Original Article

D-erythro-Sphingosine and Pregnenolonesulphate activate TRPM3 channels synergistically in INS-1E cells.

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Abstract:

Background: A group of ion channels have recently been studied to understand the pathogenesis of diabetes. The transient receptor potential (TRP) channels are thought to be involved in many cellular functions. TRPM3, a member of the melastatin-like transient receptor is mainly expressed in human kidney and brain. It is also expressed in human pancreas. Therefore, it is desirable to find compounds able to induce an increase of intracellular calcium($[Ca^{2+}]_i$) in pancreatic β cells thereby trigger insulin secretion. ***Aims:*** The aim of the study was to confirm whether D-erythro-Sphingosine and Pregnenolonesulphate activates TRPM3. Another aim was to investigate whether pancreatic β cells express TRPM3-channels. INS-1E cells were used as a model of β–cells for $[Ca^{2+}]_i$ measurement. ***Results:*** Application of endogenous neurosteroidpregnenolonesulphate (35 μM) led to a rapid Ca^{2+} influx in INS-1E cells and pancreatic beta cells. When PS was applied in the absence of extracellular Ca^{2+} the $[Ca^{2+}]_i$response to PS was completely lost. The increase in $[Ca^{2+}]_i$induced by PS was inhibited by cholesterol.Western blot data identified a protein reacting specifically with polyclonal antibodies for TRPM3. ***Conlusion:*** Our results demonstrate that both pancreatic β–cells and INS-1E cells express functional TRPM3-channels and both SPH and PS are TRPM3 agonists.

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Abbreviations:

$[Ca^{2+}]_i$, intracellular calcium concentration; TRP, Transient Receptor Potential channel; TRPM3, Transient Receptor Potential Melastatin 3 receptor; ADP, Adenosine di-phosphate; PS, Pregnenolone suhp hate; SPH, D-erythro-sphingosine; ER, Endoplasmic Reticulum; INS-1E S5, Insulinoma 1E cells S5 clone; FURA-2 AM, FURA-2 acetoxymethyl ester; CCh, Carbachol; IP3, Inositol tri-phosphate; PLC, Phospholipase C. ER; Endoplasmic reticulum.

Introduction:

Diabetes mellitus is a group of syndromes characterized by chronic hyperglycaemia, different hormonal and metabolic disturbances [1]. The most common forms of diabetes mellitus are type 1 and type 2 diabetes. Type 1 diabetes is an autoimmune disorder that occurs due to autoimmune destruction of pancreatic beta cells. Type 2 diabetes is phenotypic expression of different kinds of defects that affect lipid metabolism, insulin sensitivity to glucose level and cause insulin deficiency [2]. It is a metabolic disorder characterized by insulin resistance and by an insufficient production of insulin that trigger hyperglycaemia. This form of diabetes occurs when beta cell function fails to compensate for insulin resistance. The function of beta cells progressively deteriorates with increasing duration of diabetes[3]. Because of the diverse function of $[Ca^{2+}]_i$ in secretion in many cells, many investigators are exploring diverse aspects of Ca^{2+}-signaling in β-cells and roles of $[Ca^{2+}]_i$ homeostasis in diabetes[4]. Increase of Cytoplasmic Ca^{2+} led to increases mitochondrial Ca^{2+} thereby stimulating mitochondrial metabolism, consequently production of ATP and othersignaling substances increases that may enhance exocytosis[5]. Type 3 and type 2 inositol 1, 4, 5-triphosphate receptor and type 2 ryanodine receptors play important roles in Ca^{2+} entry through the plasma membrane[6,7]. Neurotransmitters and gut hormones increase $[Ca^{2+}_i$ in beta cells by releasing the ion from endoplasmic reticulum (ER) and stimulating capacitative Ca^{2+} entry and thereby potentiate glucose-induced insulin secretion[8]. Beta cells also have multiple molecular mechanisms for extrusion

