inflammatory drugs (NSAIDs) it has almost no anti-inflammatory activity and does not produce gastrointestinal damage. The action of paracetamol has been a mystery until recently, even though it has been used clinically for more than a century. In brain and spinal cord, paracetamol, following deacetylation to its primary amine (figure 2), is conjugated with arachidonic acid to form N-arachidonoylphenolamine, a compound known as AM404. AM404 is an endogenous cannabinoid. Acid amide hydrolase is the enzyme that is involved in the synthesis of AM404. AM404 is a TRPV1 agonist and an inhibitor of cellular anandamide uptake, (Bertolini et al., 2006).



Paracetamol → **P-aminophenol** → **AM404**

Figure 2: Schematic view of how paracetamol by deacetylation forms its primary amine, p-aminophenol, and how fatty acid amide hydrolase (+ arachidonic acid) finally forms AM404.

[6]-gingerol

Ginger has been used for more than 2500 years in China for headaches, nausea and colds. The rhizome of ginger contains a rich source of biologically active constituents including the main pungent principles, the gingerols that were identified as the major active components, and [5-hydroxyl-1-(4-hydroxy-3-methoxyphenyl)decan-3-one], ([6]-gingerol) is the most abundant constituent in the gingerol series. For treatment of travel sickness and of chronic arthritis with commercial products of ginger, gingerols has been the main active ingredient. Gingerols are thermally labile due to the presence of a β-hydroxy keto group in the structure, and undergoes dehydration readily to form the corresponding shogaols. Gingerols also has a potent inhibitory effect on prostaglandin biosynthesis. The stability of the compound in the gut, particularly in the stomach, may contribute to their overall bioavailability. As shown in figure 2 and figure 3, there are similarities in the molecule structures of [6]-gingerol and AM404, (Bhattarai et al., 2001).

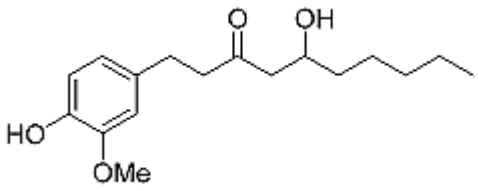


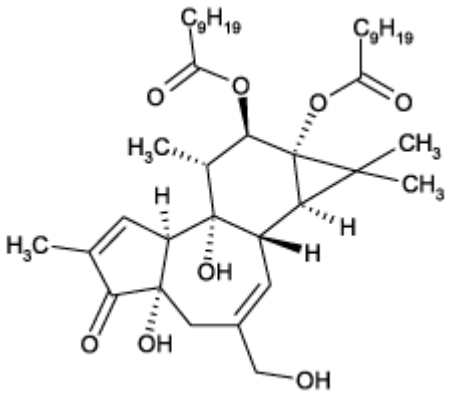
Figure 3: Molecular structure of the TRPV1 agonist [6]-gingerol, showing similarities with AM404 in figure 2.

TRPV4

TRPV4 was identified originally as a channel activated by hypotonic cell swelling, but later reports show that it can be activated also by synthetic agonists, (Vriens et al., 2004). TRPV4 is Ca^{2+} permeable and is activated by swelling and moderate heat (27 ºC – 33 ºC) as well as by diverse chemical compounds such as 4alpha-Phorbol 12,13-Didecanoate (4α-PDD), (Vriens et al., 2007). Compounds like ruthenium red and gadolinium are TRPV4 antagonists. TRPV4 is thought to be an osmoreceptor, because it is found in the circumventricular organs where osmoreceptors are supposed to be distributed. In addition TRPV4 has also shown to be sensitive to osmotic pressure in *in vitro* experiments. It is also known that TRPV4 knockout mice have abnormal osmosensitivity, (Tsushima and Mori, 2006).

4α-PDD

4α-PDD is a phorbol ester and a selective TRPV4 agonist, which promotes Ca^{2+} influx. 4α-PDD is most appropriate in studies of TRPV4, because other TRP channels have been described to be insensitive to phorbol esters, (Reiter et al., 2006). It is suggested that 4α-PDD interacts with TRPV4 through its trans-membrane segments. It is also believed that the length of the fatty acid partly determines the ligand binding affinity for the interaction between 4α-phorbol esters and TRPV4 (Vriens et al., 2007). Ruthenium red and gadolinium are TRPV4 antagonists and they can block the TRPV4 activation by 4α-phorbol esters, (Tsushima and Mori, 2006). Figure 4 shows the molecule structure of 4α-PDD.



4α-PDD

Figure 4: Molecular structure of the TRPV4 agonist 4α-PDD.

Thapsigargin and the activity of SERCA

Thapsigargin (TG) was used to inhibit the activity of Sarcoplasmic/Endoplasmic Reticulum Ca^{2+}-ATPase (SERCA) in S5 cells. TG is lipofilic and enters the cell through the cellmembrane.

TG is a sesquiterpene lactone and is obtained from the root of the plant *Thapsia garganica*, which is a tumor promoter in mammalian cells. Studies show that TG produces transient elevation of $[Ca^{2+}]_i$ and depletion of ER Ca^{2+} stores in many cells. These effects of TG are due to a potent and specific inhibition of SERCA. Since TG is a potent inhibitor of SERCA, it has become a useful tool for experimental manipulation of Ca^{2+} stores in various cell types (Rogers et al., 1995). SERCA is responsible for the maintenance of $[Ca^{2+}]_i$, which is important for the generation of Ca^{2+} mediated signalling and the correct folding and post-translational processing of proteins (Golenser et al., 2006). SERCA resides in the sarcoplasmic reticulum (SR) in muscle cells and transfers Ca^{2+} from the cytostol to the lumen of the SR at the expense of ATP hydrolysis, during muscle relaxation. SERCA plays also an important role in sequestering Ca^{2+} in to the ER. Stored Ca^{2+} can then be released again for subsequent signalling (Seth et al., 2004).

The hydrophobic interactions are the primary driving force of TG binding to SERCA, (Paula and Ball, 2004). The SERCA pump is a protein with a hydrophobic and a hydrophilic region. The hydrophobic region is integrated into the lipidic bilayer of the ER, while the hydrophilic region protrudes into the cytosol (figure 5). TG blocks SERCA and depletes the ER. ER thereby activates Ca^{2+} entry via store-operated channels (SOC) and start communicate with TRP channels to intake more Ca^{2+}. (Alvarez et al., 2006).

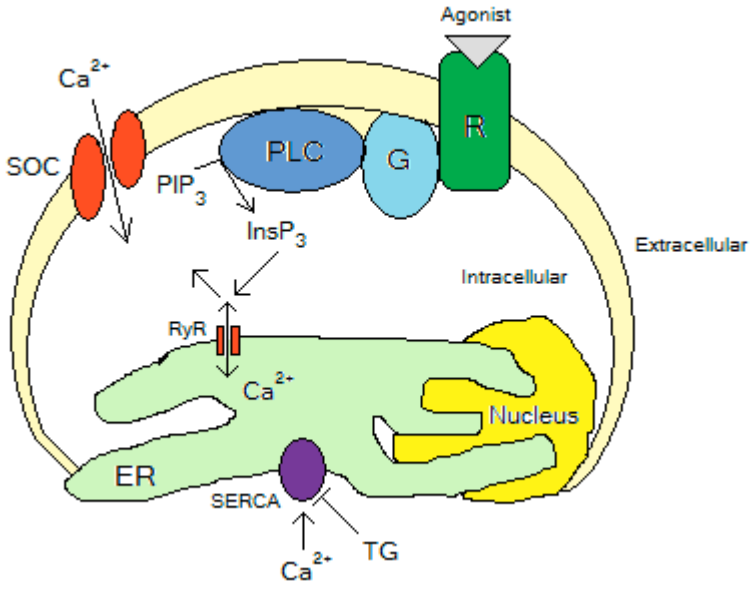


Figure 5: Thapsigargin binds to SERCA and affects the $[Ca^{2+}]_i$.
Thapsigargin blocks the SERCA pump and empties the ER which is the major store for intracellular Ca^{2+}. Depletion of ER signals to the plasma membrane to increase Ca^{2+} entry.

