Expression and association of TRPC subtypes with Orai1 and STIM1 in human parathyroid

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Abstract

The mechanism behind Ca^{2+} entry into the parathyroid cells has been widely debated, and the molecular identities of the responsible ion channels have not been established yet. In this study, we show that the parathyroid cells lack voltage-operated Ca^{2+} channels. Passive store depletion by thapsigargin, on the other hand, induces a large non-voltage-activated non-selective cation current. The increase in intracellular Ca^{2+} caused by thapsigargin is attenuated by 2-aminoethoxydiphenyl borate, a blocker of store-operated Ca^{2+} entry (SOCE). Candidate molecules for non-voltage-operated Ca^{2+} signaling were investigated. These included members of the transient receptor potential canonical (TRPC) ion channel family, as well as Ca^{2+} release-activated Ca^{2+} modulator 1 (Orai1) and stromal interaction molecule 1 (STIM1) that are key proteins in the SOCE pathway. Using RT-PCR screening, quantitative real-time PCR, and western blot, we showed expression of TRPC1, TRPC4, and TRPC6; Orai1; and STIM1 genes and proteins in normal and adenomatous human parathyroid tissues. Furthermore, co-immunoprecipitation experiments demonstrated a ternary complex of TRPC1–Orai1–STIM1, supporting a physical interaction between these molecules in human parathyroid.

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Introduction

Primary hyperparathyroidism (pHPT) is the second most common endocrine disease in the western populations (Nilsson *et al.* 2002) after diabetes. The underlying physiological disturbance is the inadequate inhibition of PTH secretion in response to elevated serum calcium. The stimulus–secretion coupling in the parathyroid cells is not completely understood. However, extracellular free calcium (Ca_o^{2+}) regulates PTH secretion from parathyroid cells by acting on a calcium-sensing receptor (CASR) expressed on the cell surface (Sherwood *et al.* 1970, Nemeth & Scarpa 1987, Brown *et al.* 1993, Hofer & Brown 2003). The CASR is a G-protein, phospholipase C (PLC)-linked, membrane-bound receptor, activation of which leads to increased levels of inositol trisphosphate (IP_3), and hence to Ca^{2+} release from intracellular stores (Hofer & Brown 2003). It is generally considered that a high concentration of cytoplasmic free Ca^{2+} (Ca_i^{2+}) mediates an inhibition of parathyroid hormone secretion (Shoback *et al.* 1984). The parathyroid cells are unique in this respect, because in most other endocrine cells, elevated Ca_i^{2+} stimulates hormone

secretion. Since radioactively labeled Ca^{2+} enters the parathyroid cells and unspecific Ca^{2+} channel blockers prevent Ca^{2+} entry (Ridefelt *et al.* 1992), a transporting mechanism must exist in the plasma membrane. It has been suggested that Ca^{2+} entry into the parathyroid cells is mediated by L-type voltage-operated Ca^{2+} channels (VOCCs; Chang *et al.* 2001). However, the presence of these channels has not been proven (Muff *et al.* 1998). The aim of this work was to investigate what type of Ca^{2+} channels can be demonstrated in normal and adenomatous human parathyroid cells. We used electrophysiology and molecular biology techniques to explore the possibility that Ca^{2+} entry is mediated by VOCCs and/or non-selective cation channels, belonging to the family of transient receptor potential canonical-type (TRPC). In other cell systems, TRPCs have been implicated in store-operated Ca^{2+} entry (SOCE), and TRPC1 associated with Ca^{2+} release-activated Ca^{2+} modulator 1 (Orai1) and stromal interaction molecule 1 (STIM1) were the core components of store-operated Ca^{2+} channels (SOCs; Ambudkar & Ong 2007, Ambudkar *et al.* 2007).

Materials and methods

Cells for electrophysiology and Ca^{2+} measurement

Small pieces of human parathyroid tissues (both normal and adenoma) were transported immediately after surgery to the laboratory in sterile tubes, and prepared according to previously published protocol (Välimäki *et al.* 2003). Parathyroid tissue collection was carried out with approval from the local ethics committee. Pancreatic β -cells were isolated from adult obese mice (*ob/ob*), and were prepared as described earlier (Bränström *et al.* 1998).

