Aus der Klinik für Augenheilkunde
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DISSERTATION

Analysis of expression and function of thermo-sensitive transient receptor potential channels in cultivated human conjunctival epithelial and human uveal melanoma cells

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1- Abstract

1-1- English

Corneal transparency maintenance is essential for normal vision. In dry eye disease (DED), which afflicts increasing number of individuals, patients experience discomfort due to ocular surface pain and inflammation. Hyperosmolarity of the tear film is one crucial pathogenic factor in the development of ocular surface inflammation in DES. Osmoprotective agents or modulation of putative osmosensitive receptors may help to prevent a hyperosmolar tear film from damaging the ocular surface. Promising receptor targets whose selective modulation may reduce inflammation and pain are transient receptor potential channels (TRPs) such as TRP vanilloid 1 (TRPV1; capsaicin receptor) and TRP melastatin 8 (TRPM8; menthol receptor). TRPs are non-selective cation channels and play a substantial role in Ca^{2+} regulation. This PhD thesis was undertaken to investigate functional TRPM8 and TRPV1 expression in human conjunctival epithelial cells (HCjEC) and ocular tumor cells such as uveal melanoma (UM). The specific aims are: 1) characterization of the osmoprotective agents through compatible solutes such as L-carnitine on Ca^{2+} regulation in HCjEC since previous data indicated a link to TRPs. 2) Similar investigations should be carried out using the thyroxine metabolites, so-called thyronamine (3-T_1AM). As a result, gene/protein and functional expression of TRPV1/TRPM8 could be detected in HCjEC and uveal melanoma cells. The functional expression of TRPM8 and TRPV1 was demonstrated by using the super cooling agent icilin (20 µM) and specific agonist capsaicin (CAP) (20 µM), respectively. During exposure to L-carnitine (1 mM), TRPV1 activation by hyperosmotic challenge (450 mOsM) or CAP suppressed Ca^{2+} influx and whole-cell currents in HCjEC. Furthermore, TRPV1 mediated shrinkage induced by exposure to a hyperosmotic challenge was blocked during L-carnitine exposure. In another set of experiments, extracellular application of 3-T_1AM (1 µM) led to an increase of intracellular Ca^{2+} in HCjEC which could be blocked by TRPM8 antagonist indicating TRPM8 activation by 3-T_1AM. Interestingly, the increases in TRPV1 functional activity induced by CAP were markedly blunted by pre-stimulating TRPM8 with 3-T_1AM. Furthermore, an endpoint of such signaling activation induced by CAP, namely, an approximate 2-fold increase in interleukin-6 (IL-6) release was clearly attenuated during exposure to 3-T_1AM. Therefore, inhibition of TRPV1 activity by possible endogenous modulators such as 3-T_1AM may suppress inflammation in DES as well as in UM. Taken together, TRPM8, TRPV1 as well as 3-T_1AM are interesting targets for the development of (adjuvant) therapies for DED and tumor diseases since (neoplastic) properties of (tumor) cells are determined by Ca^{2+}-dependent cellular mechanisms, which are regulated by TRPs.