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or sequestration of Ca^{2+}. Cyclic AMP and Ca^{2+}-signaling pathways have dramatic effects on insulin secretion[9, 10]. There is a clear correlation between insulin secretion and $[Ca^{2+}_i$. Insulin secretion depend on increase in $[Ca^{2+}]_i$[11, 13]. When $[Ca^{2+}]_i$ is measured in single islets it seems to be superimposable with insulin secretion[12]. Metabolic changes leads to periodic membrane depolarisation-repolarisation and consequent opening of the voltage-gated Ca^{2+} channels[13]. It can also cause periodic release of Ca^{2+} from the ER[14]. Oscillation of $[Ca^{2+}]_i$ is more effective in triggering insulin exocytosis[15]. It is important to study different possibilities to alter the concentration of intracellular free calcium ions $[Ca^{2+}]_i$ in the cells. Ca^{2+} induces secretion of insulin by exocytosis is of the interest in the field of diabetes. The presence of Transient receptor potential channels (TRPs) on nerve fibers innervating the Islets of Langerhans in the pancreas has opened a new door of diabetes research[16]. TRP contain CaM binding site and PEST region[17, 18]. TRP receptors are diverse group of cation channels with a wide diversity in ion selectivity, modes of activation and physiological functions. All the TRP proteins include six putative transmembrane domains and they are assumed to assemble into homo or hetero tetramers which form channels through the membrane. TRP channels are ubiquitously expressed and activated by a wide range of stimuli including intra or extracellular messengers, chemicals, mechanicals, osmotic stress and some probably by the filling state of intracellular Ca^{2+} stores (Clapham, 2003). In particular, the TRPM3 is a member of the melastatin–like TRP channel sub familyis mainly expressed in human kidney and brain.Micromolar concentrations of the steroid pregnenolone directly activate TRPM3 channel of β-cells leading to increase of $[Ca^{2+}]_i$ and augmentation of glucose stimulation insulin secretion[19, 20].This suggests that regulation of TRPM3 channel activity is depends on Ca^{2+}/Calmodulin signaling, which has not yet been substantiated experimentally.

The main aim of this work was to confirm activation of TRPM3 channels causes increased $[Ca^{2+}]_i$ in INS-1E cells and pancreatic beta cells and to investigate whether the TRPM3 channels are present inthe beta cells.

Materials and methods:

Chemicals

Fura-2 acetoxymethyl esterase (98 % pure) was from Molecular Probes Europe, Ins-1E cells and pancreatic beta cells were gift from C.B. Wollheim and P.Maechler, Geneva. Pregnenolonesulphate, Carbachol, D-erythro-sphingosine and Cholesterol were from Sigma. Cell culture materials (RPMI-1640) were purchased from Life Technologies.

Cell culture

A highly differentiated rat insulinoma cell line INS-1E, sub clone S5, was used for experiment. We used RPMI-1640 medium with fetal bovine serum (2.5%, v/v), penicillin, streptomycin (50 µg/ml), 2-mercaptoethanol (500 µM), sodium pyruvate (100 mM), and HEPES (1M). Cells were incubated at 37°C in humidified incubator in 5% CO_2. The culture medium was changed every alternative day and cells were passaged once in every week. To get healthy cells during experiment cells were passaged once in a week and cells are diluted to a one fifth in a 25 cm^2 culture flask. The culture flasks were put in a humidified incubator (37 °C, 5 % CO_2).

Preparation of coverslips for experiments

On each cover slip one drop of cell suspension (50 µL~70 µL, ~20,000 cells/ml) were placed on glass cover slips and spread out with pipette tip. Cells were allowed to grow for three to four days before use in the experiment. The coverslips were transferred to a new Petridish containing RPMI-1640 medium supplemented with 0.1% bovine serum albumin (BSA) and 2 µM of fura-2 AM and incubated for 35 minutes at 37°C. The cells were then incubated for an additional 10 minutes in the complete buffer to allow de-esterification of the calcium indicator fura-2 AM to fura-2.

Measurement of Cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) by microfluorometry

Cells were prepared on a cover slip according to the protocol described above and the coverslips were placed in a perfusion chamber. The perfusion chamber was placed on the stage of an inverted epifluorescence microscope (Olympus CK 40). A peristaltic pump and a temperature control system for maintaining temperature in plate were also connected to perfusion chamber. A thermistor for heat monitoring and a temperature probe for monitoring temperature of the plate also connected to the perfusion chamber. Temperature should be controlled because slight change of temperature alters pH of the chamber solution, which damages the cells. The temperature system is designed to maintain temperature as in vivo environment (370C).

The set temperature of the liquid administrator and the plate was 40 – 41 °C in all experiments. With this set temperature, the thermistor in the perfusion

chamber recorded a temperature in the liquid of 35 – 37 °C. A single cell was isolated optically, by means of a diaphragm and studied by using a 40× 1.3 NA oil immersion objective (40× UV APO).

The microscope was connected to a fluorescence system (M-39/2000 RatioMaster, PhotoMed) for dual wavelength excitation fluorometry. The excitation wave lengths were generated by a monochromator (DeltaRam, PTI, PhotoMed, Germany) and directed to the cell by a dichroic mirror. The background fluorescence was measured by moving the cell away from the cell field and measuring the signals without the cell. The emitted light selected by a 510 nm filter, was monitored by a photomultiplier and signals were displayed on a computer. This retiometric measurement generated two signals. The signal at 380 nm correspond to the amount of free Fura-2 while the signal at 340 nM corresponds to Fura-2 change bound to Ca^{2+}. Fluorometry system including software was purchased from Photon Technology Internatiional (PTI9, Germany. The background fluorescence was measured by moving the focus away from the selected cell and recording the signal with no cell present. This signal was subtracted from the traces before calculation of $[Ca^{2+}]_i$.

