CALCIUM TRANSPORT IN HAT-7 CELLS: A CELLULAR MODEL OF AMELOGENESIS

PhD thesis

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1. Introduction

Enamel, the hardest and most highly mineralized tissue in the human body, covers the crown of teeth and provides the mechanical resilience required for mastication. It is produced during tooth development by specialized epithelial cells, the ameloblasts, which disappear after tooth eruption. Mature enamel is acellular, avascular, inert, rigid, almost completely mineralized non-regenerative, with calcium as its predominant component. Consequently, one of the key functions of ameloblasts is the transport of calcium ions to the mineralization front. Although several Ca^{2+} channels and transporters have been identified in ameloblasts, the precise mechanisms governing Ca^{2+} transport during amelogenesis remain poorly understood. TRPM7 is a divalent cation channel, abundantly expressed in the enamel organ during tooth development and TRPM7 mutant mice show defective enamel mineralization suggesting a potential role in amelogenesis. During the maturation phase of amelogenesis - concurrent with the cyclic phenotype changes of ameloblasts - the pH in the enamel matrix oscillates between neutral and mildly acidic. Thus, it can be speculated that the acidic environment may modulate the Ca^{2+} transport process. Because ameloblasts are lost following eruption, modeling of transport dynamics requires in vitro epithelial models of polarized ameloblast-like cells. It was previously shown, that HAT-7 ameloblast cells can serve as a functional model of transepithelial bicarbonate transport in amelogenesis. This thesis summarizes my investigations into: (1) identifying the functional presence, Ca^{2+} conductance, and pH sensitivity of TRPM7 in HAT-7 cells to to clarify its putative role in Ca^{2+} uptake; and (2) examining the functional presence of purinergic and cholinergic receptors in 3D cultures of HAT-7 cells.

2. Aim and objectives

The aim of this thesis was to investigate changes of intracellular Ca^{2+} concentration in HAT-7 ameloblast cells under distinct conditions relevant to amelogenesis.

The objectives were as following:

1. To investigate the expression, functional presence and pH sensitivity of TRPM7 channels on cells grown in unpolarized culture
2. To study functional localization of TRPM7 channels in polarized cell monolayers
3. To investigate the effects of cholinergic and purinergic agonists on Ca^{2+} signal in 3D (spheroid) cell cultures

3. Methods

3.1 Cell culturing

3.1.1 Unpolarized monolayer culture

HAT-7 cells (205) were cultured in Dulbecco's modified Eagle's medium and Nutrient Mixture F-12 Ham medium (DMEM:F12; Sigma Aldrich) supplemented with 10% characterised fetal bovine serum (FBS; HyClone, Gibco), 100 U/ml penicillin and 100 mg/ml streptomycin (Sigma Aldrich) in standard conditions (37ºC, 5% CO_2). Subcultivation were performed in regular intervals using 0.25% trypsin-EDTA (Gibco).

3.1.2 Polarized monolayer (2D) culture

HAT-7 cells were grown on permeable polyester Transwell culture inserts with 0.4-μm pore size (Snapwell™ 12mm, Corning) for 2 days in standard culturing media (see above) followed by 3 days culturing in differentiation media (standard media supplemented with 2.1 M $CaCl_2$ and 10^{-5} mM dexamethasone, all Sigma). Transepithelial electrical resistance (TER) values were measured with an epithelial voltohmmeter (World Precision Instruments) daily, to monitor the paracellular permeability to electrolytes (barrier function), an important characteristic of secretory and absorptive epithelia.

3.1.3 3D (spheroid) culture

HAT-7 cells were grown within the gel layer of Matrigel® Basement Membrane Matrix (Corning). For seeding at low density to obtain single cell derived colonies within the Matrigel, approximately 80,000 HAT-7 cells were resuspended in 160 µl Hepato-STIM® (BD Biosciences), a commercially available epithelial selection medium, supplemented with 10% HyClone fetal bovine serum (Thermo Scientific), 1% l-glutamine (Sigma-Aldrich), 100 U/ml penicillin and 10 g/ml streptomycin (Bori et al., 2016). The cell suspension was mixed with 400 µl of Matrigel and 70 µl aliquots were placed in eight individual wells of a 24-well Nunclon Sphera low-attachment plate (Thermo Fisher Scientific). The plate was incubated at 37°C to allow the Matrigel to solidify, and then 1 ml of the appropriate medium was added to each well. The culture medium was changed every other day. HAT-7 spheroids were released from the Matrigel matrix with trypsin/EDTA and resuspended in physiological salt solutions for further experiments.