1-2- German

Die Erhaltung der Hornhauttransparenz ist essenziell für den Sehvorgang. Beim „Trockenen Auge“, mit wachsender Prävalenz, haben die Patienten Beschwerden, die auf Schmerzen und Entzündung am Auge zurückzuführen sind. Angesichts limitierter Therapieoptionen, die in der Regel nicht die Ursachen beheben, sind besondere Maßnahmen erforderlich um Rezeptoren zu identifizieren, welche die Symptome hervorrufen. Vielversprechende Rezeptoren sind transient Rezeptor Potenzial Kanäle wie beispielsweise TRP Vanilloid 1 Rezeptor (TRPV1; Capsaicinrezeptor) und TRP Melastatin 8 Rezeptor (TRPM8; Mentholrezeptor), dessen Modulation Entzündung und Schmerz reduzieren können. TRP-Kanäle sind nicht-selektive Kationenkanäle und spielen eine substantielle Rolle bei der Calciumregulation. In dieser Doktorarbeit soll die funktionelle Expression von TRPM8 und TRPV1 sowohl in humanen Konjunktiva Epithelzellen (HCjEC) als auch in Augentumorzellen wie uvealen Melanomzellen untersucht werden. Die speziellen Ziele dieses Projekts sind: 1) Charakterisierung des Effekts von Osmoprotektiva mit kompatiblen Soluten wie L-Carnitin auf die Calciumregulation in HCjEC da Voruntersuchungen eine Verbindung zu den TRP-Kanälen hinweisen. 2) Ähnliche Untersuchungen sollen unter Verwendungvon Schilddrüsenhormonmetaboliten wie den sogenannten Thryronaminen (T_1AM) durchgeführt werden. Daraufhin konnte die Gen-, Protein- und funktionelle Expression von TRPV1 und TRPM8 in HCjEC und uvealen Melanomzellen nachgewiesen werden. Die funktionelle TRPM8 Expression konnte speziell mit dem pharmakologisch künstlich kühlenden Icilin (20 µM) und dem TRPM8-Blocker BCTC (10 µM) gezeigt werden. Bezüglich des Nachweises der funktionellen TRPV1 Expression wurde Capsaicin (CAP; 20 µM) verwendet. Die Vorbehandlung der Zellen mit L-Carnitin (1 mM) unterdrückte die über hypertonen Stress (450 mOsM) oder über CAP (20 µM) ausgelöste TRPV1 Aktivität. Außerdem blockte die Behandlung mit L-Carnitin die TRPV1-vemittelte Senkung des relativen Zellvolumens nach hyperosmolaren Stress. In einer weiteren Versuchsserie führte die extrazellulare Applikation von 1 µM T_1AM zu einer Erhöhung des intrazellulären Calciums in HCjEC, die durch BCTC geblockt werden konnte. Dies weist auf eine TRPM8 Aktivierung über T_1AM hin. Interessanterweise konnten die durch CAP aktivierten TRPV1-Kanäle über eine Vorbehandlung der Zellen über T_1AM-induzierte TRPM8 Aktivierung unterdrückt werden. Außerdem konnte die TRPV1-induzierte Interleukin-6 (IL-6) Freisetzung durch T_1AM deutllich abgeschwächt werden. Die Unterdrückung der TRPV1 Aktivität über mögliche endogene Modulatoren wie T_1AM könnten Entzündungsprozesse beim Trockenen Auge unterdrücken als auch das Wachstum von Tumorzellen wie den UM-Zellen inhibieren. TRPM8, TRPV1 sowie 3-T_1AM sind interessante Targets für die Entwicklung von (begleitenden) Therapien des Trockenen Auges und auch Tumorerkrankungen da zell- und tumorbiologische Eigenschaften durch Kalzium-abhängige Regulationsmechanismen geprägt sind, die von TRP-Kanäle reguliert werden.

2- Abbreviations

CAP	capsaicin
CB	cannabinoid receptor
CPZ	capsazepine
DES	dry eye syndrome
ELISA	enzyme-linked immunosorbent assays
HCEC	human corneal epithelial cells
HCEC-12	human corneal endothelial cells
HCjEC	human conjunctiva epithelial cells
GPCRs	G protein-coupled receptors
IL	interleukine
La^{3+}	lanthanum-III-chloride
LNCaP	lymph node carcinoma cell line of the prostate
MMP-9	matrix metallopeptidase 9
NHC	normal human conjunctiva
PCR	polymerase chain reaction
PIP2	Phosphatidylinositol 4,5-bisphosphate
TAAR1	trace amine associated receptor 1
3-T1AM	3-iodothyronamine
TH	thyroid hormone
TNF- α	tumor necrosis factor alpha- alpha
TRPs	transient receptor potential channels
TRPM8	transient receptor potential melastatin 8
TRPV1	transient receptor potential vaniloid 1
UM	uveal melanoma

3- Introduction

The ocular surface consists of several integrated structures working together to ensure optimal functioning of the eye. The most relevant structures are the conjunctiva, corneal epithelium and tear film. Conjunctiva consists of a multilayered membrane containing mucin-producing cells (goblet cells) responsible for maintaining a lubricated ocular surface. Corneal epithelium's structure and anatomic composition are essential for the quality of vision. Tear film is crucial for the integrity of the ocular surface by ensuring protection of the corneal epithelium from dehydration, protection against infection and regulation of corneal surface [1].