Fura-2

Fura-2 is a polyamino carboxylic acid and a ratiometric fluorescent dye which binds to free intracellular Ca^{2+}. When fura-2 is excited at 340 nm and 380 nm of light, the ratio of emission at those waveleghts is directly correlated to $[Ca^{2+}]_i$. The intensity of fluorescence at these excitation wavelengths is therefore dependent upon Ca^{2+} concentration. At very high free Ca^{2+} concentrations the fluorescence recorded at 340 nm is very high and the fluorescence at 380 nm is very low and vice versa (figure 6). Because of the use of ratio, confounding variables such as cell thickness and dye concentrations can automatically be canceled, making fura-2 one of the most preferred tools to quantify Ca^{2+} concentrations. Another advantage with fura-2 is the isobestic point at wavelength around 360 nm. For certain experiments it is sometimes desirable to choose one of the excitation wavelengths at the isobestic point of the dye. Since the isobestic point is invariant with $[Ca^{2+}]_i$ it will provide a measure of events independent of the $[Ca^{2+}]_i$ such as light scatter, dye leakage or shape changes (Grynkiewicz G., 1985).

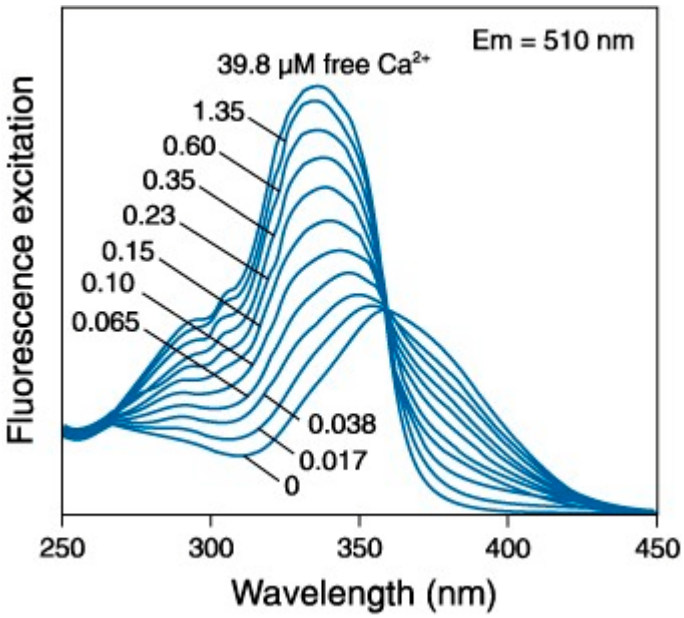


Figure 6: Excitation spectra for fura-2 fluorescence recorded at 510 nm.
The figure show when recording fluorescence emitted at 510 nm it is possible to see two peaks in excitation, one around 340 nm and the other around 380 nm.

Materials and methods

The Cells

Cell Culture

The cells used for all experiments were a sub-clone of INS-1E cells, called S5 cells. They are insulinoma cells, since they are tumorised. The cells were cultured in RPMI-1640 medium, containing fetal bovine serum (FBS) (2.5%, v/v), Penicillin (50 i.u./ml), streptomycin (50 µg/ml), β-mercaptoethanol (500 µM), HEPES (10 mM) and sodium pyruvate (1 mM). Cells were incubated at 37 ºC in a humidified incubator in 5 % CO_2. The medium was changed every other day (three times/week), and cells were passed once a week. New coverslips with cells for experiment were prepared once a week and cultured for three or four days prior to use.

Passaging of the cells

As mentioned before, the cells were passed and diluted two fifth into a new 25 cm^2 culture flask once every week. The old medium was poured off and cells were washed with two ml Ca^{2+}- and Mg^{2+}- free Hank's balanced salt solution (HBSS) for 10 seconds. Two ml of 50 % trypsin (diluted with HBSS) was added to detach the cells from the flask. To help the process, the cells were gently stern and split with a stream of medium by a pipette. After no more than two minutes, four ml of complete medium containing 2,5 % FBS with α_1 – antitrypsin, which inhibits the effect of trypsin was added. All the detached cells and medium was removed into a 15 ml plastic tube and centrifuged at 1000 rpm for two minutes. The supernatant was then poured off and the cell pellet re-suspended in five ml complete medium by pipetting. Two ml from the re-suspended cells were put in a new 25 cm^2 culture flask. Four ml complete medium, containing antibiotics, and 60 µl of β-mercaptoethanol (10 µl/ml) were also added to the flask. The culture flask was put in a humidified incubator with 5 % CO_2 at 37ºC.

Preparing cells on glass cover slips for experiments

One cover slip (Ø = 25 mm) was put in a petri dish (40 * 12 mm) and one drop (50-75 µl) of cell suspension including β- mercaptoethanol (10µl/ml) was placed on the cover slip and gently spread so that it was possible for the cells to grow apart. Then the cells were incubated for 35 minutes so that they could attach to the slip. After this period, 2 ml complete medium containing β-mercaptoethanol (10 µl/ml) was added to the petri dish. The dishes were then left in the incubator for four to five days before use (the medium was changed after three days).

Loading the cell with fura-2 AM

Loading buffer: Roswell park memorial institute medium (RPMI) and 2% bovine serum albumin (BSA) with fura-2 AM (1 μM). Fura-2 AM is a lipophilic Ca^{2+} indicator, which is designed to enter the cell through the plasma membrane. BSA is amphiphilic and was added to dissolve fura-2 AM, (without BSA it would not mix with the loading buffer). The loading buffer was incubated at 37 ºC in humidified incubator in 5 % CO_2.

Experimental methods

Procedure for the experiment

A microfluorometry- (Photon Technology Instrument (PTI)) system was used for measuring $[Ca^{2+}]_i$ from single living cells. The cells were prepared and loaded with fura-2 AM (1 μM) for 35 minutes as described before. Then the cells were washed with complete buffer (appendix A) for ten minutes. A cover slip with cells was then mounted on to a perfusion chamber that has been designed to let the fluid flow across the cover slip. The perfusion chamber was placed on an inverted epi-fluorescence microscope (CK40, Olympus, Japan) that was connected to a fluorescence system (M-39/200 Ratiomed, Photomed). The chamber was also connected to a temperature system to maintain temperature at 37 ºC. All the perfusion solutions used were kept in water bath at 37 ºC, during the experiment.

Single cell with an intact cell membrane, sharp edges and a round shapes were studied. The cell studied was isolated optically by means of a diaphragm and the fluorescence was measured by using a 40× 1.3 NA oil immersion objective (40× UV APO). The excitation wavelengths were 340 nm and 380 nm which were alternated at a frequency of 1 Hz (1 s^{-1}). The emitted light selected by a 510 nm filter was recorded by a photomultiplier tube detector and the signals could be monitored in real time in the Felix software program. When the emission signal was stable for about one minute, the compound of interest was administrated and the change in fluorescence registered. If the compound of interest activated a TRP channel, the channel opened and extracellular Ca^{2+} entered the cell loaded with fura-2. When fura-2 bound with the free intracellular Ca^{2+}, the 380 nm signal (which corresponds to the amount of free fura-2 molecules) decreased and the 340 nm signal, (corresponding to the fura-2 bound to Ca^{2+}) increased. A true $[Ca^{2+}]_i$ increase became apparent when the 340 nm signal increased and the 380 nm signal decreased. After washing out the compound, the TRP channel slowly closed and the two fluorescence signals returned to their baseline. As shown in figure 7a, the response to capsaicin (300 nM) was obtained several seconds after capsaicin was administrated. Since changes of solutions were done manually, the event markings in the plots were made when a solution was changed and not when it reached the perfusion chamber or the cell. All the reagents used for this project have been solved in DMSO. From previous work in the lab it have been showed that DMSO (0.03 %) do not induce an increase in $[Ca^{2+}]_i$.