3.2 RT-qPCR

In case of HAT-7 cells, grown as monolayers, total RNA was isolated using a GeneJET RNA Purification Kit (Thermo Scientific). Total RNA was isolated from 7-days HAT-7 spheroids using a NucleoSpin RNA XS kit (Macherey-Nagel). After RNA integrity check (1% agarose gel), RNA was reverse transcribed using a Maxima First Strand cDNA Synthesis Kit (Thermo Scientific). qPCR amplification was performed using either a ABI StepOne System or QuantStudio 5 using TaqMan Universal Master Mix II (Applied Biosystems) and TaqPath qPCR Master Mix, CG (Applied Biosystems) respectively.

The following TaqMan assays were used (all Life Technologies):

- Klk4 (Rn01498536_m1)
- Cldn4 (Rn01196224_s1)
- Tjp1/ZO-1 (Rn02116071_s1)
- Cldn1 (Rn00581740_m1)
- Cldn8 (Rn01767199_s1)
- TRPM7 (Rn01328216m1)
- Slc4a2/AE2 (Rn00566910_m1)
- Slc26a4/pendrin (Rn00693043_m1)
- Slc9a1/NHE1 (Rn00561924_m1)
- Slc4a4/NBCe1 (Rn00584747_m1)
- CFTR (Rn01455971_m1)

For internal control acidic ribosomal protein P0 (RPLP0; Rn00821065_g1) was used; each sample was measured in technical triplicates. To calculate elative fold changes the comparative Ct method ($2^{-\Delta\Delta CT}$) were used.

3.3 Immunohistochemistry

HAT-7 cells were fixed using a solution of 95% ethanol and 5% acetic acid. Hemimandibles were removed from 6-week-old C57BL/6 mice and fixed in 4% paraformaldehyde, demineralized with 8% EDTA, embedded in paraffin, and sectioned at a thickness of 5 µm. Following blocking with GeneTex Trident Universal protein blocking reagent, the tissue sections were incubated overnight at 4°C with the primary antibody - Abcam rabbit anti-TRPM7 (ab262698) at a 1:300 dilution. Subsequently, the slides were incubated for 1 hour at room temperature with the secondary antibody, FITC-conjugated goat anti-rabbit IgG (Invitrogen, 1:400 dilution). Nuclear counterstaining was performed using 1 µg/ml Hoechst 33342 (Sigma) for 5 minutes at room temperature. As a negative control, non-specific rabbit IgG was applied. Imaging was carried out using a Leica TCS SP5 (Leica) confocal microscope.

3.4 Electrophysiology

Voltage-clamp recordings were performed using the standard whole-cell configuration with an Axopatch 200B amplifier (Axon Instruments). Micropipettes were fabricated from borosilicate glass

capillaries (Harvard Apparatus) using a P-97 Flaming-Brown micropipette puller (Sutter Instrument). When filled with internal solution, pipettes had a tip resistance of 3–6 MΩ. The internal (pipette) solution contained (in mM): 120 $CsCH_4SO_4$, 20 NaCl, 10 HEPES, 2 EGTA, and 0.9 $MgCl_2$, adjusted to pH 7.2 with NaOH. In specific experiments, the intracellular Mg^{2+} concentration was modified to either 3.6 mM or approximately 0 mM (nominally Mg^{2+}-free). The standard external (bath) solution consisted of (in mM): 140 NaCl, 2.8 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, and 11 D-glucose, adjusted to pH 7.3. Solutions were delivered via continuous perfusion at 3 ml/min. When pharmacological agents were applied, recordings were taken after full exchange of the bath solution.

Whole-cell currents were recorded at a holding potential of –50 mV and during 30 ms voltage steps ranging from -100 mV to +100 mV in 20 mV increments, applied at 0.5 s intervals. Currents were corrected for pipette and whole-cell capacitance, as well as series resistance. Current-voltage relationships were constructed using stabilized current values measured 10 ms after the onset of each voltage step. To normalize for cell size, whole-cell currents were divided by membrane capacitance (typically 20–30 pF) and expressed as pA/pF. Leak subtraction was not applied, as baseline currents at the holding potential under unstimulated conditions remained below 0.5 pA/pF. Reversal potentials typically ranged from –50 to –55 mV.

Data acquisition and command protocols were managed using pClamp 11 software (Axon Instruments). Capacitative transients were compensated using analog settings, and series resistance was accepted if it remained below five times the pipette resistance. Data were analyzed using Clampfit 11 (Axon Instruments) and Microsoft Excel. All recordings were conducted at room temperature.