In the last decade, several observations detected expression of superfamily of ion channels known as transient receptor potential channels (TRPs) in the ocular tissue. Changing in osmolarity, mechanical and/or thermal stress as well as pH changes can lead to the activation of these channels. The members of TRP superfamily display extraordinary diverse activation mechanisms and participate in the plethora of physiological and pathological processes, which made them the focus of intense research in the recent years [2].

Decreased tear production or increased tear film evaporation can lead to pathophysiological changes in the ocular surface. They may result in the so-called "Dry eye syndrome" (DES). DES is one of the most common world-wide ocular health problems for which there are no therapeutic options that selectively target its pathogenesis [3]. One of the essential key factors in DES is an increase in tear osmolarity [4]. The hyperosmolarity of tear film induces shrinking of human corneal epithelial cells (HCEC) and may lead to ocular surface ulceration, tissue opacification and inflammation [5, 6].

Recently, two distinct TRP subtype channels have been documented to play an essential role in the pathophysiology of DES. Several studies demonstrated that changes in hypertonic challenge can lead to the activation of a member of TRP channels known as transient receptor potential vaniloid 1 (TRPV1; capsaicin- or heat receptor) in HCEC and human conjunctival epithelial cells (HCjEC) [7-10]. In HCEC, TRPV1 activation increases interleukin secretion which is associated with inflammation [9]. Patients suffering from DES also can experience cold-induced allodynia and increased tear fluid secretion. It has been suggested that these effects are associated with chronic exposure to hyperosmolar tears leading to

transient receptor potential melastatin 8 (TRPM8; menthol- or cold receptor) nociceptor upregulation on the nerve endings of the ophthalmic branch of trigeminal nerves [11].

Concerning these TRPs, it is known that they are also activated by G protein-coupled receptors (GPCRs), adrenergic receptors, tyrosine kinases [12], other TRPs [13, 14] as well as hormones.

Recently, 2 TRP channels (TRPV1 and TRPM8) have been documented to play an essential role in the pathophysiology of DES. Several studies demonstrated that changes in hypertonic challenge can lead to the activation of a member of TRP channels known as transient receptor potential vaniloid 1 (TRPV1; capsaicin- or heat receptor) in HCEC and human conjunctival epithelial cells (HCjEC) [7-10]. This activation in HCEC increases interleukin secretion which is associated with inflammation [9]. Patients suffering from DES also can experience cold-induced allodynia and increased tear fluid secretion. It has been suggested these effects are associated with chronic exposure to hyperosmolar tears leading to transient receptor potential melastatin 8 (TRPM8; menthol- or cold receptor) nociceptor upregulation on the nerve endings of the ophthalmic branch of trigeminal nerves [11].

TRP channel-elicited responses are modulated by their interactions with other receptors such as G protein-coupled receptors (GPCRs), adrenergic receptors, tyrosine kinases [12], other TRPs [13, 14] as well as hormones.

Treatment for DES depends on the severity of the condition. Ocular lubricants are often successful in ameliorating symptoms, especially in mild cases. Although these therapeutic approaches provide symptomatic relief in dry eye disease patients by lowering their tear osmolarity [15, 16], development of drugs that can effectively suppress receptor-mediated inflammation is limited. One strategy for an alternative treatment of patients with DES is addition of the compatible solutes into topical formulations of artificial tears in order to compensate hypertonic condition in DES. L-carnitine is one such compatible solute, due to its documented osmoregulatory activity [17]. So far, the underlying mechanisms of L-carnitine are not fully elucidated [18, 19]. It is known that TRPV1 is an osmosensor and can be stimulated during exposure to the hypertonic conditions. In this study it is postulated that the osmoprotective effects of L-carnitine against hypertonic-induced cell volume shrinkage are elicited through its interaction with TRPV1.