The measurement was made from single Fura-2 loaded S5 cells using microfluorometry. Cells were perfused with physiological solution containing 3 mM glucose (complete buffer). A sudden increase in $[Ca^{2+}]_i$ is seen at approximately 250 sec. The increase is evident when the signal at 380 nm and signal at 340 nm approach each other in the signal plot. A true $[Ca^{2+}]_i$ increase is shown as a peak in the ratio of 380 nm and 340 nm

Western Blot analysis of TRPM3:

Human islets were sonicated in a modified RIPA lysis buffer [150 mMNaCl, 20 mMTris pH 7.4, 0.25% Na-Deoxycholate, 0.1% SDS , 1% Triton X100, 1 mM EDTA and complete cocktail protease inhibitors(Roche)]. The homogenate was centrifuged at 13500 (20000) rpm for 30 min at 40°C. The supernatant containing the membrane proteins was collected, and protein concentration was measured by using PicoDrop spectrophotometer. The concentration of total protein was 40 mg/ml.

The protein was fractionated in Tris-HCl ready Gel precast Gel,10% resolving gel,4% stacking gel, 10-well (BioRad) using Mini-Protein-3 Cell gel electrophoresis System (BioRad). 80µg of the total protein of the human islets was loaded after adding loading buffer and boiled at 98°C for 10 minutes. Precision plus Protein Standards (BioRad) were used to estimate the TRPM3 size. After the electrophoresis the proteins were transferred to PVDF membrane using Mini Trans-Blot cell (BioRad). After transferring, the membrane was washed in TBS-T buffer (0.005M Tris, 0.138M NaCl, 0.0027M KCl and 0.1% Tween-20) for 10 minutes at room temperature (RT) under constant shaking.

The membrane was blocked either in TBS-T + 5% BSA + Sodium azide 0.02% or TBS-T with 5% nonfat milk over night at 40 C. This step was followed by overnight incubation at 40 C with the primary TRPM3 antibodies (Gift from Kristina Al-Khalili, KTH). The antibodies were diluted 1:500 in TBS-T with 5%BSA and sodium azide 0.02%. The primary antibodies were an affinity-purified rabbit polyclonal antibodies immunoglobin G (IgG) directed against an amino acid sequence on the C-terminal region of the TRPM3.

The PVDF membrane was washed 4-5 times, 10 minutes each with TBS-T buffer at room temperature, with shaking. Then the membrane was incubated with goat anti-rabbit IgG (cat # 170-6515, BioRad) conjugated to horseradish-peroxidase (1:5000) for one hour at RT. Membrane was washed and the immunoreactive bands were detected by enhanced chemiluminescence method Antibody–specificity was tested by using a specific blocking peptide (TRPM3 blocking peptide) (Gift from Cristina Al-Khalili, KTH).

Results:

Optimization of microfluorometry method for measurement of $[Ca^{2+}]_i$ in S5 cells.

In this set of experiments was done to establish and optimize a method for measuring Ca^{2+} in S5 cells. To test whether the method works carbacholwas used as a positive control for Ca^{2+} increase. Carbachol is a long acting analogue of acetylcholine which is known to increase Ca^{2+} in S5 cells consistently. As shown in fig 1, when 100µM carbacholcaused a rapid increase of Ca^{2+}.Ca^{2+} increase by carbachol was observed in all of the three out of three experiments. These experiments showed that the method and protocol we used were suitable for measuring Ca^{2+} from single INS-IEcells. These results also showed that carbachol is a suitable agonist that can be used as a positive control in these cells.

Effects of Pregnenolonesulphate on $[Ca^{2+}]_i$

The most well known agonist of TRPM3 channel is pregnenolonesulphate (PS). To test whether beta

cells have TRPM3 channel pregnenolonesulphate was used in different concentrations. TRPM3 is a Ca^{2+}-permeable channel, and our hypothesis was that ifINS-1Ecells have TRPM3 channel, then pregnenolonesulphate should increase $[Ca^{2+}]_i$by allowing Ca^{2+} entry through the channel.We used 3.5 µM, 7µM, 14 µM and 35 µM of pregnenolonesulphate. 35 µM PS elicited maximum increase of $[Ca^{2+}]_i$in INS-cells. Lower concentrations of PS (e.g. 3.5, 7 and 14 µM) did not increase $[Ca^{2+}]_i$(Fig 2). 35µM of PS increased$[Ca^{2+}]_i$rapidly (Fig 2A). After washout of PS, $[Ca^{2+}]_i$returned to the baseline.