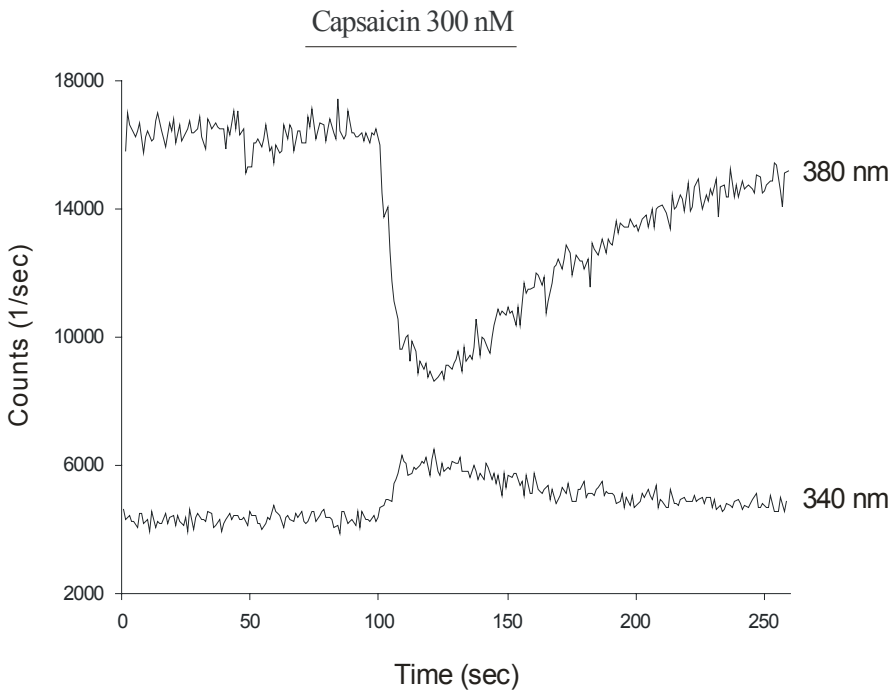


Figure 7a. The use of dual excitation ratiometric fluorometry for measuring $[Ca^{2+}]_I$ in S5 cells.. The measurement was made from a single fura-2 AM loaded S5 cell using microfluorometry. The cell was perfused with physiological solution containing 3 mM glucose and capsaicin 300 nM was added at time indicated by the horizontal bar. The $[Ca^{2+}]_i$ was stable until capsaicin reached the cell and activated the TRPV1channel.

When the experiment was completed the background fluorescence was measured by moving the cell away from the recording field and measuring the signal with no cell present in the area (figure 7b). The background was then subtracted from the original fluorescence signal at both wavelengths and a new ratio calculated. With the new ratio the $[Ca^{2+}]_i$ was calculated. The background is due to stray light from the computer and other components of the system and was usually less than 10 % of the fluorescence signal.

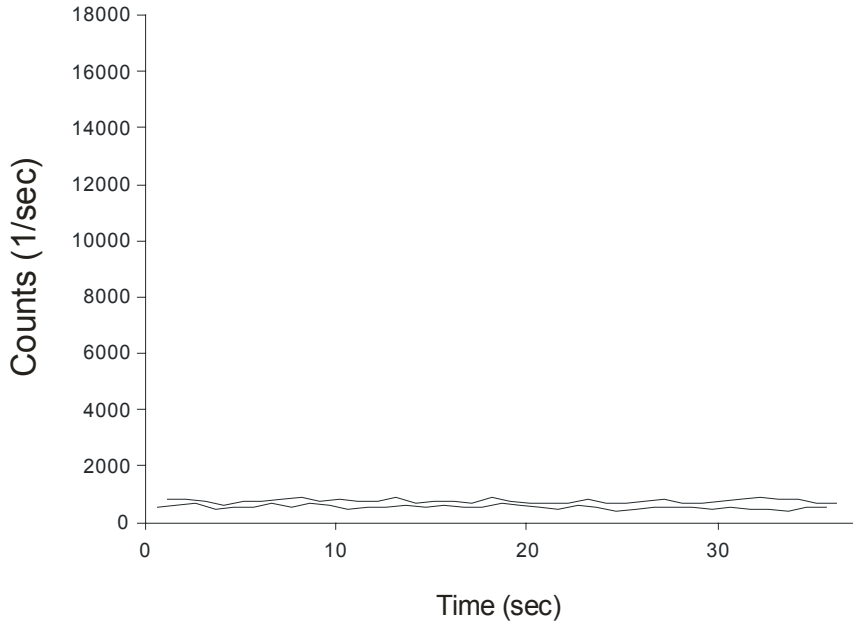


Figure 7b. Measurement of the background signal.
The background fluorescence was measured by moving the cell away from the recording field and measuring the signal with no cell present in the area. The background solution consisted of physiological solution containing 3 mM glucose. The background signal was measured for approximately 40 seconds.

With the Felix PTI software program the background signal was subtracted from the actual experiment signals and the plot of the ratio 340 nm/380 nm showed as a peak . From the ratio of the signals obtained at 340 nm and 380 nm, it was possible to calculate the $[Ca^{2+}]_i$ by using the formula below by Grynkiewicz for calculating $[Ca^{2+}]_i$ (Grynkiewicz et al., 1985). When the background was subtracted, a new ratio trace was obtained and the $[Ca^{2+}]_i$ was calculated using the calibration parameters (the experiment is presented as shown by figure 9).

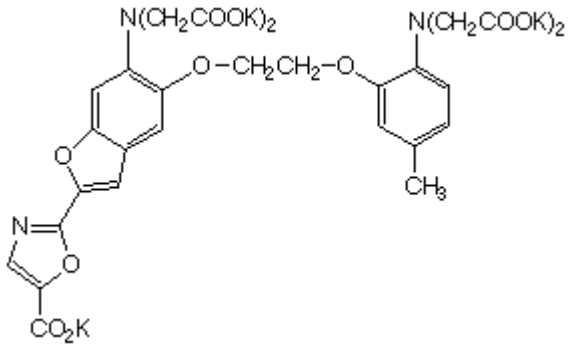
Calibration for converting fluorescence ratio to $[Ca^{2+}]_i$.

The calibration was made using protocols described before (Poenie M., 1990). The fluorescence was measured in the Krebs-Ringer bicarbonate/Hepes (KRBH) buffer containing fura-2 (5 μM). The first KRBH solution was saturated with Ca^{2+} and was used for measuring $F_{max.}$ A second KRBH solution was free from Ca^{2+} and it was used for measuring $F_{min.}$ 2 M sucrose was added to the KRBH solution to resemble the viscosity inside the cell. This was done because the fluorescent properties of fluorophores changes with the viscosity. The dissociation constant (K_d) for Ca^{2+} -fura-2 was calculated from readings obtained at 225 nM. The fluorescence ratio were converted to $[Ca^{2+}]_i$ using the following formula (Grynkiewicz G., 1985):

$$[Ca^{2+}]_i = K_d \left(\frac{R - R_{min}}{R_{max} - R} \right) \left(\frac{F_{min}}{F_{max}} \right)$$

$[Ca^{2+}]_i$ measurements with fura-2 AM

$[Ca^{2+}]_i$ was measured with the fluorescent probe fura-2 AM (acetoxymethylester) (figure 8b). The fura-2 molecule is a charged molecule and cannot enter the cell because of its hydrophilic property (figure 8a). On addition of AM groups, the fura-2 molecule becomes lipophilic and can enter the cell. Inside the cell esterases split the bond between fura-2 and AM so that the active molecule fura-2 can act as a Ca^{2+} indicator. Fura-2 has a tetracarboxylic acid core and binds Ca^{2+} in almost the same way as the Ca^{2+}-chelator EGTA. While EGTA is highly pH-sensitive, fura-2 is not, (Grynkiewicz et al., 1985), (Tsien R.Y., 1980).



Figur 8a: Molecular structure of fura-2.
Fura-2 is used as fluorescence Ca^{2+} probe due to the rich amount of aromatic structures in the molecule, while the chelating properties are due to the groups of COO^- (K).

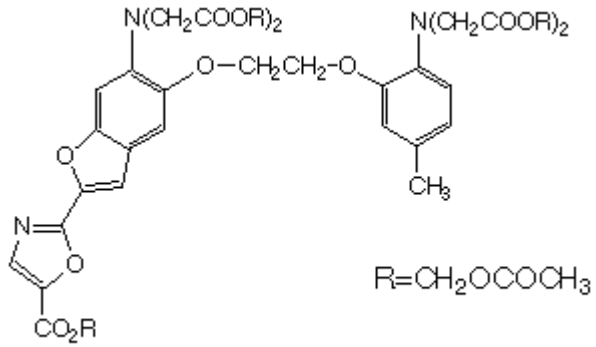


Figure 8b: Molecular structure of fura-2 AM.
When the COO- (K) is replaced by CH2OCOCH3 (R), the fura-2 molecule becomes lipophilic and can enter the cell.