Since in some tissue, TRPM8 activation leads to suppression of TRPV1 stimulation [14] , in this study, we suggested cooling agents may have therapeutic value in reducing inflammation. Treatment with the TRPM8 agonist such as icilin led to a suppression of the TRPV1-induced Ca^{2+} influx in retinal tumor cells [13]. First pilot studies using 3-T1AM indicated that it may activate TRPM8 like icilin in hepatocyte carcinoma cells (HEPG2) (unpublished observation). Therefore, such hormone may be an interesting alternative to activate TRPM8.

TAMs are decarboxylated thyroid hormone (TH) derivatives. One of them is 3-iodothyronamine (3-T1AM), which acts as a potent agonist of trace amine associated receptor 1 (TAAR1) [20]. In contrast to THs, 3-T1AM leads to severe metabolic depression such as hypothermia [21, 22]. Such an effect in the eye is associated with increases in basal tear flow and TRPM8 channel activation [11]. TAARs are endogenous receptor(s) for 3-T1AM and member of an orphan G-protein coupled receptor family [23].There is accumulating evidence that TRP channel-elicited responses are modulated by their interactions with other receptors such as GPCRs and most likely TAARs are also involved in TRPs modulation.

Uveal melanoma (UM) is both the most common and fatal intraocular cancer among adults worldwide. As with all types of neoplasia, changes in Ca^{2+} channel regulation can contribute to the onset and progression of this pathological condition. Inflammation is an important characteristic of UM and associated with bad prognosis of this disease [24, 25]. In part of this thesis, the co-expression of TRPM8 and TRPV1 in UM 92.1 cells was investigated. The crucial role of these channels in inflammatory responses may provide novel drug targets to reduce inflammation in this aggressive neoplastic disease.

4- Aim of the study

Based on the results of previous above mentioned studies, the three following aims of this thesis were proposed:

1. Characterization of gene and functional expression of TRPV1 and TRPM8 in HCjEC and UM.

2 Characterization of TRPM8 and TRPV1 co-expression in non-tumor (HCjEC) and tumor eye cells (UM) and their possible interplay.

3. Investigation of the effect of possible endogenous modulators of TRPs such as of the osmoprotectant L-carnitine and the metabolite of thyroid hormone 3-T1AM on Ca^{2+} regulation and whole-cell currents in HCjEC cells.

5- Methods

- *IOBA-NHC cell line* was used from established immortalization of normal human conjunctival epithelial cells [26]. Uveal melanoma cell a line 92.1 was also an established immortalized cell lines of UM was used in this study [27].
- *Fluorescence $[Ca2+]_i$ measurement* was performed to detect very small changes in intracellular calcium concentration. Fluorescence cell imaging was performed on cells loaded with the fluorescent Ca^{2+} indicator dye fura-2. With this dye, increases in fluorescence intensity are indicative of declines in the function in intracellular calcium regulatory mechanisms [28].
- *Patch-clamp technology* detects the changes in membrane ionic permeability and ion channel activity that precede those underlying losses in calcium homeostatic capacity. With this technique using the whole-cell configuration, drugs were applied to one single cell to assess their effect on baseline membrane permeability and channel activity [29, 30].
- *Relative cell volume:* The effect of hypertonic challenges and drugs on relative cell volume was monitored in calcein-AM loaded cells. Optical conditions were used in which there was an inverse relationship between relative increases in cell volume and increased fluorescence intensity[31].
- *ELISA:* In enzyme-linked immunosorbent assays an antigen was immobilized to a solid surface and then complexed with an antibody that was linked to an enzyme. The detection was accomplished by assessing the conjugated enzyme activity via incubation with a substrate to produce a measureable product.
- The *statistical analysis* was performed with GraphPad Prism version 5.0 and SigmaPlot software version 12.5 and the mean values are reported as means ± SEM.