Electrophysiology

For electrophysiological experiments, cells were seeded into Petri dishes (Nunc, Roskilde, Denmark), and were incubated at 37 °C and 5% CO_2 overnight in DMEM/F-12+10% FCS medium. VOCC activity was recorded using the patch-clamp technique (Hamill *et al.* 1981) with a HEKA EPC-10 patch-clamp amplifier (HEKA Elektronik, Ludwigshafen, Germany). The standard extracellular solution (i.e. bath solution) contained the following (mM): 138 NaCl, 5.6 KCl, 2.6 CaCl_2 , 1.2 MgCl_2 , and 5 HEPES–NaOH at a pH of 7.4. For whole-cell recordings of Ca^{2+} currents, the intracellular-like solution (i.e. pipette solution) consisted of the following (mM): 150 *N*-methyl-D-glucamine (NMG), 110 HCl, 1 MgCl_2 , 2 CaCl_2 , 10 EGTA, 3 MgATP, and 5 HEPES–NaOH (pH 7.15). NMG was substituted for K^+ in the pipette solution to prevent outward-directed K^+ currents. Similarly, SOC currents were recorded using whole-cell configuration. Pipette solution was composed of the following (mM): 145 CsCl, 10 EGTA, 8 NaCl, 10 MgCl_2 , and 10 HEPES (pH 7.2 with CsOH). Channel current traces were displayed according to the convention that upward deflection denotes outward currents. Pipettes were pulled from borosilicate using a P-2000 laser pipette puller (Sutter Instrument, Novato, CA, USA), and they had a resistance between 2 and 4 M Ω . All the experiments were performed at a room temperature of ~22 °C.

Measurement of Ca^{2+}

Cells were grown on glass cover slides in DMEM/F-12+10% FCS medium overnight until they attached to the slides, and were loaded with 3 μM Fura-2 AM (Invitrogen) at 37 °C for 30 min. Experiments were performed using an extracellular solution containing the following (mM): 125 NaCl, 4 KCl, 20 HEPES, 1 MgCl_2 , 0.8 NaH_2PO_4 , and 5.6 D-glucose. CaCl_2 and substances were added as indicated. Slides with cells were kept in a perfusion chamber and exposed to an

inverted fluorescence microscope (Axiovert 135 TV, Zeiss, Göttingen, Germany), and the cells were detected by a cooled charge-coupled device camera (CH250/KAF 1400, Photometrics, Tucson, AZ, USA) connected to an imaging system (Inovision, Durham, NC, USA). Fluorescence was provided by a SPEX fluorolog-2 CM1T1II spectrofluorometer (SPEX Industries, Edison, NJ, USA) with excitation wavelength at 340 and 380 nm, and emission monitored at 505 nm. Experiments were performed at ~37 °C. Fluorescence intensity was analyzed using ISEE software for UNIX (Inovision). Thapsigargin, 2-aminoethoxydiphenyl borate (2-APB), and YM-58483 (also known as BTP-2) were all obtained from Sigma.

Tissue samples of pHPT cases and controls

In this study, 20 parathyroid adenomas that were collected from patients treated surgically for pHPT at Karolinska University Hospital, Sweden, and diagnosed histopathologically as chief cell parathyroid adenomas according to the World Health Organization classification were used (DeLellis *et al.* 2004). The adenomas, the details of which have been published previously (Lu *et al.* 2008), were employed in RT-PCR, quantitative real-time (qRT)-PCR, western blot, and co-immunoprecipitation (co-IP) experiments. Clinical data for the pHPT patients are age 61.5 ± 14.7 years, serum Ca^{2+} 2.85 ± 0.20 mM (reference 2.20–2.60 mM), PTH level 269 ± 208 ng/l (reference <65 ng/l), and tumor weight 2.8 ± 2.3 g. For details, see [Supplementary Table 2](#), see section on [supplementary data](#) given at the end of this article. Two biopsies from normal parathyroid glands were obtained from patients treated for nodular thyroid lesions when parathyroid glands had to be excised and reimplanted into the sternomastoid muscle for anatomical reasons. Both these patients had normal levels of PTH, serum Ca^{2+} , and thyroid hormones preoperatively. They were both verified as histopathologically normal. In addition, anonymous normal uterine tissue was collected by an experienced pathologist, and was used as a positive control for western blot analyses. Standardized sampling procedures, tissue handling, and storage at –70 °C ensured representative tissue and good quality RNA and protein extracts of all the samples.