Results

All the protocols for the experiment presented can be found in appendix B.

TRPV1

Capsaicin was used as a positive control for TRPV1.

Capsaicin (300 nM) was applied to S5 cells and was used as a positive control. Since capsaicin is a well known TRPV1 agonist, it was used to confirm that the cell examined had indeed TRPV1 channels. Experiments with capsaicin (300 nm) induced an increase in $[Ca^{2+}]_i$ (figure 9).

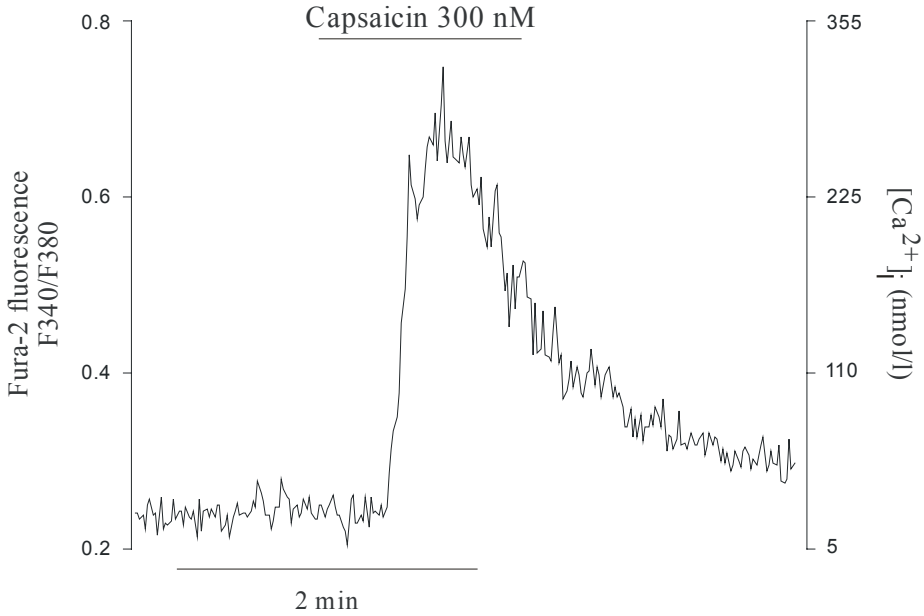


Figure 9. Capsaicin induced an increase in $[Ca^{2+}]_i$ in S5 cells.
The measurement was made from a single Fura-2 AM loaded S5 cell using microfluorometry. The cell was perfused with physiological solution containing 3 mM glucose and capsaicin 300 nM. The figure shows how $[Ca^{2+}]_i$ increases when capsaicin is administrated to a S5 cell.

AM404 induced an increase in $[Ca^{2+}]_i$ in S5 cells.

AM404 was applied to S5 cells and induced an increase in $[Ca^{2+}]_i$ in 10 out of 17 experiments. Figure 10c show that the maximal $[Ca^{2+}]_i$ increase induced by AM404 (5 μM) was 350 +/- 208 nmol/l. The response from AM404 was compared to the response of capsaicin, which was used as a positive control. In seven experiments AM404 did not induce an increase in $[Ca^{2+}]_i$ nor did capsaicin induce any increase in $[Ca^{2+}]_i$ in four of these seven experiments. This was considered to be due to biological variability. During the experiments the cells were perfused with both AM404 and capsaicin for approximately the same length of time. Capsaicin usually gave a faster and a larger response then AM404. After washout of AM404, $[Ca^{2+}]_i$ typically returned to the baseline. This demonstrated that AM404 did not damage the S5 cells under the experimental conditions.

Capsazepine inhibited $[Ca^{2+}]_i$ increase induced by AM404.

Capsazepine is a selective TRPV1 antagonist. It was investigated whether capsazepine (10 μM) could block AM404-induced $[Ca^{2+}]_i$ increase in S5 cells. Capsazpiene itself did not increase $[Ca^{2+}]_i$, but nearly completely inhibited the $[Ca^{2+}]_i$ increase induced by AM404 (5 μM). In three out of three experiments with AM404 and Capsaicin (300 nM), capsazepine inhibited the $[Ca^{2+}]_i$ increase induced by AM404. Capsazepine also inhibited the $[Ca^{2+}]_i$ increase caused by capsaicin, (figure 10b).

Capsazepine did not inhibit $[Ca^{2+}]_i$ increase induced by KCl.

KCl (30 mM) was administrated to S5 cells to test if capsazepine (10 μM) could inhibit $[Ca^{2+}]_i$ increase caused by activation of L-type voltage gated Ca^{2+} channels. KCl depolarizes membrane potential and activates voltage gated Ca^{2+} channels. As shown in figure 10d the $[Ca^{2+}]_i$ response caused by KCl (30 μM) was not inhibited by capsazepine (10 μM).

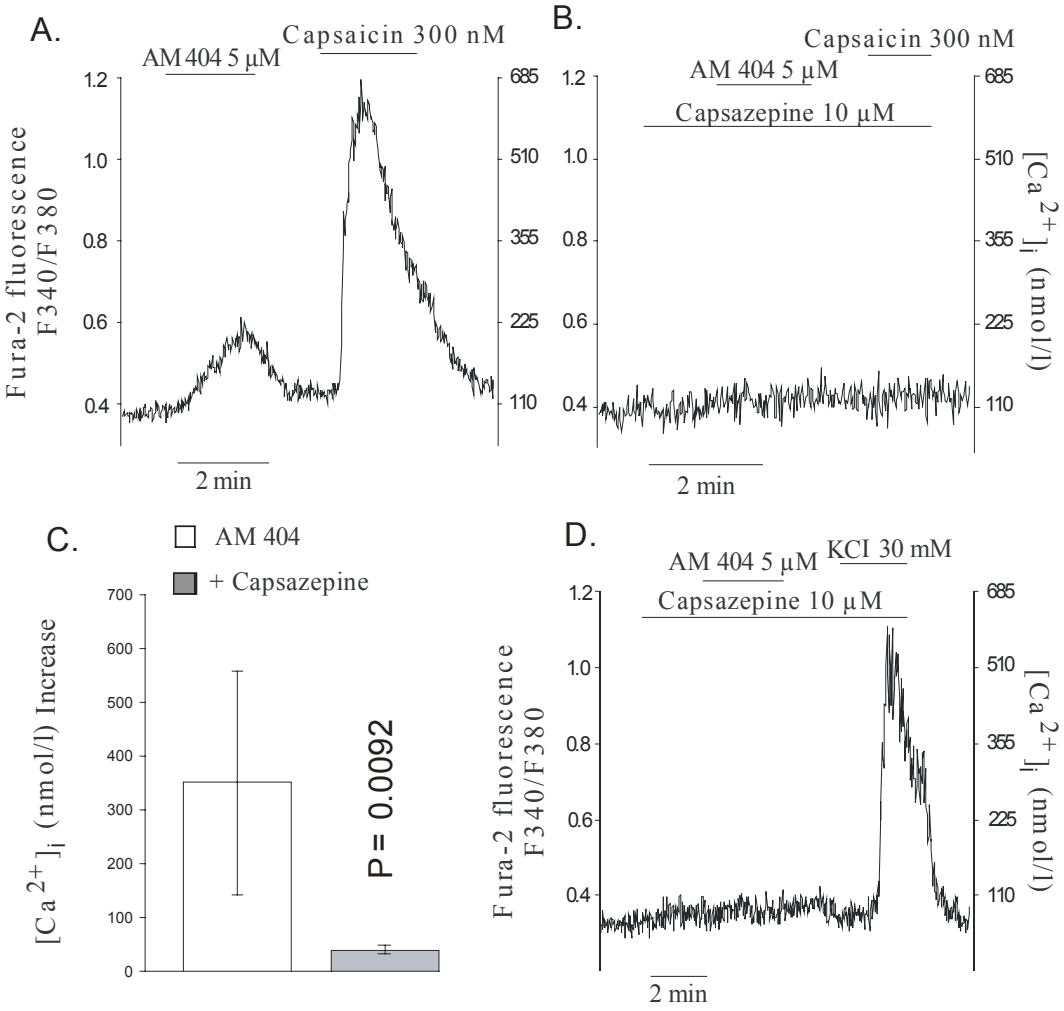


Figure 10. AM404 induced an increase in $[Ca^{2+}]_i$ in S5 cells.
The $[Ca^{2+}]_i$ was measured from single fura-2 AM loaded S5 cells using microfluorometry. The cell was perfused with physiological solution containing 3 mM glucose. AM404 (5 µM), capsaicin (300 nM), capsazepine (10 µM) and KCl (30 mM) were added as shown by the horizontal lines. Capsaicin was used as a positive control. 10a show an increase in $[Ca^{2+}]_i$ induced by AM404, representative for six experiments. The maximal $[Ca^{2+}]_i$ increase induced by AM404 was 350 +/- 208 nmol/l with p = 0.0092 and n = 11. 10b show that capsazepine (10 µM) inhibited the $[Ca^{2+}]_i$ increase induced by AM404 but was not able to inhibited the $[Ca^{2+}]_i$ increase induced by KCl in 10d. Figure 10b and 10d are representative for five respectively four experiments.