3.5 Ca^{2+} imaging

For unpolarized monolayer experiments, cells were cultured on coverslips for 2–3 days. In 3D culture experiments, spheroids were grown in Hepato-STIM medium for one week, then released from Matrigel using 0.25% Trypsin/EDTA and seeded at low density onto 25 mm coverslips pre-coated with 0.01% poly-L-lysine (Sigma-Aldrich). Cells or spheroids were incubated in a loading solution containing 4 μM fura-2 AM (Invitrogen), 0.08% F-127 (Sigma), and 1 mM probenecid (Invitrogen) in bath solution for 45–60 minutes at room temperature, followed by rinsing prior to calcium imaging. Coverslips were then mounted in a custom-built open perfusion chamber on an upright fluorescence microscope (Nikon TE600, Nikon) and continuously superfused with bath solution composed of (in mM): 137 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, and 10 glucose, adjusted to pH 7.4 with NaOH. For HAT-7 monolayer experiments, Mg^{2+} was excluded from the bath solution to eliminate

its inhibitory effect on TRPM7 channel activity.In certain experiments, a nominally Ca^{2+}-free bath solution was used to assess the source of intracellular calcium ($[Ca^{2+}]_i$) responses.

In experiments involving HAT-7 cells cultured on Snapwell™ supports loading conditions were optimized to enhance fluorescence signal. Cells were incubated with 4 μM fura-2 AM, 0.08% F-127, and 1 mM probenecid in bath solution for 75–80 minutes at 37°C, followed by an additional 20–30 minutes in bath solution containing 1 mM probenecid at 37°C to allow de-esterification of the dye. All solutions were applied simultaneously to both the apical and basolateral sides. Probenecid was included to prevent rapid fura-2 efflux. The Snapwell™ inserts were then placed into a custom-built imaging chamber designed for separate perfusion of the apical and basolateral compartments and mounted on the same fluorescence microscope described above.

Cells were illuminated alternately at 340 and 380 nm using a metal-halide lamp and an internal filter wheel (Prior Lumen 220 Pro). Imaging was conducted using either a cooled CCD camera (Retiga2000; QImaging) or an sCMOS camera (Prime BSI, Teledyne Photometrics), controlled via NIS AR software (Nikon), through a water-dipping objective (20x, NA 0.5, Nikon). The advantage of upright imaging is the reduction of background noise caused by membrane autofluorescence. Intracellular calcium changes were expressed as the ratio of fluorescence emitted at the two excitation wavelengths (F340/F380), normalized to baseline. Quantification was performed by selecting a region of interest (ROI) encompassing at least 50 cells.

3.6 Intracellular pH measurements

Real-time monitoring of intracellular pH changes was performed using microfluorometry with the pH-sensitive fluorescent dye BCECF, as previously described. In brief, cells cultured on coverslips were incubated with 4 μM BCECF-AM (Thermo Fisher Scientific) for 30 minutes. Following loading, coverslips were placed in a chamber mounted on a Nikon Eclipse TE200 (Nikon) inverted fluorescence microscope and continuously superfused at a rate of 3 ml/min with the same bath solution used in patch-clamp recordings. Fluorescence signals were recorded at 530 nm emission while alternating excitation wavelengths between 490 nm (pH-sensitive) and 440 nm (pH-insensitive), using a photomultiplier tube and amplifier system (Cairn Research). Data acquisition was controlled by DASYLab software (Measurement Computing), and the fluorescence ratio (F490/F440) was calculated every 5 seconds. Autofluorescence correction was applied at the end of each experiment by releasing intracellular BCECF with Triton X-100. The F490/F440 ratio values were then converted to pH using calibration data obtained via the high K^+/nigericin technique.

3.7 Statistical analysis

Data are expressed as mean ± SEM. Statistical significance was assessed using one-sample Student's t-tests, planned pairwise comparisons (paired or unpaired Student's two-sample t-tests), and one-way ANOVA followed by Dunnett's post hoc test. A p-value < 0.05 was considered statistically significant.

4. Results

4.1 TRPM7-mediated calcium transport in HAT-7 cells

4.1.1 TRPM7 expression in HAT-7 cells cultured as unpolrized monolayer

Quantitative gene expression analysis revealed that TRPM7 mRNA was highly upregulated in HAT-7 cells compared to other ion transporters and channels associated with amelogenesis, such as NHE1, CFTR, and pendrin. Immunostaining using a polyclonal antibody targeting the intracellular C-terminal region of human TRPM7 confirmed the presence of TRPM7 protein in HAT-7 cells. In vivo, TRPM7 exhibited a similar predominantly cytoplasmic localization in mouse maturation-stage ameloblasts.

