

THESES FOR THE DEGREE OF DOCTOR OF PHYLOSOPHY (Ph.D.)

***SIGNIFICANCE OF TRANSIENT RECEPTOR POTENTIAL
VANILLOID-1 (TRPV1) IN THE STOMATOLOGICAL
PRACTICE***

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INTRODUCTION

Capsaicin-sensitive neurons

A distinct subpopulation of primary sensory neurons, which cell-bodies are located in dorsal root (DRG) and trigeminal sensory ganglia – based on their marked sensitivity to capsaicin and to other vanilloids (e.g., resiniferatoxin (RTX) isolated from the latex of *Euphorbia resinifera*) – are generally referred to as "capsaicin-sensitive neurons". As their afferent function (via their central axons), the cells transport sensory information to the central nervous system, whereas their efferent role is to, upon activation, locally release certain neuropeptides (such as substance-P, calcitonin gene-related peptide) stored in the peripheral termini. The released substances in turn act on other cells (such as keratinocytes, mast cells, macrophages or glia cells) and participate in the local trophic, vasoregulatory, and immunomodulatory processes, the abnormal, cascade-like enhancement of which may lead to neurogenic inflammation.

The cellular action of capsaicin

The cellular effect of capsaicin and related compounds can be well characterized as three consecutive phenomena on the sensory neurones. First is the *excitation* (developing right after the application of capsaicin during), during which the permeability of the cell membrane increases to (mostly) Ca^{2+} and Na^{+} ions and, therefore, leads to the depolarization of cells. The second characteristic process is the *desensitization*, which can be described as a decreased sensitivity to capsaicin and other (chemical, thermal or mechanical) nociceptive agents. Finally, capsaicin applied at high concentrations and/or for a long duration evokes *neurotoxicity*, which is mainly assigned to the increased intracellular calcium concentration ($[\text{Ca}^{2+}]_i$) and to a subsequent activation of calcium-dependent proteases.

The vanilloid receptor (VR1 or TRPV1)

In already 1968, Jancsó postulated the existence of a so called "pain receptor", which could "prove" the capsaicin selectivity of the sensory neurons. In 1997, the molecular description of the first capsaicin-sensitive specific molecule, the vanilloid (capsaicin) receptor (VR1) was published using a rat cDNA library. The rat VR1 is a 95 kDa protein consisting of 838 aminoacids encoded by 2514 nucleotids. On the basis of its structural features, i.e., the VR1 shows marked homology to the TRP (transient receptor potential) protein of *Drosophila melanogaster*, it belongs to the TRP receptor family (TRPV1). The common features of these receptors that they possess 6 transmembrane domains and intracellular N- and C-terminals, and, most probably, that they form tetramers in the membrane. It was also shown in various structural studies that both the extracellular and intracellular sides of the TRPV1 contain various binding sites for regulatory molecules. Similarly to the native TRPV1 on the sensory neurons, the cloned TRPV1 also functions as non-specific, chiefly Ca²⁺ permeable cation channel.

The central integratory role of TRPV1 in the development of pain sensation

TRPV1 can be activated not only by exogenous vanilloids but also by various endogenous compounds ("endovanilloids") such as the low-threshold heat (~43°C), the decrease of pH (acidosis), and several inflammatory mediators (bradykinin, extracellular ATP, arachidonic acid derivatives, leukotriens, products of lipid-peroxidation). These compounds, on the one hand, may directly activate the receptor by binding to the TRPV1 (heat, acidosis), or, on the other, upon binding to their mostly metabotropic receptors, may indirectly regulate the function of TRPV1 via the modification of intracellular signal transduction pathways (kinase systems, intracellular messengers). These effects may chiefly decrease the heat threshold of the TRPV1 and thus contribute to the development of thermal hyperalgesia.

Tramadol as a potential agonist of TRPV1

Tramadol is an effective analgesic substance widely used in medical practice. Its therapeutic action was mainly attributed to the activation of μ -opioid receptors and to the inhibition of serotonin and norepinephrine re-uptake by the synaptosomes. Interestingly, however, on various cultured neuronal cell populations, tramadol was also shown to inhibit the activity of voltage-dependent Na⁺ channels, delayed rectifier K⁺ channels as well as GABA(A) and NMDA ionotropic receptors. These data strongly argue for a more complex mechanism of action of tramadol.

Of further importance, data from numerous in vivo studies also suggest that tramadol may also exert a local anesthetic agent-like effect. In light of the above findings, in the current dissertation, we therefore aimed to investigate the effect of tramadol on one of the key molecules of nociception, i.e. TRPV1.