[6]-gingerol (1 µM) did not increase $[Ca^{2+}]_i$ in S5 cells.

In previous experiments with another analog of gingerols, 6-shogaol (1 µM) induced an $[Ca^{2+}]_i$, increase in S5 cells. Even if [6]-gingerol and [6]-shogaol are very similar, [6]-gingerol (1 µM) did not induce a $[Ca^{2+}]_i$, increase in 14 out of 16 experiments (figure 11). Capsaicin (300 nM) was used as a positive control.

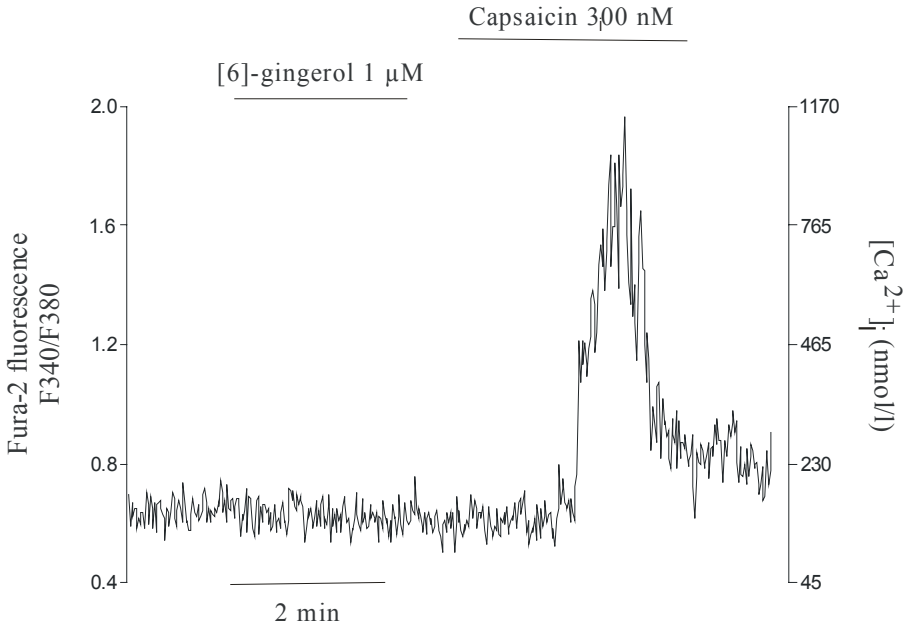


Figure 11. [6]-gingerol (1 µM) did not induce an increase in $[Ca^{2+}]_i$.
The $[Ca^{2+}]_i$ was measured from single fura-2AM loaded S5 cells using microfluorometry. The cell was perfused with physiological solution containing 3 mM glucose. [6]-gingerol (1 µM) and capsaicin (300 nM) were added as shown by the horizontal lines. This figure is representative for four experiments.

[6]-gingerol (10 µM) induced an increase in $[Ca^{2+}]_i$ in S5 cells.

To investigate whether [6]-gingerol is less potent than [6]-shogaol, [6]-gingerol (10 µM) was administrated to S5 cells and it induced a $[Ca^{2+}]_i$, increase in four out of five experiments. Figure 12c show that the maximal $[Ca^{2+}]_i$ increase induced by [6]-gingerol (10 µM) was 192 +/- 105 (nmol/l). Three out of the six experiments gave response to both [6]-gingerol (10µM) and capsaicin and the results are shown in figure 12a. Capsaicin (300 nM) was used as a positive control.

Capsazepine inhibited $[Ca^{2+}]_i$ increase induced by [6]-gingerol.

It was investigated whether capsazepine (10 µM) could block [6]-gingerol-induced $[Ca^{2+}]_i$ increase in S5 cells. Capsazpiene itself did not increase $[Ca^{2+}]_i$, but almost completely inhibited $[Ca^{2+}]_i$ increase induced by [6]-gingerol (10 µM). As shown in figure 12b, in four out of five experiments with [6]-gingerol, capsazepine (10 µM) inhibited the $[Ca^{2+}]_i$ increase induced by [6]-gingerol (10 µM). Capsazepine also inhibited the $[Ca^{2+}]_i$ increase caused by capsaicin (12b).

Capsazepine did not inhibit $[Ca^{2+}]_i$ increase induced by KCl.

KCl (30 mM) was administrated to S5 cells to test if capsazepine (10 µM) could inhibit $[Ca^{2+}]_i$ increase caused by activation of L-type voltage gated Ca^{2+} channels. KCl depolarizes membrane potential and activates voltage gated Ca^{2+} channels. As shown in figure 12d the $[Ca^{2+}]_i$ response induced by [6]-gingerol (10 µM) was inhibited by capsazepine (10 µM), while the $[Ca^{2+}]_i$ induced by KCl (30 µM) was not.