4.1.2 Intracellular Ca^{2+} response to TRPM7 activators/inhibitors

To assess whether TRPM7 channels contribute directly to Ca^{2+} uptake in HAT-7 cells beyond their proposed role in modulating store-operated calcium entry (SOCE) pathways ratiometric calcium imaging was used to directly measure intracellular Ca^{2+} responses. Cells were bathed a nominally Mg^{2+}-free extracellular solution to reduce competition between Mg^{2+} and Ca^{2+} for channel entry. Application of the TRPM7 activator naltriben (100 μM) resulted in a sustained and reversible increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) of 32.6 ± 3.3% ($n = 9$, $p < 0.05$). This increase was entirely eliminated in nominally Ca^{2+}-free extracellular environment, indicating that the rise in $[Ca^{2+}]_i$ was attributable to extracellular Ca^{2+} influx. Furthermore, co-application of the TRPM7 inhibitor NS8593 (20 μM) reduced the naltriben-induced $[Ca^{2+}]_i$ response by 56% ($n = 5$, $p < 0.01$).

In a separate set of experiments, stimulation with a different TRPM7 activator, mibefradil (50 μM) increased $[Ca^{2+}]_i$ by 52 ± 5% ($n = 12$, $p < 0.05$). Unlike naltriben, this response was only partially reduced by ~60% to 20 ± 2% ($n = 6$, $p < 0.004$) in a nominally Ca^{2+}-free extracellular solution, suggesting a partial involvement of intracellular store release. Depletion of intracellular Ca^{2+} stores with SERCA inhibitor thapsigargin (100 nM) completely abolished the mibefradil-induced $[Ca^{2+}]_i$ increase and even led to a transient decrease of 1.8 ± 0.2% below baseline ($n = 4$, $p < 0.05$).

4.1.3 TRPM7-like ion currents in HAT-7 cells

A well-established electrophysiological feature of TRPM7 channels is an outwardly rectifying cation current - typically recorded as a Cs^+ current - which is enhanced by depletion of intracellular Mg^{2+}. Whole-cell patch-clamp recordings from HAT-7 cells, conducted under conditions designed to suppress other ionic currents, revealed a modest outwardly rectifying current (4.7 ± 0.14 pA/pF at +80 mV, n = 14) when the intracellular pipette solution contained a physiological Mg^{2+} concentration (0.9 mM). Increasing intracellular Mg^{2+} to 3.6 mM led to a statistically significant reduction in current amplitude (3.6 ± 0.2 pA/pF at +80 mV, n = 14, $p < 0.05$). In contrast, complete removal of Mg^{2+} from the intracellular solution caused a pronounced, approximately six-fold increase in current (29.8 ± 2.4 pA/pF at +80 mV, n = 14, $p < 0.0001$).

Additional evidence were gathered using two established TRPM7 inhibitors: NS8593 and FTY720 which were tested on the Mg^{2+}-depletion-induced currents. In the absence of intracellular Mg^{2+}, application of 20 μM NS8593 reduced the current measured at +80 mV by approximately 60%, from 26.1 ± 3.0 to 10.4 ± 1.8 pA/pF (n = 5, $p < 0.05$) while treatment with 2 μM FTY720 produced an even greater inhibition, reducing the current by 90% (from 31.4 ± 5.2 to 3.4 ± 0.8 pA/pF, n = 5, $p < 0.001$).

The stimulatory effects of mibefradil and naltriben, were also tested: under physiological intracellular Mg^{2+} conditions (0.9 mM). Application of extracellular mibefradil (50 μM) led to an approx. tenfold increase in outward current, rising from 4.0 ± 0.4 to 41.5 ± 9.7 pA/pF at +80 mV (n = 5, $p < 0.05$). Increasing the intracellular Mg^{2+} concentration to 3.6 mM significantly attenuated this mibefradil-induced current by approx. 75%, reducing it to 10.4 ± 3.6 pA/pF (n = 4, $p < 0.05$).

Application of 50 μM naltriben also significantly enhanced the outward current at +80 mV, increasing it from 2.7 ± 0.3 to 26.7 ± 3.5 pA/pF (n = 4, $p < 0.0001$). This naltriben-induced current was largely suppressed by the TRPM7 inhibitor NS8593 (20 μM), which reduced the current by approx. 80% to 5.2 ± 0.8 pA/pF (n = 4, $p < 0.05$).