6- Results

6-1- TRPV1 and TRPM8 gene and protein expression in IOBA-NHC and UM 92.1 cells

RT-PCR and quantitative real-time PCR analysis confirmed TRPV1 gene expression in IOBA-NHC as well as UM 92.1 cells. Furthermore, TRPM8 gene expression could be detected for the first time in both cell lines. Specifically, the anticipated PCR products for TRPV1 (285 bp) and TRPM8 (621 bp) were detected in this thesis and were identical to those found in different cell lines used as positive controls. In this thesis TRPV1 gene expression also was

detected in healthy human uvea. While, no TRPM8 gene expression was detected in this cell type. As positive controls, human corneal endothelial cells (HCEC-12)[32], human corneal epithelial cells (HCEC) and the prostate carcinoma cell line LNCaP were used in which TRPV1 and TRPM8 gene expression is documented [33, 34]. Moreover, quantitative real-time PCR (qPCR) confirmed TRPV1 and TRPM8 expression in all aforementioned cell types. In addition, immunofluorescence staining confirmed corresponding TRPV1 and TRPM8 protein expression. The immunostaining pattern indicates that this expression in both cases is detectable in plasma membrane as well as cytoplasmic region.

6-2- Functional TRPV1 and TRPM8 expression in IOBA-NHC and UM 92.1 cells

To assess TRPV1 function in IOBA-NHC cells, Ca^{2+} influx was measured using the selective TRPV1 agonist capsaicin (CAP; 20 µM) and a hypertonic challenge (450 mOsM). CAP as well as hypertonic challenge induced an irreversible increase in the f_{340}/f_{380} ratio indicating a rise of intracellular Ca^{2+} concentration. Preincubation with the selective TRPV1 channel blocker capsazepine (CPZ; 20 µM) negated the rise in $[Ca^{2+}]_i$.

To investigate the functional expression of TRPV1 in UM 92.1 cells heating and CAP were used. Elevating the bath temperature to more than 43 °C as well as using CAP (20 µM) caused the large increase in f_{340}/f_{380} ratio. Preincubation with non-selective TRP channel blocker La^{3+} (500 µM) and CPZ (10 µM) prevented this response.

Functional TRPM8 activity was demonstrated based on moderate cooling of the bath solution (below 23 °C). In addition, the TRPM8 cooling agent icilin (20 µM) was used in the pharmacological approach. Moderate cooling as well as application of icilin increased the f_{340}/f_{380} ratio. This increase could be clearly suppressed by the specific TRPM8 blocker BCTC (10 µM), indicating functional TRPM8 expression in both IOBA-NHC and UM 92.1 cells.

6-3- Effect of L-carnitine on TRPV1-mediated Ca^{2+} regulation and whole-cell currents

To characterize TRPV1-mediated Ca^{2+} regulation, further calcium imaging experiments were carried out and CPZ was replaced with L-carnitine. Interestingly, preincubation with L-carnitine (1 mM) for 15 to 60 min in isotonic medium had the same effect as CPZ on the hypertonic challenge. The fluorescence ratio decreased in the presence of L-carnitine following exposure to CAP and hypertonic challenge. This equivalence between the

inhibitory effects of L-carnitine and CPZ on the $[Ca^{2+}]_i$ suggests that L-carnitine and CPZ elicit $[Ca^{2+}]_i$ reversal through interacting with TRPV1.