The expression of TRPV1 is not restricted to neuronal tissues

One of the most intriguing results of research on the TRPV1 field was the unambiguous identification of the presence of TRPV1 on numerous non-neuronal cell types. We and others have found that functional TRPV1 is expressed, for example, on various epithelial cells such as human skin keratinocytes, bronchial epithelium, urothelium, cells of the gastrointestinal tract, as well as on mast cells, glial cells, etc. Moreover, it was also shown that the activation of TRPV1 on these cells may result in changes e.g. in proliferation, apoptosis, differentiation, and/or cytokine release.

In relation to these mostly in vitro functional data on regulation of cell growth, it is also of great importance that TRPV1 is expressed at various levels in certain malignancies. For example, elevated TRPV1 expression was identified in carcinomas of the human prostate, colon, pancreas, or urinary bladder. Moreover, certain data also indicate that the level of TRPV1 may alter in relation to the degree of malignancy; e.g. positive (prostate cancer) or negative

(bladder carcinoma, glioma) correlations were equally found with increasing grades of the respective tumours.

Although sparse reports indicate that dietary capsaicin may inhibit tongue carcinogenesis in rats, intriguingly, we lack data on the existence of the “capsaicin receptor” TRPV1 on structures of the oral cavity which comprise the primary “target” of the regularly consumed TRPV1 agonist capsaicin.

The epidemiology of oral tumors

Tongue tumors pose a significant medical problem worldwide. Malignant growths arising from precancerous lesions (such as leukoplakia) compromise 30-40% of malignant oral tumors, 95% of which originate from the squamous epithelium. Although prevalence varies widely around the globe, both incidence and mortality is sadly on the rise in most countries. In view of these parameters Hungary is ranked last among European countries, since both mortality and incidence of lip and oral carcinomas is highest here. According to data from the Hungarian Central Statistical Office and the National Cancer Registry cancers of the lip and oral cavity (C00-C14) are ranked fifth among tumor localizations associated with high mortality. The mortality of oral carcinomas showed an appallingly dynamic growth at the end of the previous century by effectively quadrupling. Although the dramatic growth of mortality has ceased by the turn of the century, we have not managed to cause a significant decrease in this area in the last 7 years.

Oral squamous cell carcinomas are typically associated with poor prognosis. The five year survival rate of tumors diagnosed in an advanced stage has not improved significantly from the 50-55% value in spite of improvements in surgical techniques, chemo- and radiotherapy over the last decades. Unfortunately we lack easily applicable prognostic markers to track the disease’s progress and prognosis to this day. This is why great emphasis must be placed not only on screening and preventive programs but also on the discovery of diagnostic molecules, prognostic factors and chemo preventive agents that

could help in the early discovery and the assessment of aggressiveness as well as the planning and execution of the supportive treatment of surgical therapy.

RATIONALE AND AIMS OF THE STUDY

In the first phase of our experiments we examined the possible role of TRPV1 in the analgesic effect of tramadol, which is widely used in the stomatological practice.

1. At first we were interested if tramadol has any effect on the intracellular calcium homeostasis of a TRPV1 overexpressing heterologous expression system (TRPV1-CHO cells).
2. We were also interested if this effect happens through TRPV1, and if so if it is inhibitory or stimulatory in character.
3. We examined furthermore the specific TRPV1-mediated cell responses' kinetic parameters, comparing them with the transients elicited by capsaicin, the receptor's most well described agonist.
4. Finally we examined if the effect of tramadol is dose-dependent.

In the second phase of our experiments we analyzed the expression of TRPV1 in tongue tumors and their precancerous lesion (leukoplakia).

1. We first examined TRPV1 expression in both dorsal and ventral healthy human tongue epithelial cells.
2. Next we analyzed if the expression level of the receptor changes in precancerous lesions and different grade malignant tongue squamous epithelial carcinomas.
3. We furthermore evaluated if the changes in TRPV1 expression in tumorous tissue show any correlation with the tumor's histopathological attributes.
4. Finally, in search of a model for later functional tests, we examined the TRPV1 expression of a human tongue squamous epithelial carcinoma-derived cell line (CAL27).

MATERIALS AND METHODS

Expression system, cell culturing

The expression system was generated as cDNA of the rat TRPV1 was subcloned into pUHG102-3 and was transfected into CHO cells carrying the pTet Off Regulator plasmid (TRPV1-CHO cells). In these cells, expression of the pUHG plasmid (hence TRPV1) is repressed in the presence of tetracycline and is expressed upon removal of the antibiotic. Therefore, cells were routinely cultured in Ham F-12 medium. Before calcium imaging, cells were seeded on 25-mm glass coverslips or 96-well plates and were switched to tetracycline-free Ham F-12 medium and cultured at 34.5 °C for 48 h. To evaluate the efficacy of the induction of TRPV1 expression, Western blot analysis was performed.