D-erythro-Sphingosine and Pregnenolonesulphate work in synergy.

The experiments showed that 35 µM PS had effect on $[Ca^{2+}]_i$. Further we then examined whether SPH and PS act synergically. When SPH was applied together with PS the resulting $[Ca^{2+}]_i$ increases was more than that of when PS and SPH applied individually. It was previously shown (Fig 2) that PS in lower concentrations cannot increase$[Ca^{2+}]_i$. 10 µM sphingosine phosphate did not have any effect on $[Ca^{2+}]_i$. Experiments were then performed with both PS 10 µM and SPH 10 µM. When combined, PS 10 µM and SPH 10 µM induced large $[Ca^{2+}]_i$increase (Fig 3).From previous experimentswith 35 µM PS it was shown that at this concentration PS induced increased $[Ca^{2+}]_i$. Also, 20 µM SPH increased $[Ca^{2+}]_i$ (Fig 4A). When 35 µM PS was applied together with 20 µM SPH they induced $[Ca^{2+}]_i$increase in an oscillatory manner in all experiments (n=3). Combined effects of PS and SPH (Fig 4C) were more than that of individual effects of PS and SPH (Fig 4A and 4B). This result indicates that PS 35 µM and SPH 20 µM work in synergy in inducing an increase in $[Ca^{2+}]_i$.

Cholesterol inhibited $[Ca^{2+}]_i$increase by PS

PS 50 µM was administered either alone or together with 100µMcholesterol. In three out of four experiments, PS caused increased $[Ca^{2+}]_i$(Fig 5A).Three experiments with PS plus cholesterol were performed (Fig 5B). Cholesterol was first administered alone to see whether it affects $[Ca^{2+}]_i$by itself. Cholesterol itself did not alter $[Ca^{2+}]_i$ (fig5B). When cholesterol was applied together with PS, the latter did not induce an increase in $[Ca^{2+}]_i$(fig 5C). In figure 5C there was no changes in 340 and 380 signals indicating that there was no change in $[Ca^{2+}]_i$. But PS alone induced $[Ca^{2+}]_i$ increase (5A). These results indicate that cholesterol inhibited $[Ca^{2+}]_i$increases induced by PS.

Western blot of TRPM3:

Total protein was extracted to do Western blot. TRPM3 specific polyclonal antibodies IgGwere used. Western blot data showed a very clear and distinct band of about 70 KDa (Fig.6 column 1). Two repeated experiments showed identical results with the same antibody. After stripping and re-probing the same membrane with blocked TRPM3 antibodies with specific peptide of TRPM3 C-terminal region, a weaker band at the same position i.e. 70 KDa was observed (Fig 6 column 2). The intensity of the appeared band was 60% less than the original band. No other bands was appeared

Discussion:

Pregnenolone is a neuroactive steroid and increases neurogenesis in the hippocampus[19]. Recently it has been shown that TRPM3, a divalent-permeable cation channel, is rapidly and reversibly activated by extracellular pregnenolonesulphate. Also pregnenolonesulphate activates endogenous TRPM3 channels in insulin-producing β cells[19].

If Ca^{2+} increases in the cell when SPH and PS are administered to it, it is an indication that TRPM3 is present in the cell. Whether pregnenolonesulphate is a TRPM3 agonist and if cells expressed functional TRPM3, was divided into two questions. These are:

1. Dose pregnenolonesulphate change $[Ca^{2+}]_i$by activating TRPM3?

2. Which concentration of pregnenolonesulphate is effective in increasing in increasing $[Ca^{2+}]_i$in INS-1E cells?

To investigate whether pregnenolonesulphate could induce an increase in $[Ca^{2+}]_i$by activating the TRPM3, experiments were performed with pregnenolonesulphate and using INS-1E cells. Cells were perfused with physiological solution containing 3 mM glucose and different concentration of pregnenolonesulphate. To check cell function, 100 µM carbachol was used as a positive control. In this concentration carbachol produces IP3 which releases Ca^{2+} from the ER and thereby causes large $[Ca^{2+}]_i$increase. The experiments were carried out with different concentrations of pregnenolonesulphate to find out which concentration gave maximal $[Ca^{2+}]_i$increase. We tested if relatively low concentrations of pregnenolonesulphate could increase $[Ca^{2+}]_i$. At low concentration (3.5 µM, 7 µM and 14 µM), pregnenolonesulphate did not induce $[Ca^{2+}]_i$increase. When pregnenolonesulphate was applied in the absence of extracellular Ca^{2+} (fig: 2C) the $[Ca^{2+}]_i$ response to PS was completely lost. This confirmed

that in INS-1E cells there were TRPM3 channels in the plasma membrane which mediated the response to PS by allowing extracellular Ca^{2+} to enter into the cell.