4.1.4 Effect of SOCE inhibiton on Ca^{2+} response triggered by different TRPM7 activators

Souza Bomfim et al. (2020) have recently suggested the involvement of store-operated calcium entry (SOCE) in TRPM7 activation-induced Ca^{2+} influx. Thus, responses to the TRPM7 activators were also investigated in the presence of the SOCE inhibitor BTP2.

To assess SOCE activity, HAT-7 cells were pretreated with thapsigargin in a calcium-free extracellular solution to deplete ER calcium stores and the effect of extracellular Ca^{2+} repletion was then measured in the presence and absence of BTP2. Pretreatment (20 μM for 45 minutes) with and

continuous application of BTP2 in the perfusate effectively inhibited SOCE-mediated calcium influx in unstimulated cells - resulting in a 92% reduction ($n = 3$, $p < 0.001$).

We then evaluated the impact of BTP2 on Ca^{2+} influx triggered by naltriben (100 µM) and mibefradil (50 µM) in the absence of prior ER store depletion. Under normal extracellular Ca^{2+} conditions, naltriben-induced Ca^{2+} influx was almost completely abolished by BTP2, showing an 88% reduction ($n = 4$, $p < 0.001$). In contrast, the Ca^{2+} influx elicited by mibefradil was completely unaffected by BTP2 ($n = 5$). These findings indicate that naltriben-induced Ca^{2+} entry is largely dependent on the SOCE pathway, whereas mibefradil-induced Ca^{2+} influx more likely occurs directly through the TRPM7 channel itself.

4.1.5 Effect of pH on TRPM7 currents and Ca^{2+} influx

During enamel maturation, hydroxyapatite crystal formation generates large amounts of protons and a cyclic acidification of the enamel matrix is observed. Therefore, we have also investigated the pH sensitivity of the observed TRPM7-related $[Ca^{2+}]_i$ responses and currents.

Whole-cell recordings from HAT-7 cells showed that lowering extracellular pH to 4.3 led to a seven-fold increase in outward current, from 6.6 ± 0.7 to 48.2 ± 4.6 pA/pF at +80 mV ($n = 4$, $p < 0.05$). This acidification-induced current was reduced by about half in the presence of the TRPM7 inhibitor FTY720 (2 µM), decreasing to 21.8 ± 1.3 pA/pF ($n = 4$, $p < 0.05$ vs. pH 4.3). A milder acidification from pH 7.3 to 6.3 - comparable to physiological conditions in maturation-stage ameloblasts - also caused a small but significant increase in current, from 4.3 ± 0.2 to 6.6 ± 0.6 pA/pF at +80 mV ($n = 5$, $p < 0.05$).

Furthermore, we have also tested the effects of both extracellular acidification and alkalization on $[Ca^{2+}]_i$. Acidification to pH 6.9 resulted a mild, but highly significant decrease in $[Ca^{2+}]_i$ (10 ± 1.6%, $n = 8$, $p < 0.001$), while a mild alkalinization (pH 7.9) increased the $[Ca^{2+}]_i$ by 33 ± 8% ($n = 5$, $p < 0.05$). However, such changes in the extracellular millieau did not alter the mibefradil-triggered $[Ca^{2+}]_i$ responses.

Since cytosolic acidification disrupted the stability of the membrane-glass seal, we were unable to study the effect of intracellular acidification on TRPM7 currents using whole-cell recording. Hence, we were able to assess the pH sensitivity of TRPM7-mediated Ca^{2+} influx using the ammonium prepulse technique, a well-established method of intracellular pH manipulation.

Application of 20 mM NH_4Cl for 2 minutes induced a transient intracellular alkalinization, followed by rapid acidification upon NH_4^+ withdrawal, as demonstrated by real-time BCECF fluorescence measurements. To prevent proton extrusion via Na^+/H^+ exchange, extracellular Na^+ was subsequently

replaced with the impermeant cation $NMDG^+$, leading to a sustained intracellular acidification to approximately pH 6.4. In separate calcium imaging experiments under the same conditions the NH_4^+-induced alkalinization caused a 35 ± 4.2% increase in $[Ca^{2+}]_i$ ($n = 9$, $p < 0.05$), which was abolished in a nominally Ca^{2+}-free extracellular solution (data not shown). The ensuing intracellular acidification triggered a modest but significant decrease in $[Ca^{2+}]_i$ of 12 ± 2% ($n = 6$, $p < 0.05$).