The CAL27 cells were cultured in Dulbecco's Modified Eagle's Medium supplemented with 10 % foetal bovine serum, Glutamine and antibiotics. Medium was changed every other day and cells were subcultured at 80 % confluence.

Measurements of $[Ca^{2+}]_I$ using single cell calcium imaging

TRPV1-CHO cells were cultured on 25-mm glass coverslips and a calcium sensitive probe was introduced into the intracellular space by incubating the cells with 2 μ M fura-2 AM. Before each measurement, the cells were kept at room temperature in normal Tyrode's solution for 30 min to allow homogeneous distribution of the dye. The coverslips, containing the fura-2-loaded cells, were then placed on the stage of an inverted fluorescence microscope. Excitation was altered between 340 and 380 nm using a dual wavelength monochromator. The emission was monitored at 510 nm with a photomultiplier at an acquisition rate of 10 Hz per ratio, and the fluorescence ratio (F340/F380) values were determined. Cells were continuously washed by Tyrode's solution using a slow background perfusion system, whereas the agents investigated (capsaicin,

tramadol and capsazepine) were applied through a rapid perfusion system positioned in close proximity to the cell measured. Analyses of the $[Ca^{2+}]_i$ transients were performed by a PTI analysis program developed by us which measures 1) maximal amplitude of the transient above the baseline (in fluorescence ratio, F340/F380); 2) the time to peak value (time interval between the start of the application of the drug and the maximal value of the elevation, in s); and 3) the rate of rise value (slope of the ascending phase measured between the onset and peak of the transient, in ratio/s).

Microfluorimetric measurements of $[Ca^{2+}]_i$

Cells were seeded in 96-well black-well/clear-bottom plates at a density of 40,000 cells per well in Ham F-12 medium, supplemented as above, and cultured at 34.5 °C for 48 h. The cells were then incubated with Ham F-12 medium containing the cytoplasmic calcium indicator 2 μ M Fluo-4 AM at 34.5 °C for 40 min. The cells were washed four times with and finally cultured in Hank's solution containing 1% bovine serum albumin and Probenecid for 30 min at 34.5 °C. The plates were then placed to a FlexStation II384 fluorimetric image plate reader and changes in $[Ca^{2+}]_i$ (reflected by changes fluorescence; IEX=494 nm, IEM=516 nm) was recorded. When calculating dose-response curves, data were fitted to the Hill equation.

Immunocytochemistry and confocal microscopy

CAL27 cells growing on glass coverslips were fixed in acetone and blocked. Cells were first incubated with the appropriate anti-TRPV1 antibody and then with a fluorescein isothiocyanate (FITC)-conjugated IgG. Cell nuclei were counterstained by 4,6-diamidino-2-phenylindole (DAPI). Confocal microscopy images were acquired using a Zeiss LSM 510 microscope and images were stored for further analysis.

Human tissues

The study was approved by the Institutional Research Ethics Committee and written consent was obtained from all patients. Seven normal adult (healthy) tongue epithelial tissue samples were removed for routine diagnosis. The control patients had no history of pre- or malignant oral mucosal lesions. Eight epithelial leukoplakia lesions and 18 tongue SCC samples were involved in the study and were verified by histopathological evaluations by expert pathologists. Neither the leukoplakia patients, nor the SCC patients had previous or contemporary oral malignancies.

Human tissue sample preparation

In general, the fresh tissue specimens were divided into two parts. One part of the samples was fixed in 4% paraformaldehyde, embedded in paraffin, and processed for histopathology grading and for immunohistochemistry. From the second part epithelial tissue-enriched samples were made and frozen in liquid nitrogen. Finally the sections were processed for either quantitative “real-time” PCR (Q-PCR) or Western blot analysis.

Immunohistochemistry

The expression of TRPV1 was determined by horseradish-peroxidase (HRP) based method using diaminobenzidine (DAB) as a chromogene. In brief, paraffin-embedded sections, after antigen retrieval and endogen peroxidase block, were first incubated with a primary anti-TRPV1 antibody. Sections were then incubated with a HRP-polymer-conjugated secondary antibody. Immunoreactions were finally visualized using DAB-substrate and the sections were counterstained by hematoxylin. In addition, for negative controls of the labelling procedure, antibodies were either omitted from the procedure or were pre-absorbed by control blocking peptides provided by the manufacturers. For positive controls, human skin and prostate tissues were employed.