D-erythro-sphingosine is a product of de novo synthesis of cellular sphingolipids. TRPM3 is the first TRP superfamily member described to be activated by SPH and SPH analogs. It has been reported that SPH activates TRPM3 channel [21]. In our experiments, 20 μM SPH induced $[Ca^{2+}]_i$increase. This confirms the report of Grimm et al. [21] who used a HEK-293 cell lines. Our result shows that 20 μM SPH effective in activating TRPM3 channel. The magnitude of Ca^{2+} increase by 20 μM SPH was less than that by 35 μM PS. We also showed that the $[Ca^{2+}]_i$increase by (fig: 2A &4A) 20 μM SPH was slower compared to that by 35 μM PS. Later on, we examined whether PS and SPH acted synergistically. We found that combined application of SPH and PS triggered a large $[Ca^{2+}]_i$ increase in the INS-IE cells (fig. 3). This result suggested that these two compounds work in synergy to induce a $[Ca^{2+}]_i$increase. In some experiments, there was no response to either pregnenolonesulphate or D-erythro-sphingosine when the substances were used alone. This probably means that the cell tested lacked TRPM3. Since PS and SPH can work in synergy, it is probable that they share a part of their activation mechanism.

$[Ca^{2+}]_i$response to PS and SPH show great variability in individual cells. This heterogeneity of response could be due to differences in the level of expression of TRPM3 in the cells which are in different stages of their maturation. The amount of functional TRPM3 channels in the cell membrane is probably of great importance in determination the $[Ca^{2+}]_i$response. All these results confirmed that functional TRPM3 channels are present in INS-1E cells.

Cholesterol is a precursor of all steroid hormones. Recently it has been reported that cholesterol can be act an inhibitor of TRP superfamily. Our aim was to test whether cholesterol has inhibitory effects on TRPM3 channel. Cells were perfused with a physiological solution containing 3mM glucose. First 100 μM cholesterol was added as a positive control to test whether the cell responds by an increase of $[Ca^{2+}]_i$. When 100 μM cholesterol was added, there was no change in the fluorescence signal. That means cholesterol has no stimulatory effects on $[Ca^{2+}]_i$. To test whether cholesterol has inhibitory effect 100 μM cholesterol was added in combination with 50 μM PS. Previously we showed that PS induced $[Ca^{2+}]_i$in INS-IE cells. When cholesterol was applied together with pregnenolonesulphate there was no increase in $[Ca^{2+}]_i$, cholesterol inhibited $[Ca^{2+}]_i$increase by PS. This might be due to structural similarity between cholesterol and PS. They share a common structure i.e. the cholesterol ring. Later in this experiment carbachol 100 μM was applied,to insure that experiments with cholesterol were performed on a viable cell.

Western blot is considered to be a sensitive immunological method to identify proteins using specific antibodies [22,23]. This method was applied here to investigate whether TRPM3 channel is present in human pancreatic beta cells. The total protein used in this experiment was extracted from human islets received from (Geneva Hospital, Switzerland). TRPM3 channel has been identified in different mammalian organs in mouse, rat and human. In human, the major organs where TRPM3 exists are brain, kidney, testis and spinal cord [24]. Recently, TRPM3 expression in human Beta cells has been reported but in at a very low level [21, 20, 24]. Several splice variants of TRPM3 has also been reported. They are nine variants in all and according to the database (NCBI) [25] they are; splice variant 1 with 177.33 kDa and splice variant 9 with 194.74 kDa, variant 2 with 178.88kDa, variant 3 with 176.25 kDa, variant 4 with 177.80 kDa, variant 5 with 180.12 kDa, variant 6 with 179.10 kDa, variant 7 with 25.20 kDa, and variant 8 with 28.00 kDa. Although the results obtained from the calcium signaling experiments (fig: 2 A-C, 4A-C) strongly indicate that TRPM3 channel exists in human islets, however, the size of the protein we observed (70kDa) corresponding to the channel is less than what is reported in the literature (176-194 kDa)[26]. On the other hand, the antibodies used in this project were generated from an amino acid sequence on the C-terminal region of the TRPM3. The consistency of the results we obtained twice signifies some specificity between the antibodies and the 70kDa band. The results of the blocking of the antibodies with the specific peptide support the idea that the 70 kDa band could be a candidate of TRPM3 channel. More work needs to be done to clarify this result.

Conclusion:

TRPM3 agonists, like pregnenolonesulphate and d-erythro-sphingosine, induce an increase in $[Ca^{2+}]_i$ in INS-1E cells. Also when the TRPM3 inhibitor cholesterol was administered together with an agonist for TRPM3 there was no increase in $[Ca^{2+}]$

$_i$. Future work could include experiment with both cholesterol and d-eryhtro-sphingosine in Ca^{2+} free buffer. Calcium free buffer is a buffer nominally free from Ca^{2+} and could be used to determine the location of TRPM3 in INS-1E cells. By western blot one isoform of TRPM3 was identified. It can be confirm by doing sequencing of TRPM3 protein. Then it would make us confirm that the identified band was a TRPM3 isoform.