When applied under the intracellular acidification phase, both mibefradil (50 μM) and naltriben (100 μM) significantly enhanced the TRPM7-mediated Ca^{2+} influx beyond what was observed at normal pH_i. Specifically, mibefradil increased $[Ca^{2+}]_i$ by 78 ± 3.9% at low pH_i, compared to the 43 ± 6% measured at normal pH_i resulting an 81% relative increase ($n = 6$, $p < 0.05$). Similarly, naltriben induced a 46 ± 16% relative increase in Ca^{2+} influx under acidic conditions ($n = 5$, $p < 0.05$), suggesting that intracellular protons potentiate TRPM7 activation. In this context it is important to note that changes in extracellular pH from 7.3 to 6.3 - mimicking physiological fluctuations at the apical surface of ameloblasts - can also cause a moderate and reversible intracellular acidification of 0.38 ± 0.032 pH units after 10 minutes ($n = 6$, $p < 0.05$), as measured by BCECF fluorescence.

In summary, these findings demonstrate functional TRPM7 channels in HAT-7 ameloblasts and suggest that, beyond modulating SOCE, TRPM7 may potentially act as a pH-sensitive pathway for Ca^{2+} uptake.

4.1.6 Polarization of TRPM7 activity in HAT-7 cells

To determine the functional polarization of TRPM7 channels, we used HAT-7 cells cultured on Transwell membranes under differentiating conditions as previously reported by Bori et al. (2016) For calcium imaging experiments a custom-made chamber was constructed, that enabled the separate perfusion of apical and basolateral surfaces of Snapwell™ inserts under an upright fluorescent microscope.

Similarly, to previous experiments, fura-2 ratiometric calcium imaging was used to directly measure intracellular Ca^{2+} responses. Mibefradil (50 μM) was applied separately either on the apical or on the basolateral side. Stimulation on the apical side increased $[Ca^{2+}]_i$ by 20.1 ± 1.8% ($n = 4$, $p < 0.002$), while no significant increase was observed by basolateral application suggesting that TRPM7 channels are functionally present primarily on the apical side (apical v. basolateral, $n=4$, $p < 0.001$).

4.2 Purinergic and cholinergic signalization in 3D cultured HAT-7 cells

4.2.1 3D culture of HAT-7 cells

Cells suspension (Hepato-STIM media) of HAT-7 cells were mixed with Matrigel matrix and seeded in low-attachment plate wells. Spheroid development was monitored over a period of two weeks. After three days of culture a multicellular spherical appearance was observable and many spheroids reached 100 μm in diameter within one week. Interestingly, further prolongation of culturing period resulted in signs of disintegation. For further experiments, spheroids grown for one week in Hepato-STIM media were used.

4.2.2 Transporter expression profile of HAT-7 cells in 3D culture

RNA expression levels of several tight junction proteins (claudin 1, 4, 8, and ZO-1), electrolyte transporters (NHE1, AE2, NBCEe1, pendrin, CFTR) as well as maturation-stage ameloblast marker KLK4 were compared between 2D monolayer and 3D culture conditions. *KLK4* had the highest expression level in spheroids, also the increase was approximately 70-fold compared to the monolayer culture. Spheroid cultures showed significant downregulation of tight junction proteins claudin-1, claudin-4, and ZO-1, with a concomitant increase in claudin-8. Among HCO_3^- transporters, pendrin (*SLC26A4*) and *CFTR* were significantly reduced, while minor decreases in *NHE1*, *AE2*, and *NBCe1* expressions were not statistically significant.

4.2.3 Intracellular calcium responses to adrenergic and cholinergic stimuli

Previous studies have shown that electrolyte secretion of HAT-7 cells in 2D culture is activated by agonists known to mediate their effects by elevating intracellular cAMP and Ca^{2+}, such as forskolin and ATP, respectively. To test the presence of purinergic signalization in the spheroids, we measured the intracellular Ca^{2+} concentration $[Ca^{2+}]_i$ in response to purinergic agonists ATP (50 μM) and UTP (50 μM). Both ATP and UTP induced similar biphasic Ca^{2+} responses consisting of an initial transient peak and a sustained component provided that external Ca^{2+} (2 mM) was present. The absence of external Ca^{2+}, however, resulted only in transient Ca^{2+} responses for both agonists, suggesting that a Ca^{2+} influx from the extracellular space caused the sustained component. Since UTP does not stimulate ionotropic P2X receptors, we speculate that Ca^{2+} entry was due store-operated Ca^{2+} channels (SOCCs) activated by the depletion of intracellular Ca^{2+} stores.

Acetylcholine (ACh) has been reported to evoke $[Ca^{2+}]_i$ increase in freshly isolated rat ameloblasts. In our experiments, however, ACh analog, carbachol (100 μM), did not elicit $[Ca^{2+}]_i$ reponse

suggesting that neither muscarinic nor nicotinic functional ACh receptors are expressed in HAT-7 spheroids.