Image analysis

Immunohistochemical images were captured and digitalized. Digitalized images were then analyzed using Image Pro Plus 4.5 image analysis software. The intensity of TRPV1-ir was measured at 10 randomly placed, equally areas of interest (AOI) and the average of immunopositive pixels of the 10 AOI was determined.

Western blotting

Tissues and cells were homogenized in lysis buffer and the protein content of samples was measured. The samples were subjected to SDS-PAGE, transferred to nitrocellulose membranes, and then probed with the primary anti-TRPV1 antibody. HRP-conjugated secondary antibodies were then employed and the immunoreactive bands were visualized by enhanced chemiluminescence. To quantitatively assess the immunosignals, immunoblots were finally subjected to densitometric analysis using an Intelligent Dark Box and the Image Pro Plus 4.5.0 software.

Quantitative “real-time” Q-PCR

Q-PCR was carried out on an ABI PRISM 7000 Sequence Detection System by using the 5' nuclease assay according to our previous reports. Briefly, frozen tissues were pulverized under liquid N₂ and total RNA was isolated using TRIzol. Three µg of total RNA were then reverse transcribed into cDNA by using AMV reverse transcriptase and random primers. PCR amplification was carried out by using the TaqMan primers and probes using the TaqMan Universal PCR Master Mix Protocol. As internal controls, transcripts of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were determined, and the amount of TRPV1 transcripts were normalized to those of GAPDH using the Δ CT method.

Statistical analysis

Statistical analysis was carried out using SPSS software version 13. For statistical analysis, a two-tailed un-paired t-test was employed and $p < 0.05$ values were regarded as significant differences.

RESULTS

I.

I. The effect of tramadol on TRPV1

Tramadol induces transient elevation of $[Ca^{2+}]_i$ in TRPV1-CHO cells

We first investigated the effect of capsaicin on TRPV1-CHO cells. Confirming previous results, 1 μ M capsaicin induced a transient rise in $[Ca^{2+}]_i$ which, upon repeated applications (in a 300 s long interval), showed no tachyphylaxis. This effect was mediated by TRPV1 since capsaicin was unable to modify $[Ca^{2+}]_i$ on empty-vector expressing CHO cells (in contrast to ATP which elevated $[Ca^{2+}]_i$ on 73% of the cells investigated, $n = 11$) and since the TRPV1 antagonist capsazepine (5 μ M) effectively abrogated the action of capsaicin on TRPV1-CHO cells.

We then intended to investigate the effect of tramadol on the capsaicin-evoked $[Ca^{2+}]_i$ transients. However, intriguingly, we observed that 1 μ M tramadol alone induced a transient elevation in $[Ca^{2+}]_i$ which, again similarly to the action of capsaicin, was not observed on CHO cells lacking TRPV1. These data strongly argued for that tramadol, surprisingly, rather acted as a TRPV1 agonist in our system.

The tramadol-induced Ca-transients are distinct from those evoked by capsaicin and exhibit profound tachyphylaxis upon repeated applications.

To further assess this issue, we have characterized the effect of tramadol on a high number of TRPV1-CHO cells. Similar to capsaicin, 1 μ M tramadol was able to induce transient elevations in $[Ca^{2+}]_i$ in 72 % of the TRPV1-CHO cells investigated ($n = 41/57$). These transients were characterized by medium amplitudes (1.2 ± 0.1 elevation in the fluorescence ratio), time to peak values of 20.9 ± 1.2 s, and rate of rise values of 0.27 ± 0.04 ratio/s. Although these parameters were comparable to those observed with the application of 1 μ M

capsaicin, the maximal amplitude and rate of rise values were significantly smaller, whereas the time to peak values were significantly greater in the case of the tramadol-induced responses. In addition, we also found that a similar fraction of transients (76 % with capsaicin, 79 % with tramadol) returned to the baseline value after the cessation of administration of the drugs.

The most striking difference was found when we compared the phenomenon of tachyphylaxis. It is well-known, that in TRPV1-CHO cells, the repeated application of 1 μ M capsaicin resulted in an insignificant decrease in the amplitude of the subsequent $[Ca^{2+}]_i$ transients. In contrast, upon the repeated administration of 1 μ M tramadol (in 300 s intervals), the amplitude of the second $[Ca^{2+}]_i$ transient was 63.4 ± 5.4 % (n = 41) of the first (control) one, whereas the amplitude of the third $[Ca^{2+}]_i$ transient was 46.3 ± 3.8 % (n = 41) of the second one.