Antibodies

The following primary antibodies were used for western blot and immunoprecipitation experiments: rabbit polyclonal anti-TRPC1 and control antigen (Cat. No. ACC-010, Alomone Labs, Jerusalem, Israel); rabbit monoclonal anti-TRPC1 (EP1417Y; Cat. No.

NB110-57630, Novus Biologicals, Littleton, CO, USA); rabbit polyclonal anti-TRPC4 and control antigen (Alomone Labs, Cat. No. ACC-018); rabbit polyclonal anti-TRPC6 and control antigen (Alomone Labs, Cat. No. ACC-017); mouse monoclonal anti-Orai1 (Abcam, Cat. No. ab54076); mouse monoclonal anti-Stim1 (BD Biosciences, Cat. No. G72120-050); and mouse monoclonal anti- β -actin (Cat. No. ab6276, Abcam, Cambridge, UK).

Reverse transcription-PCR

RT-PCR was used to screen for TRPC expression in normal and three pHPT samples. Total RNA was extracted using TRIzol reagent (Invitrogen) and was quantified using a spectrophotometer, and the purity and quality of the RNA were further confirmed by analysis in an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Total RNA (3 μ g) from each sample was reverse transcribed into 40 μ l cDNA applying the SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen, Cat. No. 18080-051). Two microliters of each cDNA were used for PCR amplification of TRPC1, TRPC3, TRPC4, TRPC5, TRPC6, and TRPC7 transcripts, which was carried out using optimized gene-specific primers (Table 1) and the Platinum Taq DNA polymerase high-fidelity kit (Invitrogen). The program was constituted of i) 94 °C for 2 min and ii) 40–45 cycles of 94 °C for 30 s, 55 °C for 30 s, and 68 °C for 1 min. Human brain cDNA (Invitrogen, Cat. No. B1110033) was used as a positive control since all *TRPC* genes have been detected previously in human brain tissue (Riccio *et al.* 2002). The PCR products were subsequently size-verified by agarose gel electrophoresis, observed, and photographed under u.v. light. After purification using the PCR purification kit (Qiagen, Cat. No. Q28104), the products were confirmed by DNA sequencing with the assistance of KJSeq, Karolinska Institutet.

Quantitative real-time PCR

Expression of TRPC1, TRPC4, and TRPC6; Orai1; and STIM1 was determined in 2 normal parathyroid and 20 adenoma samples (Supplementary Table 3, see section on supplementary data given at the end of this article). Five micrograms of total RNA from each sample were reverse transcribed into 40 μ l cDNA using the methodology described above, which gave a final concentration of 125 ng/ μ l. Gene expression was quantified using TaqMan Gene Expression Assays (Applied Biosystems) with gene-specific primers and TaqMan probes for TRPC1 (Hs00608195_m1), TRPC4 (Hs00175753_m1), and TRPC6 (Hs00211805_m1) Orai1 (Hs00385627_m1) and STIM1 (Hs00963373_m1) (for details see Supplementary Table 1, see section on supplementary data given at the end of this article), and an ABI real-time PCR 7900HF Fast System (Applied Biosystems). The real-time PCRs were performed in a final volume of 10 μ l with 4.5 μ l cDNA (~20 ng), 0.5 μ l TaqMan Gene Expression Assay (20 $\times$), and 5 μ l TaqMan Universal PCR Master Mix. The amplification program included i) 95 °C for 10 min and ii) 40 cycles of 95 °C for 15 s and 60 °C for 1 min. For each gene, a standard curve was created by serial dilution (1:10, 1:50, 1:250, 1:1250, and 1:6250) of human brain cDNA (Invitrogen). To normalize the individual mRNA level, the house-keeping gene *RPLP0* (Hs99999902_m1) was amplified in parallel as an endogenous control. A no-template sample served as a negative control. Triplicate measurements were performed for all samples, and the experiments were performed on two separate occasions with consistent results.