5. Conclusions

1. HAT-7 ameloblast cells showed strong TRPM7 expression at both mRNA and protein levels.
2. TRPM7 activators mibefradil and naltriben elicited Ca^{2+} influx that was further potentiated by intracellular acidification, suggesting the functional presence and pH sensitivity of TRPM7 channels in unpolarized HAT-7 cells.
3. HAT-7 cells exhibited TRPM7-like transmembrane ion currents measured in whole-cell configuration of voltage clamp experiments. The currents were responsive to both stimulation and inhibition of TRPM7 channels, as well as were potentiated by extracellular acidification.
4. In polarized HAT-7 cells mibefradil elicited a polarized Ca^{2+} response, suggesting that functional TRPM7 channels are present predominantly in the apical membrane.
5. Spheroid (3D cultures) of HAT-7 cells showed intracellular Ca^{2+} responses characteristic to metabotropic P2Y2 and/or P2Y4 receptors, while there was no evidence of functional muscarinic or nicotinic ACh receptors.

In summary, HAT-7 ameloblast cells abundantly express functional TRPM7 channels. Under specific conditions these channels possess Ca^{2+} conductance that is sensitive to changes in extra- and intracellular pH suggesting a potential connection between environmental pH changes and Ca^{2+} transport during amelogenesis. In HAT-7 cells cultured as a polarized monolayer, mibefradil-evoked Ca^{2+} signal is localized to the apical membrane suggesting polarized distribution of TRPM7 channel proteins. HAT-7 cells cultured as spheroids exhibited functional metabotropic purinergic signaling, whereas no evidence was found for the presence of functional cholinergic receptors. These findings suggest that HAT-7 ameloblast cells could serve as a suitable model for investigating calcium transport mechanisms during amelogenesis.

6. Bibliography of the candidates's publications

Publications related to the thesis

Kadar K, Juhasz V, Foldes A, Racz R, Zhang Y, Lochli H, Kato E, Koles L, Steward MC, DenBesten P, Varga G, Zsembery A. TRPM7-Mediated Calcium Transport in HAT-7 Ameloblasts. **INTERNATIONAL JOURNAL OF MOLECULAR SCIENCES** 22: 8 Paper: 3992, 14 p. (2021) SJR indikátor: D1, **IF: 6,208**

Foldes A, Sang-Ngoen T, **Kadar K**, Racz R, Zsembery A, DenBesten P, Steward MC, Varga G. Three-Dimensional Culture of Ameloblast-Originated HAT-7 Cells for Functional Modeling of Defective Tooth Enamel Formation. **FRONTIERS IN PHARMACOLOGY** 12 Paper: 682654, 14 p. (2021) SJR indikátor: Q1, **IF: 5,988**

∑IF: 12,196

Publications unrelated to the thesis

Toth BE, Takacs I, Valkusz Z, Jakab A, Fulop Z, **Kadar K**, Putz Z, Kosa JP, Lakatos P. Effects of Vitamin D3 Treatment on Polycystic Ovary Symptoms: A Prospective Double-Blind Two-Phase Randomized Controlled Clinical Trial. **NUTRIENTS** 17: 7 Paper: 1246, 20 p. (2025) SJR indikátor: D1, **IF: 5,0**

Koles L, Ribiczey P, Szebeni A, **Kadar K**, Zelles T, Zsembery A. The Role of TRPM7 in Oncogenesis. **INTERNATIONAL JOURNAL OF MOLECULAR SCIENCES** 25: 2 Paper: 719, 31 p. (2024) SJR indikátor: D1, **IF: 4,9**

Mathe D, Szalay G, Cseri L, Kis Z, Palyi B, Foldes G, Kovacs N, Fulop A, Szepesi A, Hajdrik P, Csomos A, Zsembery A, **Kadar K**, Katona G, Mucsi Z, Rozsa BJ, Kovacs E. Monitoring correlates of SARS-CoV-2 infection in cell culture using a two-photon-active calcium-sensitive dye. **CELLULAR AND MOLECULAR BIOLOGY LETTERS** 29: 1 Paper: 105, 16 p. (2024) SJR indikátor: D1, **IF: 10,2**

Toth BE, Takacs I, **Kadar K**, Mirani S, Vecsernyes M, Lakatos P. Safety and Efficacy of Loading Doses of Vitamin D: Recommendations for Effective Repletion. **PHARMACEUTICALS** 17: 12 Paper: 1620, 24 p. (2024) SJR indikátor: Q1, **IF: 4,8**