The effect of tramadol to increase $[Ca^{2+}]_i$ is mediated by TRPV1

To further assess the TRPV1-specificity of the tramadol-induced $[Ca^{2+}]_i$ responses, we also investigated the effect of the TRPV1 antagonist capsazepine. In these experiments, due to the above tachyphylaxis, the following protocol was employed. First, 3 consecutive 10 s-long 1 μ M tramadol “pulses” were administered, but now in 600 s intervals, and then the rate of tramadol-induced tachyphylaxis on numerous cells was determined. This relatively long inter-administration period was chosen since 1) as was shown above, it took approximately 2-300 s for the tramadol-induced $[Ca^{2+}]_i$ transients to return to the baseline; and 2) we intended to measure the effect of a 300 s-long capsazepine application. Statistical analysis has shown that, under these conditions, the amplitude of the second $[Ca^{2+}]_i$ tramadol-evoked transient was 76.6 ± 7.8 % (n = 10) of the first (control) one, whereas the amplitude of the third $[Ca^{2+}]_i$ transient was $78,2 \pm 8.4$ % (n = 10) of the second one.

Subsequently, we repeated the above protocol; however, in this case, 300 s after the initiation of the second tramadol “pulse”, cells were pre-incubated with 5 μM capsazepine for 300 s, and the third 1 μM tramadol challenge was administered in the presence of capsazepine. Consistent with the above findings, the presence of the TRPV1 antagonist almost fully abrogated the effect of tramadol to induce $[\text{Ca}^{2+}]_i$ elevation. Statistically, the amplitude of the third tramadol-induced $[\text{Ca}^{2+}]_i$ transients in the presence of capsazepine was only $12.7 \pm 2.8 \%$ ($n = 10$) of those (third) elevations which were recorded in the lack of the TRPV1 antagonist. Finally, this inhibition of the tramadol-induced responses by capsazepine was reversible since another tramadol application (600 s after the third one) again resulted in significantly higher $[\text{Ca}^{2+}]_i$ transients than those evoked in the presence of the antagonist.

The effect of tramadol is dose-dependent

Finally, we investigated the dose-dependence of tramadol on TRPV1-CHO cells. Due to the above marked tachyphylaxis, we were unable to employ the single-cell Ca-image technique to record the effects of various tramadol doses on the very same cell. Therefore, the measurement of the dose-response curve of tramadol was performed using FLIPR. Tramadol (similar to the single-cell data shown above) did not alter the $[\text{Ca}^{2+}]_i$ of control (empty-vector expressing) CHO cell. In contrast, on TRPV1-CHO cells, it was able to increase $[\text{Ca}^{2+}]_i$ in a dose-dependent fashion; mathematical analysis by fitting the measured values to the Hill equation resulted in an EC_{50} value of $0.08 \pm 0.03 \mu\text{M}$.

II. The expression of TRPV1 in various oral diseases

The expression of TRPV1 on epithelial cells of healthy human tongue

Using immunohistochemistry, a minor TRPV1-specific immunoreactivity (ir) was identified on epithelial cells of healthy (control) human tongue. On the

ventral surface of the tongue, this faint immunosignal was exclusively localized to the most upper layers of the stratum (str.) superficiale. However, on the specialized epithelium of the dorsal surface of the tongue, mostly intracellular TRPV1-ir (with characteristic granular pattern) was found in the basal epithelial cells of the str. basale.

The expression of TRPV1 increases in premalignant and malignant lesions of the tongue

Intriguingly, the epithelium of the premalignant leukoplakia samples exhibited an intense and characteristic TRPV1-ir when compared to the healthy tissues. On all cells of the str. basale and str. spinosum, TRPV1-ir was clearly localized to the cell membrane. Furthermore, this immunopositivity was also identified on degenerated cells of the str. superficiale as well as on the hyperorthokeratotic surface.

On human SCC samples, on tumour epithelial cells infiltrating the submucosa, we observed a markedly increased TRPV1-ir when compared to the control. It was also evident that, besides the weak surface membrane-localized immunosignals, the dominant TRPV1-specific staining pattern (i.e. intracellular/granular localization) highly resembled to those found on cells on the str. spinosum in the control tissues.

Investigation of the epithelium surrounding the tumour invasion revealed another intriguing phenomenon. Namely, on this thickened epithelium which still possesses the characteristic morphological appearance of that of the “healthy” tissues, TRPV1-ir was also dramatically increased (when compared to the control) on all layers of the epithelium. Basal cells of the str. basale and str. spinosum, similar to described above, exhibited intracellular/granular staining pattern. However, TRPV1-ir was rather localized to the cell membrane on cells of the more apical (str. spinosum and superficiale) layers. Moreover, the

intensity of TRPV1-ir gradually increased towards the surface and reached in maximal values in the upper layers of the str. superficiale.