Western blot

Total proteins were extracted and quantified with a dye-binding assay (Bradford 1976). The yield of the total protein samples was used for western blot and immunoprecipitation experiments. Protein samples

Table 1 Details of primers used for RT-PCR analyses of human *TRPC* genes

Gene	Accession no.	Orientation	Sequence (5'–3')	Amplicon length (bp)
<i>TRPC1</i>	NM_003304	Forward	ACAGCAAAGCAATGATACCT	620
		Reverse	AAGTCCGAAAGCCAAGTAA	
<i>TRPC3</i>	NM_003305	Forward	GTTGTGGAATGTGCTTGACT	722
		Reverse	TGAAAGGTGGAGGTAATGTT	
<i>TRPC4</i>	NM_016179	Forward	CCTGGACATTTTGAAGTTTC	356
		Reverse	CTGCATGGTCAGCAATCAGT	
<i>TRPC5</i>	NM_012471	Forward	GTGGCCTATGTCAAGTATAA	214
		Reverse	GGCAGTAGATAAAGAGGAAT	
<i>TRPC6</i>	NM_004621	Forward	AGGATGACGCTGATGTGGAG	454
		Reverse	TCCTTCAGTTCCTTCGTT	
<i>TRPC7</i>	NM_020389	Forward	TGGGATTATTAGTTGTGAATG	742
		Reverse	GATATCTTTCACAGTTCTCCC	

were denatured by heating at 95 °C for 5 min, electrophoresed in 10% Tricine gels, and transferred onto nitrocellulose membranes. A Ponceau Red stain was used to assess the presence of protein after blotting. After blocking non-specific binding sites with 5% fat-free milk, the membranes were incubated overnight at 4 °C with appropriate primary antibodies for rabbit polyclonal anti-TRPC1 (dilution 1:200), anti-TRPC4 (1:200), anti-TRPC6 (1:200), anti-Orai1 (1:100), and anti-STIM1 (1:250), followed by incubation with secondary antibodies (goat anti-mouse IgG-HRP or goat anti-rabbit IgG-HRP) at a dilution of 1:20 000 for 1 h at room temperature. Immunoreactive bands were detected using enhanced chemiluminescence, and were exposed to a hyperfilm. Subsequent incubation with anti- β -actin was used as a control of protein loading and quality. To confirm signal specificity, primary antibodies were pre-incubated with peptide antigen (i.e. blocking peptide) for 1 h at room temperature and for 2 h at 4 °C.

Co-immunoprecipitation

Co-IP was used to identify possible associations between TRPC1, Orai1, and/or STIM1 proteins. Precleared protein was obtained by incubating 1 mg parathyroid adenoma protein (patient no. 18, see [Supplementary Table 2](#)) with 15 μ l protein G-Sepharose bead slurry for 1 h at 4 °C to reduce non-specific binding of proteins to the Sepharose beads. After centrifugation, 5 μ l of rabbit monoclonal anti-TRPC1 (EP1417Y), anti-Orai1, or anti-STIM1 antibody were added to the supernatant and incubated under rotation for 6 h at 4 °C. Subsequently, 50 μ l of protein G-Sepharose beads were added, and rotation was continued for 4 h. The beads were washed three times with ice-cold modified RIPA buffer, followed by resuspension of the Sepharose beads in 2 $\times$ SDS sample buffer. The samples were heated at 95 °C for 5 min before electrophoresis and blotted, and signal visualization was done using TRPC1 (rabbit polyclonal), Orai1, or STIM1 primary antibody respectively. As a negative control, protein samples were mock-immunoprecipitated with an irrelevant antibody and treated in the same way as described above. In all the co-IP experiments, the respective antibodies recognized a band of the same and expected size in both the lysate and the immunoprecipitated samples, but not in the mock IP sample.

Statistical analysis

All data are given as mean $\pm$ s.d. If not indicated, experiments were repeated at least three times. Statistical significance was analyzed using paired and

unpaired *t*-tests or ANOVA for multiple groups, as appropriate. A *P* value of <0.05 was considered significant.