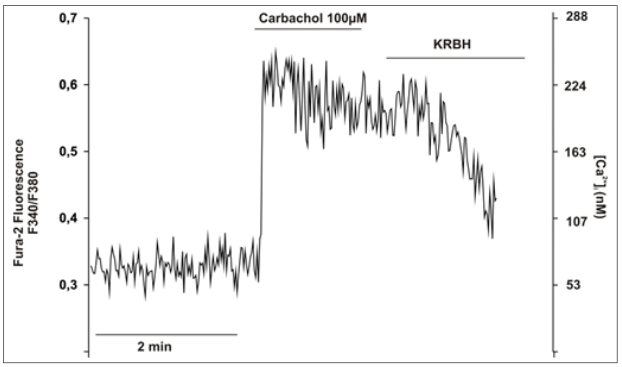
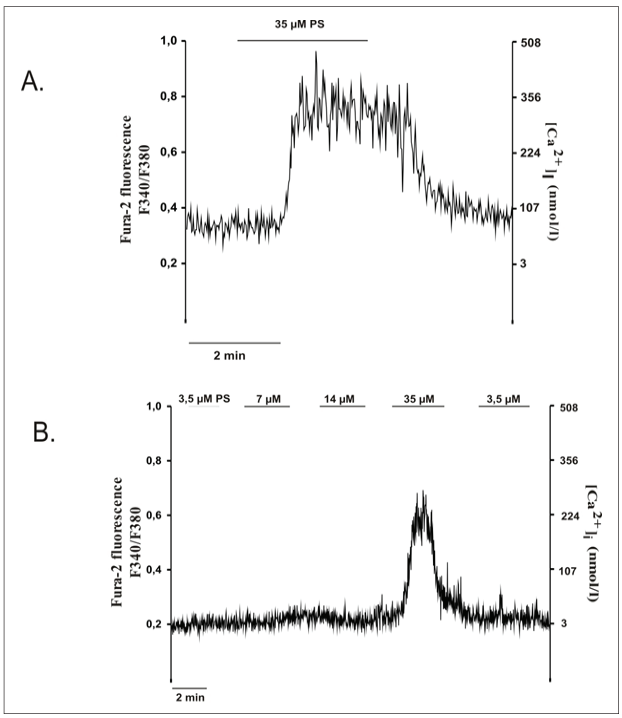


Figure 1: Effect of Carbachol on [Ca2+]iin INS-1E cells. [Ca2+]iwas measured by microfluorometry in single Fura-2 loaded INS-1E cells

Cells were excited at 340 and 380 nm wavelengths alternately. Emitted light at 510 nm was recorded by a photomultiplier tube detector. Carbachol 100µM was added at times indicated by the horizontal bar. Carbachol increased [Ca2+]i. The trace is representative of at least three experiments.



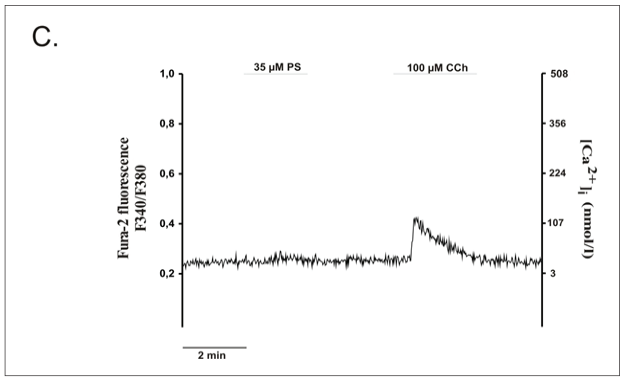


Figure 2: Effect of Pregnenolonesulphate on [Ca2+]iin INS-1E cells. [Ca2+]i was measured by microfluorometry in single Fura-2 loaded INS-1E cells. [A. B. C]

Cells were perfused with physiological solution containing 3 mM glucose. Cells were excited at 340 and 380 nm wavelengths alternately. Emitted light at 510 nm was recorded by a photomultiplier tube detector. A) Pregnenolonesulphate 35 µM induced $[Ca^{2+}]_i$increases in S5 cells. B) Pregnenolonesulphate 3 µM, 7 µM, 14 µM has no effects on $[Ca^{2+}]_i$. C) PS 35 µM applied with Ca^{2+} free buffer. Carbachol was used as a positive control.