The intensity of immunosignals was then quantitated by image analysis software. Comparison of the average intensity values revealed that TRPV1-ir was markedly and significantly higher in sections from leukoplakia, at the site of SCC invasion, and on the superficial epithelium surrounding the tumours when compared to those of the dorsal epithelium of the control samples (which exhibited the strongest immunosignals on the healthy tongue). In addition, mutual comparison of the diseased samples also defined significant differences between TRPV1-ir values of the precancerous leukoplakia and the SCC samples and of the epithelium surrounding the tumours.

Although these results clearly indicated the overexpression of TRPV1 in human tongue SCC, due to the rather semi-quantitative nature of the above technique, we also investigated the level of TRPV1 in SCC samples using Western blot (followed by quantitative densitometry analysis) and Q-PCR techniques. These two complementary techniques concordantly revealed that the expression of the TRPV1-specific mRNA transcripts and protein, yet exhibiting marked inter-individual variations, was higher in all tumour samples investigated. Statistical analysis of densitometry and Q-PCR values of all SCC samples indicated that this elevation was significantly different in the grade 1 and grade 2 SCC groups, when compared to the control. Although (at least) a similar increase in TRPV1 expression was also identified in grade 3 tumour samples, the low number of the available tumour samples ($n = 2$) made it impossible to perform statistical analysis. Of further importance, we found statistically insignificant differences when respective values of the tumour samples with various grades were mutually compared to one another.

The expression of TRPV1 increases confluence-dependently on the human SCC-derived cell line CAL27

Finally, we investigated the expression of TRPV1 at the cellular levels using the human SCC-derived cells line CAL27. As assessed by immunocytochemistry followed by confocal microscopy analysis, TRPV1-ir was localized both to the surface membrane and in the cytoplasm of the cells, similar to tumour cells in situ. In addition, quantitative Western blot and Q-PCR analyses also revealed that the expression of TRPV1 (both at the protein and mRNA levels) significantly and, of importance, gradually increased in parallel to the accelerated growth rate (hence the confluence) of the cell cultures.

DISCUSSION

Tramadol acts as an agonist of the TRPV1

Based on both the data in the literature and my own personal experiences in the University of Debrecen's Oral Surgical Department, the treatment of mandibular fractures and oral carcinomas (the removal of which often requires extensive head-and-neck dissection) postoperatively applied tramadol is extremely effective in the treatment of even such extensive surgeries. This extraordinary effective analgesic facility (beyond the activation of μ -opioid receptors) can also be explained by the simultaneous blockage of other voltage and ligand ion channels.

In this dissertation, we investigated the effect of a potent analgetic agent tramadol on the function of TRPV1. Using a heterologous expression system, we found that 1) tramadol significantly elevated $[Ca^{2+}]_i$ of TRPV1-CHO cells in a dose-dependent fashion; 2) its effect was reversibly prevented by the TRPV1 antagonist capsazepine; and 3) tramadol did not modify $[Ca^{2+}]_i$ in control (empty vector expressing) CHO cells. These findings strongly support the intriguing novel concept that tramadol, surprisingly, acts as an agonist of TRPV1.

As was detailed in the Introduction, tramadol (besides stimulating μ -opioid receptors) exerts a wide-array of inhibitory actions of numerous voltage- and ligand-gated neuronal channel populations, underlying its robust effect to mitigate pain. In light of these previous reports, our data presented in the current manuscript immediately invite a key question: How would the unexpected *activation* of the "pain-receptor" TRPV1 "fit" to the *in vivo* analgesic action "pattern" of this popular therapeutic agent? One straightforward explanation could be that the activation of TRPV1 by tramadol is rapidly followed by the desensitization of the sensory afferents (a phenomenon that is well-characterized by vanilloid administration to nociceptive neurons) which, in turn, would lead to the cessation of action potential firing and hence pain sensation. This idea may

be supported by that, in our system, tramadol induced a much stronger tachyphylaxis than capsaicin.

However, it is also well-established that the activation of TRPV1 also results in the local release of various peptides (e.g. substance P, calcitonin gene-related peptide) from the sensory ending. These neuropeptides, in turn, act on various neighboring cell types of the given tissue (e.g. mast cells, vessels, keratinocytes) and initiate numerous local regulatory mechanisms such as vasodilation, immunomodulation, cytokine and mediator release, etc. It is conceivable, therefore, that if tramadol (e.g. upon local application) stimulates TRPV1-expressing sensory afferents, the initiation of the “efferent” function of the nerve endings would result in such local responses.