Results

Lack of functional VOCCs and demonstration of store-operated cation currents in parathyroid cells

The possible existence of functional VOCCs in parathyroid cells was first addressed by voltage-clamping using the whole-cell configuration. Single parathyroid cells were voltage-clamped at -70 mV, and were subsequently depolarized in steps of 10 to $+70$ mV. As illustrated in [Fig. 1A and B](#), this did not result in an

Figure 1 Patch-clamp experiments demonstrating the absence of VOCCs in parathyroid cells. Whole-cell Ca^{2+} current (I_{Ca}) was not recorded in normal (A) or adenoma (B) parathyroid cells after applying a depolarizing voltage protocol (between -70 and $+70$ mV in 10 -mV increments). Top trace and filled circles show results obtained with a total of 20 mM Ca^{2+} in the extracellular solution, and open circles represent results obtained with 20 mM Ba^{2+} . (C) Typical Ca^{2+} current in pancreatic β -cells after applying the same depolarizing voltage protocol.

inward-directed current in normal parathyroid or adenoma cells. Application of the same protocol to pancreatic β -cells, used as a positive control, resulted in inward-directed currents, verifying that our solutions and patch-clamp protocol were suitable for recording of VOCCs (Fig. 1C). These experiments demonstrated that human parathyroid cells do not express a functional VOCC in the plasma membrane. In addition, human parathyroid cells (both normal and adenoma) were voltage-clamped at 0 mV, and every 2 s, a voltage ramp from -80 to $+80$ mV was applied (rise time 400 ms). In the pipette solution, Cs^+ replaced NMG. As shown in Fig. 2A–C, addition of thapsigargin activated a non-selective cation current. The effect of thapsigargin on whole-cell current was rapid and slowly partly reversible (Fig. 2C).

Measurement of Ca_i^{2+}

Activation of SOCE was assessed by depletion of intracellular Ca^{2+} store with thapsigargin. In Fig. 3A, Ca^{2+} was withdrawn from extracellular solution, and

Figure 2 Patch-clamp measurement of SOC currents in human parathyroid cells. SOC currents were estimated using whole-cell configuration and voltage ramp from -80 to $+80$ mV every 2 s. Bath solution contained standard extracellular solution (see Materials and methods) supplemented with 17.4 mM CaCl_2 (final concentration 20 mM CaCl_2). (A) Ramp current before, during, and after the addition of 2 μM thapsigargin. (B) Summary of ramp current at -80 mV (indicated by arrow in A) in the absence and presence of thapsigargin ($n=3$). Plotting of ramp current at -80 mV showed a rapid and partly reversible effect on whole-cell current after the addition of thapsigargin (C). $**P<0.01$.

the cells were subsequently exposed to 1.5 mM Ca_o^{2+} , prior to and after the addition of thapsigargin. Addition of thapsigargin, in the absence of Ca_o^{2+} , caused a small increase in Ca_i^{2+} in $\sim 50\%$ of the cells studied. In all the experiments, a larger rise in Ca_i^{2+} was seen with 1.5 mM Ca_o^{2+} after exposure to thapsigargin. In Fig. 3B, 2-APB caused a transient increase in Ca_i^{2+} , followed by a lowering of Ca_i^{2+} . The lower level was kept even when 1.5 mM Ca_o^{2+} was added. A sharp elevation of Ca_i^{2+} was seen after the removal of 2-APB, suggesting that the effect of 2-APB on Ca^{2+} entry is reversible. YM-58483, also known as BTP-2, which has been shown to inhibit TRCP3 and 5, but not TRCP1, did not affect the elevation of Ca_i^{2+} caused by thapsigargin (Fig. 3C).

Gene expression of TRPC1, TRPC4, and TRPC6; Orail; and STIM1 in human parathyroid

RT-PCR products of expected lengths corresponding to TRPC1, TRPC4, and TRPC6 were detected in the normal parathyroid and adenoma samples used for screening of TRPC expression (Fig. 4A). No products were obtained for TRPC3, TRPC5, or TRPC7 in any of these samples. In support of the specificity and sensitivity of the assay, all TRPCs were readily amplified from human brain cDNA that was used as a positive control, while no product was detected in the RT-negative control. Furthermore, all PCR products were verified by sequencing, which revealed 100% identity to published human sequences.