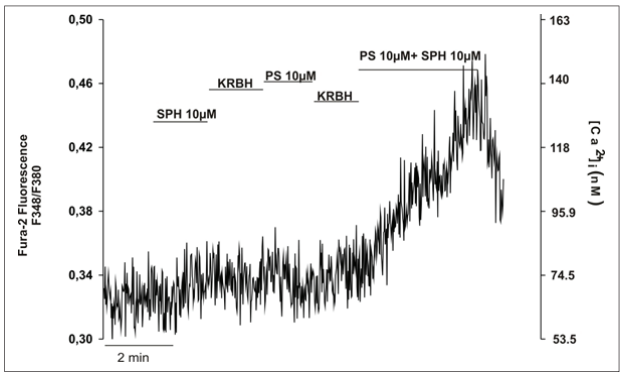


Figure 3: Effect of Pregnenolonesulphate and D-erythro-sphingosine on [Ca2+]iin INS-1E cells. [Ca2+]iwas measured by microfluorometry in single Fura-2 loaded INS-1E cells.

Cells were excited at 340 and 380 nm wavelengths alternately. Emitted light at 510 nm was recorded by a photomultiplier tube detector. Pregnenolonesulphate 10µM and D-erythro-sphingosine 10 µM were added at times indicated by the horizontal bars. Pregnenolonesulphate 10 µM increased $[Ca^{2+}]_i$ in one out of three experiment done.

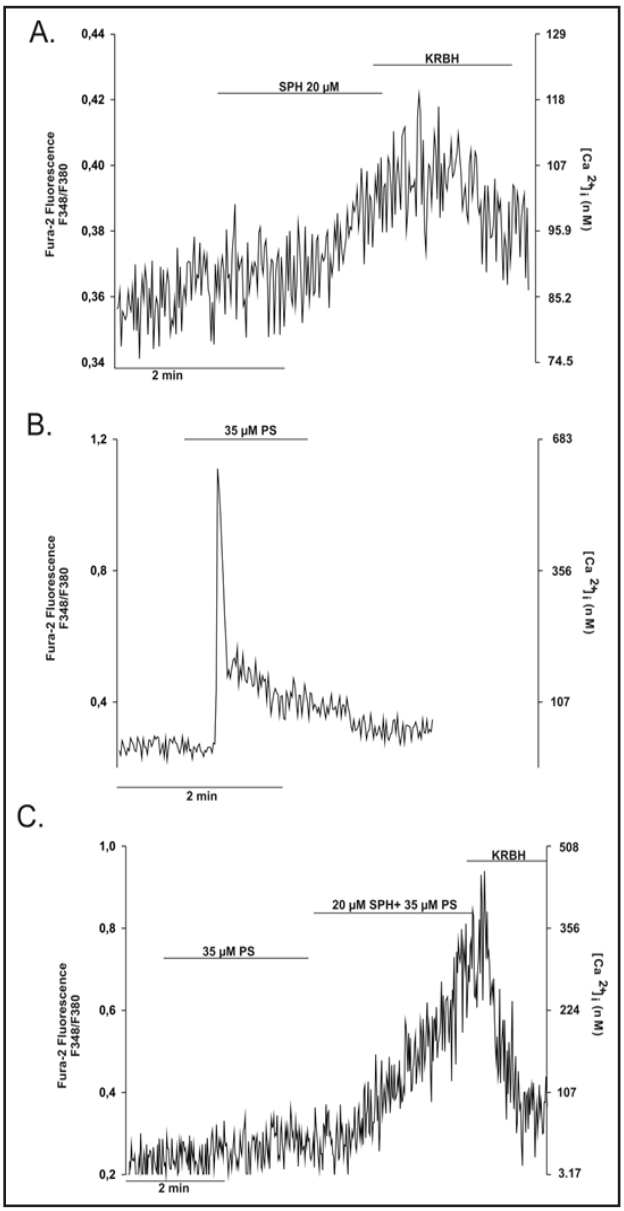


Figure 4: Effect of pregnenolonesulphate and D-erythro-sphingosine on [Ca2+]iin INS-1E cells. [Ca2+]iwas measured by microfluorometry in single Fura-2 loaded INS-1E cells.