As a support for this argument, in various human studies, local intradermal application of tramadol (besides inducing a local anesthetic effect similar to that of lidocain) resulted in skin erythema, flare, and urticaria. Of further importance, intradermal tramadol injection also initiated burning skin sensation and pain. Likewise, when the local anesthetic effect of tramadol was investigated following short (1 min) venous retention of the agent, in 31% of the patients, transient burning pain sensation and skin erythema developed distally from the place of occlusion along the affected veins. These *in vivo* results further argue for that tramadol may indeed activate TRPV1.

Collectively, our presented findings (along with the above *in vitro* and *in vivo* data) suggest that tramadol (besides the aforementioned multiple targets) may indeed act as a “classical” agonist of TRPV1. Namely, tramadol may first excite sensory neurons (calcium influx and transient burning pain sensation), then initiate neuropeptide release (skin erythema and flare), and finally induce desensitization (tachyphylaxis) and analgesia. Hence, although further studies (e.g. using gene deficient mice) are to be performed to exactly define the *in vivo* role of TRPV1 in mediating the action of tramadol, the presented concept of

“triple response” by tramadol may equally explain both the desired analgesic/anesthetic as well as the “unexpected” side-effects of the agent.

The possible role of TRPV1 in human tongue SCC

Previous studies have clearly identified the existence the “capsaicin receptor” TRPV1 on parietal cells of the human stomach, and on certain epithelial cell types of this organ system. However, to our best knowledge, our current study provides the first evidence that TRPV1 is expressed (both at the mRNA and protein levels) on the primary “target” of capsaicin, i.e. on epithelial cells of the human tongue.

Mutually complementary immunohistochemical, Western blot and Q-PCR analyses have also shown that the relatively low level of TRPV1 expression (localized mostly to the basal layers of the epithelium in healthy tongue tissues) was markedly increased in all grades of human tongue SCC samples. These intriguing data suggested that TRPV1 – similarly to findings on other epithelial cells such as human skin keratinocytes, bronchial epithelium, and urothelium – may participate in the growth control of the cells. This idea was further supported by showing that the expression of TRPV1 gradually increased with the accelerated growth rate of the human tongue SCC-derived cell line CAL27. According to the experiments performed since the publishing of the article that served as a basis of the current publication the TRPV1 agonists capsaicin really did influence the growth of CAL27 cells. Using an MTT-based colorimetric proliferation assay we determined that capsaicin dose-dependently decreased the cells’ proliferation. Since the MTT assay determines the living cell count based on the activity of the mitochondrial dehydrogenase enzyme it can be supposed that the activation of TRPV1 leads to the cell’s death.

Although further (both in vitro and in vivo) studies are invited to clarify the growth-modulatory role of TRPV1 (similar to work of Tanaka et al. (2002) suggesting that dietary capsaicin may inhibit tongue carcinogenesis in rats), our

findings identify TRPV1 as a novel, promising target molecule in the putative supportive treatment of human tongue SCC.

Intriguingly, the above evaluations have also revealed that the elevated TRPV1 expression in SCC tissues of all grades did not correlate with the degree of malignancy of the tumours. It appears therefore, that in contrast to findings on prostate and bladder carcinomas as well as on gliomas where definite correlations were described, TRPV1 may not serve as a prognostic factor in the clinics of human tongue SCC.

Nonetheless, several lines of evidence argue for that TRPV1 may rather act as a novel diagnostic molecule in human tongue transformation. Namely, in the current study, we also present that TRPV1 is highly overexpressed already in the grade 1 (low malignancy) SCC group. Moreover, of further importance, markedly elevated levels of TRPV1 were identified in the precancerous leukoplakia samples and also in the “healthy” epithelium surrounding the tumour invasion. Our hypothesis is supported by preliminary findings that show that lichen planus (which was considered to be a precancerous lesion, but is lately seen as a precancerous state) also expresses increased levels of TRPV1. Although the tendency of lichen to malignise is still disputed in the literature, these data suggest that the overexpression of the molecule may be a relatively early step in the process of tumour genesis; hence, determination of TRPV1 levels may hold out a promise for the benefits of early diagnosis.