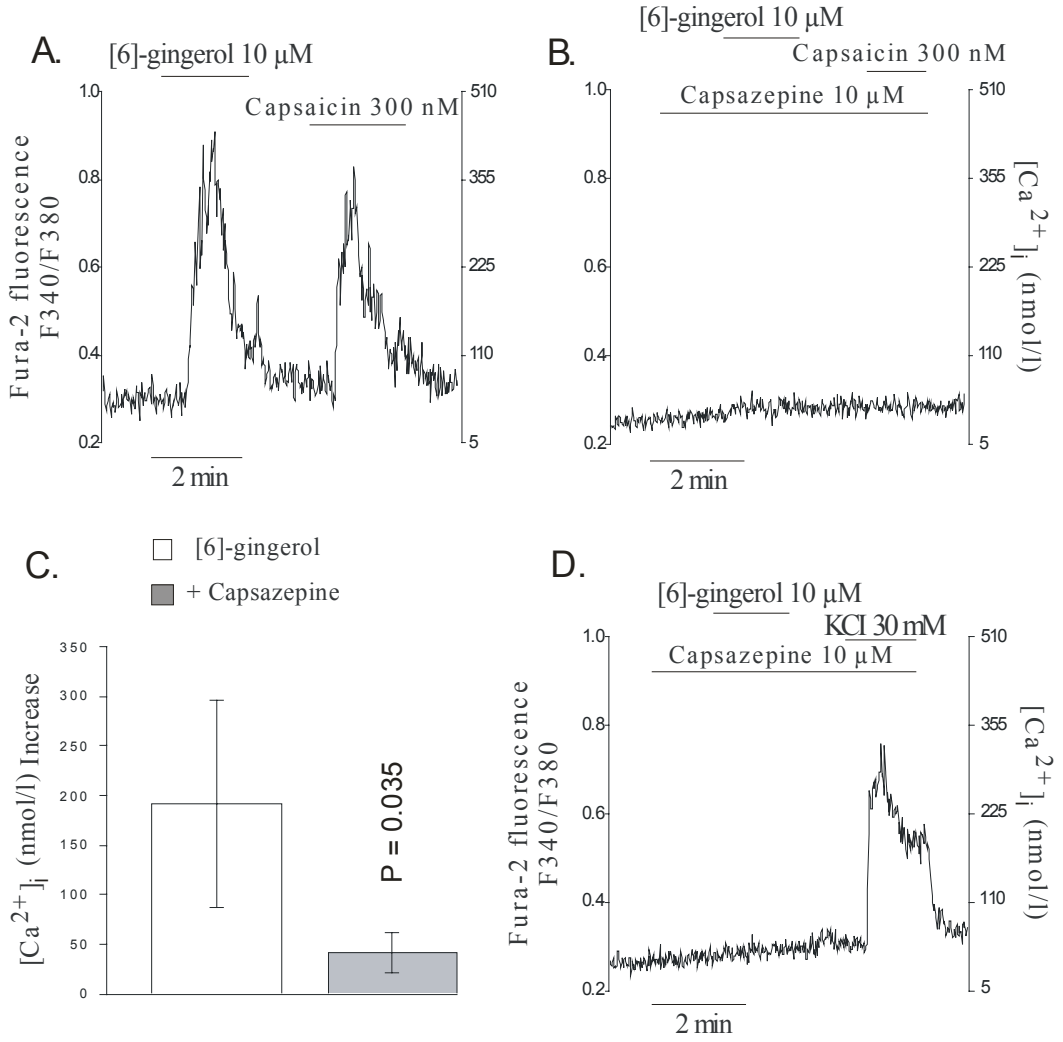


Figure 12. [6]-gingerol (10 µM) induced an increase in $[Ca^{2+}]_i$ in S5 cells.
The $[Ca^{2+}]_i$ was measured from single fura-2 AM loaded S5 cells using microfluorometry. The cell was perfused with physiological solution containing 3 mM glucose. [6]-gingerol (10 µM), capsaicin (300 nM), capsazepine (10 µM) and KCl (30 mM) were added as shown by the horizontal lines. Capsaicin was used as a positive control. 12a is representative for three experiments and show an increase in $[Ca^{2+}]_i$ induced by [6]-gingerol (10 µM). Figure 12c show that the maximal $[Ca^{2+}]_i$ increase induced by [6]-gingerol was 192 +/- 105 nmol/l with p = 0.035 and n = 7. 12b show that capsazepine (10 µM) inhibited the $[Ca^{2+}]_i$ increase induced by [6]-gingerol but was not able to inhibited the $[Ca^{2+}]_i$ increase induced by KCl in 12d. Figure 12d is representative for one experiment.

TRPV4

4α-PDD induced an increase in $[Ca^{2+}]_i$.

4α–PDD (10 µM) was administrated to S5 cells. Since TRPV4 is activated by lower temperatures than TRPV1, three experiments with 4α-PDD (10 µM) were performed in 37 ºC (figure 13d), and three experiments in room temperature (23-25 ºC), (figure 13c) to investigate at what temperature the highest $[Ca^{2+}]_i$ increase was observed. Only experiments at 37 ºC induced an increase in $[Ca^{2+}]_i$. Experiments with 1 µM and 5 µM of 4α-PDD in 37 ºC were also performed and are shown in figure 13a and 13b. In five out of five experiments with 4α-PDD (1 µM) there was no increase in $[Ca^{2+}]_i$. In three out of three experiments with 4α-PDD (5 µM) there was no increase in $[Ca^{2+}]_i$. Since 4α-PDD is the most specific TRPV4-agonist, no other substance was used as a positive control for TRPV4 activation. Experiments with capsaicin 300 nM were performed on the same day as control experiments before the actual experiment with 4α-PDD, showed that the cells were in good condition and responded to any TRPV - agonist.

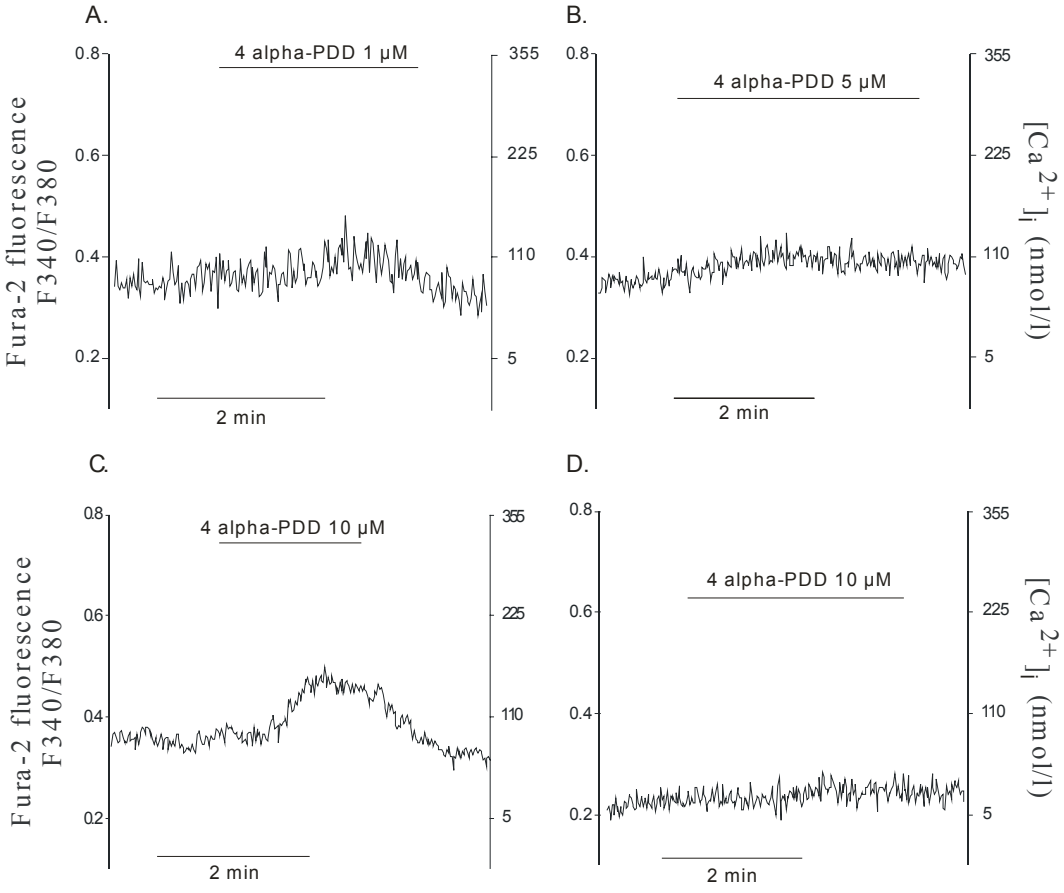


Figure 13. 4α-PDD induced an increase in $[Ca^{2+}]_i$ in S5 cells in 37 ºC.
The $[Ca^{2+}]_i$ was measured from single fura-2 AM loaded S5 cells using microfluorometry. The cell was perfused with physiological solution containing 3 mM glucose. 4α-PDD (1, 5 and 10 µM), was added as shown by the horizontal lines. Figure 13c is representative for three experiments and show a increase in $[Ca^{2+}]_i$ induced by 4α-PDD (10 µM) in 37 ºC. The maximal $[Ca^{2+}]_i$ increase induced by 4α-PDD (10 µM) in 37 ºC was 80 +/- 17 nmol/l.

Effect of Thapsigargin on $[Ca^{2+}]_i$.

TG (5 µM) was administrated to the S5 cells. One out of three cells responded by an increase in $[Ca^{2+}]_i$. Since the response due to TG is not mediated by any specific ion channel, no positive control was used, but a control experiment with capsaicin was made to ensure the wellness of the cell.

The $[Ca^{2+}]_i$ increase due to TG (5µM) is represented in figure 14 and shows that the signal did not return back to baseline after TG was administrated. Instead, $[Ca^{2+}]_i$ remained elevated in the form of a platue. This plateau is due to Ca^{2+} entry through the SOCs in the plasma membrane. If the same experiment is performed with no Ca^{2+} outside the cell the plateau stage should be eliminated meaning that Ca^{2+} outside the cell enters the cell through SOC. After a short time the plateau stage remains as a straight line because of the Ca^{2+} coming in to the cell and leaving the cell achieves an equivalent flow.