Gene expression in parathyroid tissues was further analyzed for TRPC1, TRPC4, and TRPC6 in an extended series of 20 parathyroid adenoma and 2 normal parathyroid samples by TaqMan quantitative real-time PCR (Fig. 4B). Expression of Orail and STIM1 was similarly studied. All genes were found expressed in the normal parathyroid and adenomas studied (complete qRT-PCR results may be found in Supplementary Table 3). No statistical comparison was made between normal and adenoma samples since only two normal parathyroids were available for analysis, whereas the expression of TRPC1 was significantly larger than that of TRPC4 and TRPC6. These findings confirm the results obtained from RT-PCR screening, and further demonstrate that *TRPC1*, *TRPC4*, and *TRPC6*; *Orail*; and *STIM1* are the generally expressed genes in parathyroids.

Protein expression of TRPC1, TRPC4, and TRPC6; Orail; and STIM1 in parathyroid tissue

Protein expression of TRPC1, TRPC4, and TRPC6; Orail; and STIM1 was subsequently determined in parathyroid adenomas. Positive expression of the expected molecular weights was detected in all the 20

Figure 3 Recordings of Ca^{2+}_i using Fura-2. A step increase in Ca^{2+}_o from 0 to 1.5 mM caused an increase in Ca^{2+}_i . The response was much enhanced in the presence of thapsigargin (A). The effect of an increased Ca^{2+}_o was inhibited by 2-APB (B). The elevated level of Ca^{2+}_i after step increase in Ca^{2+}_o to 1.5 mM and the addition of thapsigargin was blocked by 50 μ M 2-APB but not by 5 μ M YM-58483 (C). At least five cells were tracked and recorded in each experiment in parallel, and at least three experiments were conducted for each protocol with a similar result.

Figure 4 (A) RT-PCR analyses demonstrating mRNA expression of TRPC1, TRPC4, and TRPC6 in human parathyroid. Agarose gel electrophoresis showing amplified products of the expected sizes for TRPC1 (620 bp), TRPC4 (356 bp), and TRPC6 (454 bp) in normal parathyroid (normal), parathyroid adenoma (adenoma), and human brain cDNA used as a positive control (control). (B) qRT-PCR analysis using Taqman showed expression of TRPC1, TRPC4, and TRPC6; Orai1; and STIM1 in both normal (black bar, $n=2$) and adenomatous (greyed bar, $n=20$) human parathyroid. The results are presented as mean $\pm$ s.d. and normalized to housekeeping gene *RPLP0* expression level. *** $P < 0.001$.

adenomas studied, i.e. 100 kDa for TRPC1, 95 kDa for TRPC4, 110 kDa for TRPC6, 30 kDa for Orai1, and 85 kDa for STIM1 (Fig. 5A–C and Supplementary Figure 1, see section on supplementary data given at the end of this article). The level of expression varied between the individual samples for all proteins. In the control experiments, the primary antibodies were pre-incubated with blocking peptides before incubation with the western blot membranes. In all cases, this resulted in loss of the expected protein product, thus confirming the specificity of the findings. Furthermore, total protein extracts from human uterus, rat brains, or rat pancreas served as a positive control, which revealed a strong signal at the expected molecular weight in all cases.

Demonstration of TRPC1–Orai1–STIM1 ternary complex in human parathyroid

Possible interactions between TRPC1, STIM1, and/or Orai1 were further examined by co-IP experiments. To verify a true association between the proteins, TRPC1, Orai1, or STIM1 antibodies were used as bait in the co-IP, and the same antibodies were then used to detect the proteins in each IP fraction. The IP fraction