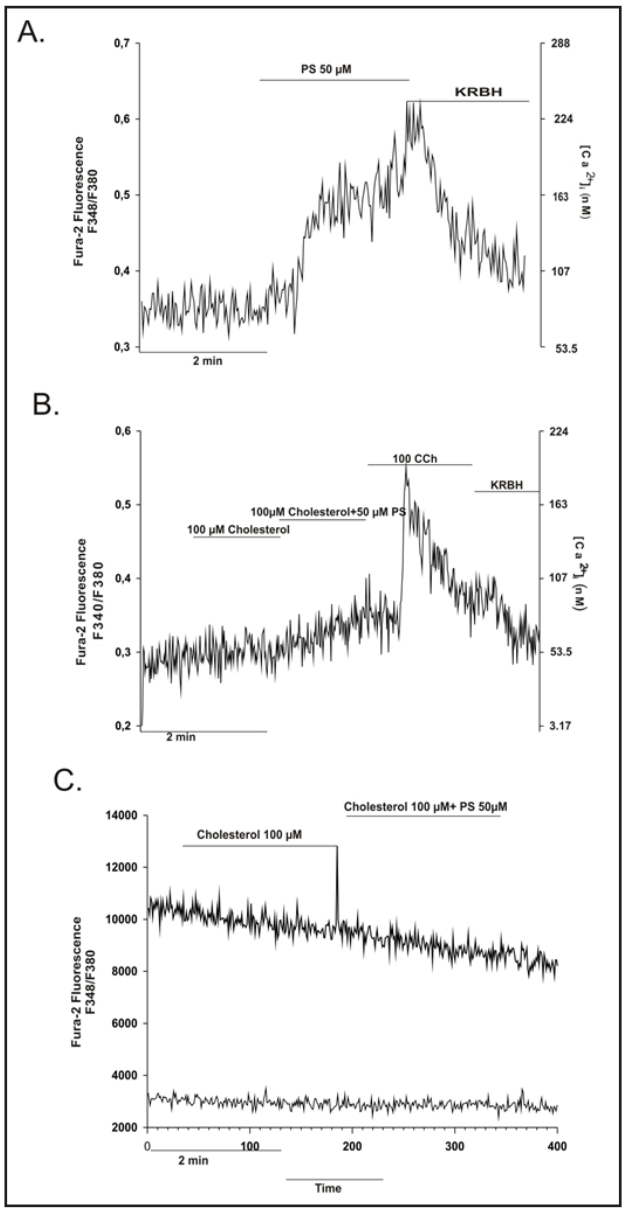


Figure 5: Effect of Pregnenolonesulphate and cholesterol on [Ca2+]iin INS-1E cells. [Ca2+]i was measured by microfluorometry in single Fura-2 AM loaded INS-1E cells.

Cells were excited at 340 and 380 nm wavelengths alternately. Emitted light at 510 nm was recorded by a photomultiplier tube detector. Pregnenolonesulphate 35µMandpregnenolonesulphate(35µM)+D-erythro-sphingosine (20 µM) were added at times indicated by the horizontal bars. Pregnenolonesulphate increased $[Ca^{2+}]_i$ only slightly. When pregnenolonesulphate was applied together with D-erythro-sphingosine, there was a large increase of $[Ca^{2+}]_i$. The trace is representative of three experiments done.

Cells were excited at 340 and 380 nm wavelengths alternately. Emitted light at 510 nm was recorded by a photomultiplier tube detector. Cholesterol 100 µM was added at times indicated by the horizontal bar. A) PS 50 µM induced $[Ca^{2+}]_i$increase in three out of four experiments. B) Cholesterol by itself did not increase $[Ca^{2+}]_i$. But cholesterol inhibited $[Ca^{2+}]_i$increase by 50µM PS in three out of three experiments. Carbachol was used as a positive control and increase $[Ca^{2+}]_i$indicating that the cell was functional. C) There was

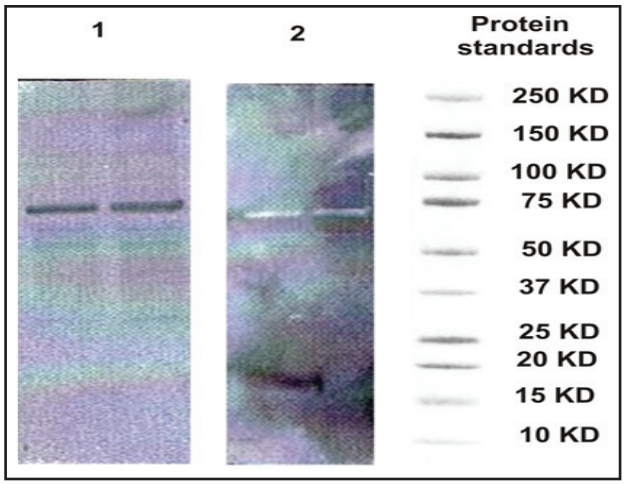


Figure 6: Column 1 shows the result of the western blot of the total protein extracted from human islets using TRPM3 primary antibodies.

no change in 340 and 380 signals which indicated that there was no change in $[Ca^{2+}]_i$

A band with a molecular weight of about 70 KD appeared clearly. Column 2 shows the same membrane after striping the primary antibodies. The membrane was then treated with blocking peptide together with the primary antibodies. A significantly weaker band appeared in the same position.

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Conflicts of interest: There is no conflict of interest.

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