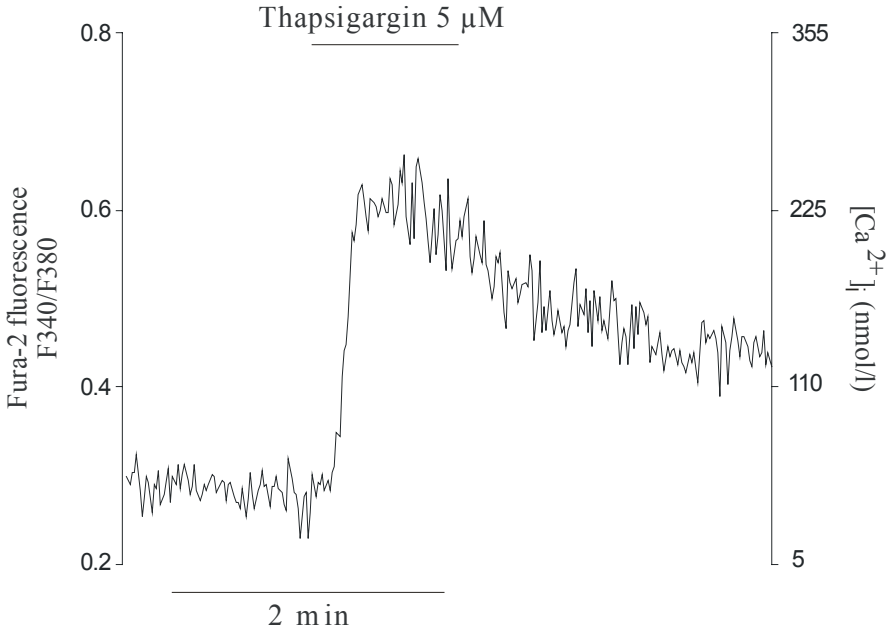


Figure 14. Thapsigargin induced an increase in $[Ca^{2+}]_i$ in S5 cells.
The $[Ca^{2+}]_i$ was measured from single fura-2 AM loaded S5 cells using microfluorometry. The cell was perfused with physiological solution containing 3 mM glucose. Thapsigargin (5 µM) was added as shown by the horizontal line. This figure is representative for one experiment with Thapsigargin (5 µM) and show that the $[Ca^{2+}]_i$ increase was 170 nmol.

Discussion

TRPV1

To investigate if S5 cells express TRPV1 channels, experiments with AM404 and [6]-gingerol were performed. To be certain that the responses obtained from AM404 and [6]-gingerol were due to TRPV1, experiments with capsazepine was performed.

The first step in the investigation of TRPV1 was to find out if AM404 and [6]-gingerol could increase the concentration of cytoplasmatic calcium. $[Ca^{2+}]_i$. When that first question was positively answered, the second step was to try to inhibit the increase in $[Ca^{2+}]_i$ caused by AM404 and [6]-gingerol with the selective TRPV1 antagonist capsazepine. Not only did capsazepine inhibit AM404 and [6]-gingerol-induced Ca^{2+} increase, but it also inhibited capsaicin-induced Ca^{2+} increase. To be sure that the capsazepine did not inhibit other Ca^{2+}-channels than TRPV1, the effect of KCl (30 mM) on the cells was studied. KCl is known to stimulate cellular uptake of Ca^{2+}-by a non-TRPV1-dependent system. The result showed that capsazepine was not able to inhibit the increase in $[Ca^{2+}]_i$ obtained by KCl.

Taken together, all the results with AM404, [6]-gingerol, capsaicin, capsazepine and KCl strongly suggest that S5 cells express TRPV1 channels.

In addition to the investigation of the presence of TRPV1, we could show that [6]-gingerol was less potent than [6]-shogaol. [6]-shogaol, which is related to gingerol, is able to induce an increase in $[Ca^{2+}]_i$ at ten times lower concentration than the concentrations needed for [6]-gingerol (data not shown).

TRPV4

To investigate if S5 cells express the ion channel TRPV4, experiments with 4α-PDD were performed. We obtained a mixed result with an increase of $[Ca^{2+}]_i$ in only one out of four cells. Since 4α-PDD is the best known selective TRPV4 agonist, no other control substance was used in experiments with 4α-PDD.

There are no selective antagonists for TRPV4. Ruthenium red and gadolinium are non-specific inhibitor of TRPV4. Even so, if the obtained increase in $[Ca^{2+}]_i$ due to 4α-PDD was shown to be inhibited by ruthenium red or gadolinium, that would have provide support to the conclusion about the presence of TRPV4. Unfortunately the instrument was sent away for repair for about 3-4 weeks, which meant that there was no time for further investigations along these lines.

Before concluding that TRPV4 is present in S5 cells further experiments with 4α-PDD and selective TRPV4 antagonists as well as many other additional experiments should be performed by future students.

Despite the time lacking for the investigation of TRPV4, there was one interesting result with 4α-PDD. It is claimed that TRPV4 is activated in temperatures between 27-33 ºC, but our result showed that the highest $[Ca^{2+}]_i$ increase due to 4α-PDD was obtained from experiments at 37 ºC. In addition, it was more difficult to get the intact cell to stay attached during experiments at lower temperature than 37 ºC. If a cell floated away during an experiment, the total $[Ca^{2+}]_i$ increase dropped . Therefore, experiments with cells floating away were excluded. This also contributed to a lower number of experiments.

Thapsigargin

TG increased $[Ca^{2+}]_i$. A plateau-stage that was formed instead of the signal returning back to the baseline after administration of TG. This is probably due to Ca^{2+} entry through the SOCs in the plasma membrane. The result show that TG does block SERCA in S5 cells.

Conclusion and future work

The results obtained suggest that the following conclusions can be made:

1. S5 cells express the TRPV1 channel.
2. S5 cells probably express the TRPV4 channel.
3. SERCA is blocked by TG in S5 cells.

***Future work*:**

More experiments with 4α-PDD need to be done. In particular, experiments with TRPV4 antagonist needs to be performed. A dose-response curve should be done for both [6]-gingerol and 4α-PDD to find out their most effective concentrations. (We already have a dose-response curve for AM404).

All the experiments should also be performed on human cells, since the aim of the project is to be able to provide better treatment for patients with diabetes.

I believe that a second and complementary method to ensure the presence of TRPV1 and TRPV4 in S5 cells is desirable. One possible strategy is using antibodies for TRPV1 and TRPV4 and by Western blot identify the channel-proteins in S5 cells.

Acknowledgements

First I would like to express my gratitude to Md. Shahidul Islam's group at Forskningcentrum, Karolinska Institutet, for giving me the opportunity to work with them and for all the knowledge in the area of microfluorometry they have given me.

I would particularly like to thank:

My supervisors Md. Shahidul Islam and Amanda Jabin Gustafsson for providing support, guidance, and the knowledge in microfluorometry technique. I would also like to thank Peter Frykestig who taught me the practical steps in the area of microfluorometry and cell culturing.

My examiner Magnus Neumüller from Mälardalen University.

I also want to thank the rest of the staff at forskningcentrum, and another master thesis student Linnea Ericsson for all the good times in the lab.

A special thanks to my mother, my father, my two lovely brothers and my best friend and fiancé for giving me invaluable support throughout this project.

References

Articles

1. Alvarez D.F., King J.A., Weber D., Addison E., Liedtke W., Townsley M.I., *Transient receptor potential vanilloid 4-mediated disruption of the alveolar septal barrier: a novel mechanism of acute lung injury*. Circulation research, Vol. 99, No. 9, 2006, p. 988-995.

2. Bertolini A., Ferrari A., Ottani A., Guerzoni S., Tacchi R., Leone S., *Paracetamol: New vistas of an old drug*. CNS Drug Review, Vol. 12, No. 3-4, 2006, p. 250-275.

3. Bhattarai S., Tran V.H., Duke C.C., *The stability of Gingerol and Shogaol in Aqueous Solutions*. Journal of Pharmaceutical Science, Vol. 90, No. 10, 2001, p. 1658- 1664.

4. Golenser J., Waknine J.H., Krugliak M., Hunt N.H., Grau G.E., *Current perspectives on the mechanism of action of artemisinins*. International journal for parasitology, Vol. 36, No. 14, 2006, p. 1427-1441.

5. Grynkiewicz G., Poenie M., Tsien R.Y., *A new generation of Ca^{2+} indicators with greatly improved fluorescence properties*. Journal Of Biological Chemistry, Vol. 260, 1985, p. 3440-3450.

6. Gustafsson Jabin, A., Islam, M.S, *Cellens Kalciumjonsignalering – från grundforskning till patientnytta*, Läkartidningen, Vol. 102, Nr: 44, 2005, p. 3214-3219.