Figure 5 Western blot analysis of TRPC1 (A) and TRPC4 (B) expression in a panel of parathyroid adenoma tissues and human uterus used as a positive control (C). A protein product of the expected size (~100 kDa, TRPC1, and ~95 kDa, TRPC4) was revealed, and the specificity was verified by peptide neutralization (peptide) of the antibody prior to membrane incubation. β-Actin served as a loading control, and 'N' denotes normal parathyroid tissue. (C) A representative western blot of TRPC6 (~110 kDa), Orai1 (~30 kDa), and STIM1 (~85 kDa) showing expression in parathyroid adenoma tissue. Numbers above western blot lane represent patient numbers. (D) Co-immunoprecipitation analysis showing interaction between TRPC1, Orai1, and STIM1. Each row under WB represents the result revealed from western blot using an appropriate antibody. Input indicates cell lysates without pull-down performance, e.g. 'IP:TRPC1' represents the immunoprecipitate using TRPC1 antibody to pull down interacting proteins using the same 'input' cell lysate as the starting material. Western blot analysis of TRPC1, TRPC4, TRPC6, Orai1, and STIM1 was performed using rabbit polyclonal anti-TRPC1 antibody (1:200), rabbit polyclonal anti-TRPC4 antibody (1:150), rabbit polyclonal anti-TRPC6 antibody (1:200), mouse monoclonal anti-Orai1 antibody (1:250), and mouse monoclonal anti-STIM1 antibody (1:100) respectively.

revealed a distinct signal at the expected weight from all the three proteins evaluated (Fig. 5D), but not from the mock IP (data not shown). The strongest signals in the IP fraction were revealed using Orai1 antibody as bait. Using STIM1 antibody revealed a clear but rather weaker signal compared with the other antibody that was used. The interaction was demonstrated and verified using any of the corresponding antibodies as bait. The co-IP was repeated twice with identical results. The concordant results from the co-IP experiment show that a TRPC1–Orai1–STIM1 ternary complex does exist in human parathyroid tissues.

Discussion

Intracellular Ca^{2+} is likely to have a pivotal role in the stimulus–secretion coupling in the parathyroid cells, since alterations in Ca_i^{2+} have a major impact on hormone secretion (Shoback *et al.* 1984, Mihai *et al.* 2000). Alterations of extracellular Ca^{2+} levels and addition of Ca^{2+} channel blockers have indicated that Ca^{2+} influx is probably an important source for

changes in Ca_i^{2+} . However, many studies have attempted to characterize the mechanism(s) controlling Ca_i^{2+} (Wallace *et al.* 1983, Muff *et al.* 1998), but the nature of the mechanism transporting Ca^{2+} into the parathyroid cells is still uncertain. One issue debated during the past two decades has been whether or not the parathyroid cells express a functional VOCC in the plasma membrane (Hove & Sand 1981, Wallace *et al.* 1983). High potassium, which decreases Ca_i^{2+} (Shoback & Brown 1984), stimulated PTH secretion, a finding which does not support the presence of VOCC, even though L-type calcium channel in parathyroid cells has been suggested (Chang *et al.* 2001, Yokoyama *et al.* 2009). In this study, we could not demonstrate any VOCC in parathyroid cells using a protocol that unequivocally demonstrates the presence of VOCC in the pancreatic β-cells. Therefore, non-selective cation channels that mediate SOCE have to be considered, even though the presence or absence of VOCC does not rule out the coexistence of non-selective cation channels.

SOCE is stimulated by depletion of Ca^{2+} from intracellular calcium stores, and is mediated by activation of specific plasma membrane channels,

termed SOCs. This activation produces a biphasic increase in Ca_i^{2+} , starting with a rapid Ca_i^{2+} release from ER stores, followed by a slower Ca_i^{2+} entry from outside the cell. This pattern, typical of PLC-coupled receptors (e.g. CASR), has also been demonstrated in parathyroid cells (Mihai & Farndon 2000). Thapsigargin, a specific inhibitor of Ca^{2+} -ATPase on intracellular stores, has been widely used as a tool to activate SOCE. Our electrophysiological studies showed a current which increased sixfold in the presence of thapsigargin, indicating the activation of SOCs. Even though thapsigargin irreversibly blocks Ca^{2+} -ATPase activity, whole-cell current decays with time, most likely reflecting channel 'run-down'. The electrophysiological findings, in combination with recordings of Ca_i^{2+} showing a large rise in Ca_i^{2+} by 1.5 mM Ca^{2+} after thapsigargin treatment, are a strong indication for the presence of SOCs in human parathyroid cells.