Sramko B, Foldes A, **Kadar K**, Varga G, Zsembery A, Pircs K. The Wisdom in Teeth: Neuronal Differentiation of Dental Pulp Cells. **CELLULAR REPROGRAMMING** 25: 1 pp. 32-44. (2023)

SJR indikátor: Q3, **IF: 1,2**

Zsembery A, **Kadar K**, Jaikumpun P, Deli MA, Jakab F, Dobay O. Bicarbonate: An Ancient Concept to Defeat Pathogens in Light of Recent Findings Beneficial for COVID-19 Patients? (26 Apr, 2026) Available at SSRN: https://ssrn.com/abstract=3589403 or http://dx.doi.org/10.2139/ssrn.3589403| Csak repozitóriumban hozzáférhető közlemény (Egyéb) | Tudományos

Fabian Z, **Kadar K**, Patonay L, Nagy K. Application of 3D printed biocompatible plastic surgical template for the reconstruction of a nasoalveolar cleft with preoperative volume analysis. **MATERIALE PLASTICE** 56: 2 pp. 413-415. (2019) SJR indikátor: Q3, **IF: 1,517**

Nagy K, Lang O, Lang J, Perczel-Kovach K, Gyulai-Gaal S, **Kadar K**, Kohidai L, Varga G. A novel hydrogel scaffold for periodontal ligament stem cells. **INTERVENTIONAL MEDICINE AND APPLIED SCIENCE** 10: 3 pp. 162-170. (2018) SJR indikátor: Q3

Foldes A, **Kadar K**, Keremi B, Zsembery A, Gyires K, Zadori S, Varga G. Mesenchymal Stem Cells of Dental Origin-Their Potential for Antiinflammatory and Regenerative Actions in Brain and Gut Damage. **CURRENT NEUROPHARMACOLOGY** 14: 8 pp. 914-934. (2016) SJR indikátor: Q1, **IF: 3,365**

Racz GZ, **Kadar K**, Foldes A, Kallo K, Perczel-Kovach K, Keremi B, Nagy A, Varga G. Immunomodulatory and potential therapeutic role of mesenchymal stem cells in periodontitis. **JOURNAL OF PHYSIOLOGY AND PHARMACOLOGY** 65: 3 pp. 327-339. (2014) SJR indikátor: Q2, **IF: 2,386**

Kiraly M, **Kadar K**, Horvathy DB, Nardai P, Racz GZ, Lacza Z, Varga G , Gerber G. Integration of neuronally predifferentiated human dental pulp stem cells into rat brain in vivo. **NEUROCHEMISTRY INTERNATIONAL** 59: 3 pp. 371-381. (2011) SJR indikátor: Q2, **IF: 2,857**

Kiraly M, Porcsalmy B, Pataki A, **Kadar K**, Jelitai M, Molnar B, Hermann P, Gera I, Grimm WD, Ganss B, Zsembery A, Varga G. Simultaneous PKC and cAMP activation induces differentiation of human dental pulp stem cells into functionally active neurons. **NEUROCHEMISTRY INTERNATIONAL** 55: 5 pp. 323-332. (2009) SJR indikátor: Q2, **IF: 3,541**

Kadar K, Porcsalmy B, Kiraly M, Molnar B, Jobbagy-Ovari G, Somogyi E, Hermann P, Gera I, Varga G. Humán fogbél eredetű őssejtek izolálása, tenyésztése és jellemzése [Isolating, culturing and characterizing stem cells of human dental pulp origin]. **FOGORVOSI SZEMLE** 102: 5 pp. 175-181. (2009) SJR indikátor: Q4

Kadar K, Kiraly M, Porcsalmy B, Molnar B, Racz GZ, Blazsek J, Kallo K, Szabo EL, Gera I, Gerber G, Varga G. Differentiation potential of stem cells from human dental origin - promise for tissue engineering. JOURNAL OF PHYSIOLOGY AND PHARMACOLOGY 60: Suppl 7 pp. 167-175. (2009) SJR indikátor: Q2, **IF: 1,489**

Molnar B, **Kadar K**, Kiraly M, Porcsalmy B, Somogyi E, Hermann P, Grimm WD, Gera I, Varga G. Emberi foggyökérhártya eredetű őssejtek izolálása, tenyésztése és jellemzése [Isolation, cultivation and characterisation of stem cells in human periodontal ligament]. **FOGORVOSI SZEMLE** 101: 4 pp. 155-161. (2008) SJR indikátor: Q3

∑IF: 41,255