Finally, we have also observed that – similar to other neuronal and non-neuronal cell types such as e.g. sensory neurons, mast cells, various skin cells, hepatoblastoma cells, – the specific TRPV1-ir was not restricted to the plasma membrane of the cells but intracytoplasmic staining patterns were also found. Moreover, here we also show that the subcellular localization pattern of the receptor is markedly different in the various diseased samples; e.g. mostly intracytoplasmic staining in the healthy epithelium and in the submucosal SCC islets whereas prominent surface membrane TRPV1-ir in the leukoplakia

samples, in the more superficial layers of the SCCs, and in the epithelium surrounding the SCC. Although further studies are invited to define the exact functional role of the intracellular TRPV1 in SCC-derived cells, these data argue for that TRPV1 may have a central role in the transformation of the epithelium of the human tongue leading to unwanted growth. This hypothesis is supported by previous findings showing that the intracellularly localized TRPV1 indeed functions as Ca-release channel and hence may act as a key regulator of cell morphology, viability, and migration.

Collectively, our current findings identify TRPV1 as a novel, promising target molecule in the supportive treatment and diagnosis human tongue SCC.

SUMMARY

In our current study we examined the role of the transient receptor potential vanilloid-1 (TRPV1) ion channel in the stomatological practice. In the first part of our experiments we wished to determine the effect of tramadol (a drug widely used in the clinical practice) on TRPV1. In our heterologous expression system, which is composed of TRPV1 expressing CHO cells, we observed that tramadol – similarly to capsaicin – dose-dependently increased the $[Ca^{2+}]_i$, while on the cells that did not express TRPV1 (cells transfected with an empty vector) we detected no change in $[Ca^{2+}]_i$. We also found that tramadol, in contrast with capsaicin, caused significant tachyphylaxis. It also became clear that the TRPV1 antagonist capsazepine markedly but reversibly blocked the changes in $[Ca^{2+}]_i$ caused by tramadol. These data suggest that the analgesic drug tramadol is capable of specifically activating TRPV1.

In the second half of our experiments we examined the expression of TRPV1 on tongue epithelial cells originating from healthy, leukoplakic and tumorous samples. We found that TRPV1 is expressed in healthy human tongue epithelial cells. We also found that TRPV1 expression is significantly increased in both the precancerous lesions and in different grade squamous cell carcinomas. It also became clear that the degree of increased TRPV1 expression shows no correlation with the tumor grade. We further showed that the TRPV1 expression of human tongue squamous cell carcinoma derived CAL27 cells increases in parallel with their confluence. Based on these data it can be hypothesized that TRPV1 plays an important part in the regulation of proliferation and survival of the cells. Our data also suggest that TRPV1 can be a new, promising target molecule in both the early diagnosis and supportive treatment of tongue squamous cell carcinomas.

PUBLICATIONS

The thesis was built on the following *in extenso* publications:

1. **Marincsák R.**, Tóth B.I., Czifra G., Szabó T., Kovács L., Bíró T. (2008):
The analgesic drug, tramadol, acts as an agonist of the transient receptor potential vanilloid-1. *Anesth Analg.* 106(6):1890-1896. **IF: 2,214**
2. **Marincsák R.**, Tóth B.I., Czifra G., Márton I., Rédl P., Tar I., Tóth L., Kovács L., Bíró T. (2009): Increased expression of TRPV1 in squamous cell carcinoma of the human tongue. *Oral Dis.* [Epub ahead of print] **IF:1,945**

Other in extenso publications:

1. Lázár J., Szabó T., **Marincsák R.**, Kovács L., Blumberg P.M., Bíró T. (2004): Sensitization of recombinant vanilloid receptor-1 by various neurotrophic factors. *Life Sci.* 75(2):153-163. **IF: 2,158**
2. Czifra G., Tóth I.B., **Marincsák R.**, Juhász I., Kovács I., Ács P., Kovács L., Blumberg P.M., Bíró T. (2006): Insulin-like growth factor-I-coupled mitogenic signaling in primary cultured human skeletal muscle cells and in C2C12 myoblasts. A central role of protein kinase Cdelta. *Cell Signal.* 18(9):1461-1472. **IF:4,881**
3. Bíró T., Tóth B.I., **Marincsák R.**, Dobrosi N., Géczy T., Paus R. (2007): TRP channels as novel players in the pathogenesis and therapy of itch. *Biochim Biophys Acta.* 1772(8):1004-1021. **IF:4,041**
4. Czifra G., Varga A., Nyeste K., **Marincsák R.**, Tóth B.I., Kovács I., Kovács L., Bíró T. (2009): Increased expressions of cannabinoid receptor-1 (CB1) and vanilloid receptor-1 (TRPV1) in human prostate carcinoma. *J Cancer Res Clin Oncol.* 135(4):507-514. **IF:2,366**

The total impact factors of the publications: **17,605**