Interestingly, the characteristics of SOCs in different cell types are quite distinct, suggesting diversity in the channel components (Liu *et al.* 2004, Beech 2005, Parekh & Putney 2005). The canonical-type family of TRP channels is different from other groups of ion channels in displaying a huge diversity in ion selectivities, modes of activation, and the permeability to cations. All six members of the TRPC-type TRP channel (TRPC1, 3–7) have been proposed to be involved in both the receptor-operated and SOCE, which is linked to the PLC activation and inositol phospholipid breakdown signaling cascade (Pedersen *et al.* 2005). Emptying intracellular Ca^{2+} store with thapsigargin has been shown to activate Ca^{2+} influx through the plasma membrane presumably via TRPC (Ambudkar *et al.* 2007). Among the TRPC homologs most consistently associated with SOCs is TRPC1, which is required for SOCE in many cell types, e.g. salivary gland, keratinocytes, and smooth muscle cells (Liu *et al.* 2004, Cai *et al.* 2006, Dietrich *et al.* 2006). Overexpression of TRPC1 has been shown to have a pathological impact in other tissues, e.g. arterial smooth muscle (Bergdahl *et al.* 2005).

The CASR is expressed in plasma membrane, and is postulated to be the key player of Ca^{2+} signaling in the parathyroid cells (Brown *et al.* 1993). CASR is a G-protein, PLC-linked receptor, and has previously been shown to activate non-selective cation channels (e.g. TRPC) in breast cancer cells (Pedersen *et al.* 2005). A recent study has demonstrated the need for TRPC1 in the CASR-mediated Ca^{2+} oscillation in human embryonic kidney cells (Rey *et al.* 2006). Here, we demonstrated endogenous expression of TRPC1, TRPC4, and TRPC6 genes and proteins in normal parathyroid and pHPT. Concordant results were obtained by RT-PCR, qRT-PCR, and sequencing of PCR products. Expression of the corresponding protein was verified by western blot analyses. Blocking

experiments with corresponding immunizing peptides confirmed the specificity of our western blot findings. Since qRT-PCR results show that TRPC1 has a higher expression level compared with TRPC4 and TRPC6 in parathyroid cells, we assume that TRPC1 is likely to be a key protein for SOCs in human parathyroid cells. Since no specific TRPC1 channel blockers are available, 2-APB and YM-58483, two commonly used SOC blockers, were used. Recordings of Ca_i^{2+} show that 2-APB totally blocks Ca_i^{2+} entry in parathyroid cells, whereas YM-58483 has only a mild, if any, effect. 2-APB has been shown as a reliable SOC blocker (Bootman *et al.* 2002). It has no effect on VOCC, and has the capability to block different TRPC isoforms, including TRPC1, TRPC3, TRPC6, and TRPC7. YM-58493, on the other hand, is a new potential SOC blocker (He *et al.* 2005), shown to have blocking effect on TRPC3 and TRPC5 (Ishikawa *et al.* 2003). The lack of effect of YM-58483 on Ca_i^{2+} is most likely due to the absence of TRPC3 and TRPC5 in human parathyroid cells. The potent effect of 2-APB on Ca^{2+} entry further supports the existence of SOCs in human parathyroid.

A recent study has demonstrated that STIM1 and Orai1 are required for TRPC1-dependent SOC function, and that STIM1, located in junctional ER, is associated with Orai1 and TRPC1 in specific plasma membrane microdomains during activation of SOCE (Ong *et al.* 2007). In our study, we demonstrated an endogenous expression of the *Orai1* and *STIM1* genes and corresponding proteins in parathyroid tissues. To further clarify a putative interaction and thereby elucidate possible cellular signaling pathways, we performed co-IP using antibodies raised against TRPC1, STIM1, or Orai1. In all the three different co-IP setups, the TRPC1, STIM1, and Orai1 proteins were pulled down together. IP products were detected in all co-IP fractions at the expected size although at varying intensities. The strongest signal was revealed using the Orai1 antibody as the co-IP bait. A clear but weaker signal became visible using STIM1 antibody, probably due to low antibody-binding capacity. To evaluate the quantitative contribution of individual SOCs in the parathyroid, a comprehensive investigation is needed, in which the siRNA approach would seem to be best suited. The novel data presented here will provide a useful guide in this regard.

In summary, our study provides evidence for a close association between TRPC1, Orai1, and STIM1, which was demonstrated for the first time in human parathyroid.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1677/JME-09-0138>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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