7. Kornfeldt T., *Därför känns mint kallt och chilli hett*, DN Vetenskap, July 2007, p. 24 – 25.

8. Montell C., Rubin G.M., *Molecular characterization of the Drosophila trp locus: a putative integral membrane protein required for phototransduction*. Neuron, Vol. 2, No. 4, 1989, p. 1313-1323.

9. Nagata K., *TRP channels as target sites for insecticides: physiology, pharmacology and toxicology*. Invert Neurosci, Vol. 7, No. 1, 2007, p. 31-37.

10. Nunemaker C.S., Satin S.L., *A tale of two rhythms: a comparative review of the pulsatile endocrine systems regulating insulin and GnRH secretion*. Cellscience Reviews, Vol 2, No. 1, 2005, ISSN: 1742-8130.

11. Paula S., Ball W.J. Jr., *Molecular determinants of thapsigargin binding by SERCA Ca2+-ATPase: a computational docking study*. Proteins, Vol. 56, No. 3, 2004, p. 595-606.

12. Ponie M., *Alteration of intracellular Fura-2 fluorescence by viscosity: A simple correction*. Cell Calcium, Vol. 11, 1990, p. 85-91.

13. Reiter B., Kraft R., Günzel D., Zeissig S., Schulzke J.D, Fromm M, Harteneck C. *TRPV4-mediated regulation of epithelial permeability*. FASEB J, Vol. 20, No. 11, 2006, p. 1802-1812.

14. Rogers T.B., Inesi G., Wade R., Lederer W.J., *Use of Thapsigargin to study Ca^{2+} Homeostasis in Cardiac Cells*. Bioscience Reports, Vol. 15, No. 5, 1995, p. 341-349.

15. Seth M., Sumbilla C., Mullen S.P., Lewis D., Klein M.G., Hussain A., Soboloff J., Gill D.L., Inesi G., *Sarco(endo)plasmic reticulum Ca2+ ATPase (SERCA) gene silencing and remodeling of the Ca2+ signaling mechanism in cardiac myocytes*. Proceedings of the National Academy of Sciences of the United States of America, Vol. 101, No. 47, 2004, p. 16683-16688.

16. Szallasi A., Cortright D.N., Blum C.A., Eid S.R., *The vanilloid receptor TRPV1: 10 years from channel cloning to antagonist proof-of-concept*. Nat Rev Drug Discov, Vol. 6, No. 5, 2007, p. 357-372.

17. Tsien R.Y., *New calcium indicators and buffers with high selectivity against magnesium and protons: Design, synthesis and properties of prototype structures*. Biochemistry, Vol. 19, 1980, p. 2396-2404.

18. Tsushima H., Mori M., *Antidipsogenic effects of a TRPV4 agonist, 4alpha-phorbol 12,13-didecanoate, injected into the cerebroventricle*. Am J Physiol Regul Integr Comp Physiol. Vol. 290, No. 6, 2006, p. R1736-1741.

19. Vriens J., Watanabe H., Janssens A., Droogmans G., Voets T., Nilius B., *Cell swelling, heat, and chemical agonists use distinct pathways for the activation of the cation channel TRPV4*. PNAS, Vol. 101, No. 1, 2004, p. 396- 401.

20. Vriens J., Owsianik G., Janssens A., Voets T., Nillius B., *Determinants of 4 alpha-phorbol sensitivity in transmembrane domains 3 and 4 of the cation channel TRPV4*. Journal of Biological Chemistry, Vol. 282, No. 17, 2007, p. 12796-12803.

Websites for the molecular structures

Paracetamol;
http://landarzt.wordpress.com/2007/07/27/paracetamol/

p-aminophenol;
www.rsc.org/images/Scheme%201_tcm18-34907.gif

AM404;
www.axxora.com/files/formula/ALX-340-032.gif

Fura-2:
http://www.fura-2.com/fura2.gif

http://www.invitrogen.com/etc/medialib/en/images/ics_organized/brands/molecular-probes.Par.85588.Image.-1.0.1.gif

Fura-2-AM:
http://w3.uniroma1.it/MEDICFISIO/FURA2.HTM

[6]-gingerol:
http://www.dalton.com/images/6-gingerol.gif

4a-pdd;
www.alexis-biochemicals.com/Viral-Signalling....

Gadolinium;
http://commons.wikimedia.org/wiki/File:Gadolinium-Diethylentriaminpentaacetat.svg

Ruthenium red;
http://journals.prous.com/journals/dof/20032808/html/df280787/images/316263.gif

TG;
www.alexis-biochemicals.com, → "Thapsigargin"

WHO (diabetes);
http://www.who.int/mediacentre/factsheets/fs312/en/index.html

Appendix A

Buffers and source of reagents

Modified Krebs Ringers Hepes Buffer

Modified Krebs Ringers Hepes buffer (KRBH) was a 3 mM glucose solution that was made as a stem solution. To make KRBH all chemicals in table 1 were weigh in and solved in Milli-Q-water except $CaCl_2$, which was added and solved last.

Table 1: Chemicals needed to make 1000 ml modified KRBH

Chemical	Molecular weight	Concentration (mM)	Amount (g)
NaCl	58.44	140	8.1810
KCl	74.56	3.6	0.268
NaH_2PO_4	137.99	0.5	0.0689
$MgSO_4 * 7H_2O$	246.48	0.5	0.123
Hepes	238.3	10	2.383
$CaCl_2$	147.02	1.5	0.220

Complete buffer

Complete buffer was made fresh on the day of experiments. Complete buffer was used for washing the cells after fura-2 AM incubation and for solving the chemicals to be experimented with. Modified KRBH was used to make complete buffer.

Table 2: Chemicals needed to make 100 ml complete buffer.

Chemical	Molecular weight	Concentration	Amount
$NaHCO_3$	84.01	2 mM	0.017 g
Glucose	180.2	3 mM	0.054 g
BSA		0,1 %	2 ml
KRBH			(100 ml - 2 ml BSA) = 98 ml

Reagents purchased from Cayman Chemical
AM404

Reagents purchased from Sigma Aldrich
Capsazepine
Capsaicin
6-gingerol
Ruthenium red
Thapsigargin
DMSO

Reagents purchased from ALEXIS Biochemical's
4α-PDD

Reagents purchased from Gibco, Invitrogen
RPMI 1640
Sodium Pyruvate
HBSS
Hepes
β-mercaptoethanol
Penicillin
Fetal Bovine Serum
Albumin Serum
Fura-2
Fura-2 AM

Reagents solved in DMSO
AM404
Capsazepine
[6]-gingerol
Thapsigargin

Appendix B

Protocols

Test of capsaicin (used as a control).

1)	3 mM glucose	100 sec
2)	3 mM glucose + 300 nM Capsaicin	100 sec
3)	3 mM glucose	100 sec

Test of AM404

1)	3 mM glucose	100 sec
2)	3 mM glucose + 5 μM AM404	200 sec
3)	3 mM glucose	100 sec
4)	3 mM glucose + 300 nM Capsaicin	100 sec
5)	3 mM glucose	100 sec

Test of AM404 and capsazepine

1)	3 mM glucose	100 sec
2)	3 mM glucose + 10 μM Capsazepine	100 sec
3)	3 mM glucose + 5 μM AM404 + 10 μM Capsazepine	200 sec
4)	3 mM glucose + 10 μM Capsazepine	100 sec
5)	3 mM glucose + 300 nM Capsaicin	100 sec
6)	3 mM glucose	100 sec

Test of [6]-gingerol

1)	3 mM glucose	100 sec
2)	3 mM glucose + 1 μM and 10 μM [6]-gingerol	200 sec
3)	3 mM glucose	100 sec
4)	3 mM glucose + 300 nM Capsaicin	100 sec
5)	3 mM glucose	100 sec

Test of thapsigargin

1)	3 mM glucose	100 sec
2)	3 mM glucose + 1, 3 and 5 μM Thapsigargin	200 sec
3)	3 mM glucose	100 sec

Test of 4α-PDD

1)	3 mM glucose	100 sec
2)	3 mM glucose + 1, 5 and 10 μM 4α-PDD	200 sec
3)	3 mM glucose	100 sec
4)	3 mM glucose + 300 nM Capsaicin	100 sec
5)	3 mM glucose	100 sec