To further characterize the effect of L-carnitine another high-sensitive functional assay such as the planar patch-clamp technique was used. After hyperpolarization to -60 mV (from a holding potential of 0 mV) in the whole-cell configuration, 20 µM CAP strongly increased inward currents confirming Ca^{2+} influx since the internal bath solution is Ca^{2+} free and 1.5 mM Ca^{2+} was in the external solution. After depolarization to +130 mV, typical TRP-like outwardly rectifying currents could be detected which significantly increased in the presence of CAP. In contrast, in- and outward currents could be suppressed in the presence of L-carnitine (1 mM) in the external solution. Therefore, L-carnitine clearly suppressed increases in whole-cell currents mediated by TRPV1 activation (Fig. 1).

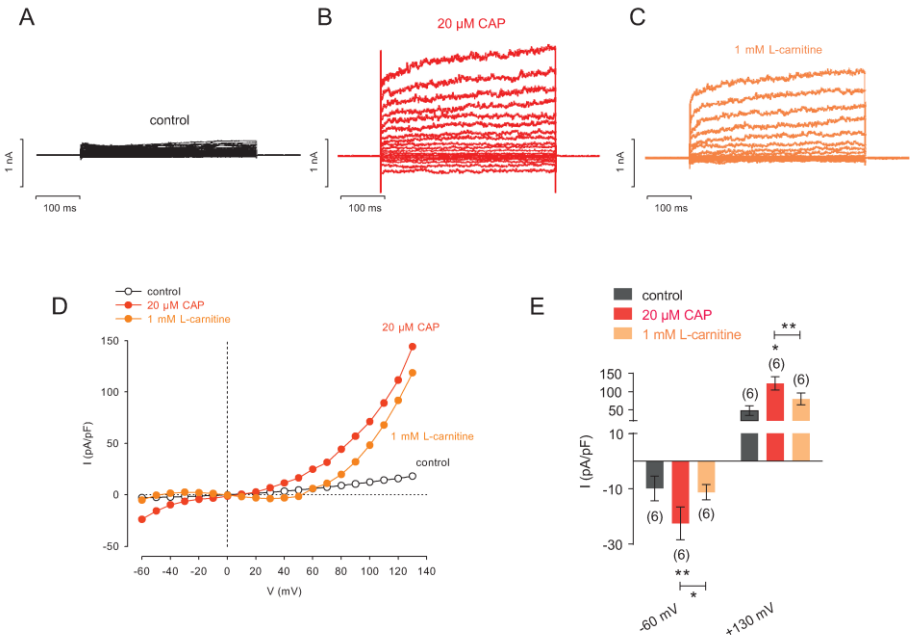

Fig. 1. Effect of CAP and L-carnitine on whole-cell currents. A-B: Whole-cell currents following voltage stimulation from -60 to +130 mV in 10 mV steps for 400 ms each. 20 µM CAP increased in- and outward whole-cell channel currents. C: 1 mM L-carnitine suppressed both in- and outward currents. D: Effects of CAP and L-carnitine are summarized in a current/voltage plot (I/V plot). For the current/voltage relation, maximal peak current amplitudes were plotted against the voltage (mV). The upper trace (red filled circles) was obtained in the presence of 20 µM CAP and the lower trace (orange filled circles) in the presence of 1 mM L-carnitine. Controls without application of drugs had no effect on whole-cell currents (open circles). E: Summary of the experiments with CAP and L-carnitine (n = 6) [7].

6-4- Effect of L-carnitine on cell volume regulation

The effects of L-carnitine on relative cell volume were monitored in calcein-AM loaded cells. Exposure to 400 mOsM led to the declining signal indicative of cell shrinkage due to dye quenching. While during the exposure to 1 mM L-carnitine, the signal remained unchanged suggesting that this osmolyte is internalized to equilibrate extracellular and intracellular osmolarity. To assess the involvement of TRPV1 in this osmoregulatory response to a hypertonic challenge, it was determined whether L-carnitine in TRPV1 gene silenced cells affected the decline in fluorescence intensity induced by exposure to a hypertonic challenge. Relatively large cell volume shrinkage was detected in TRPV1 gene silenced cells and the best protection against shrinkage was obtained in non-transfected cells exposed to L-carnitine (Fig. 2)

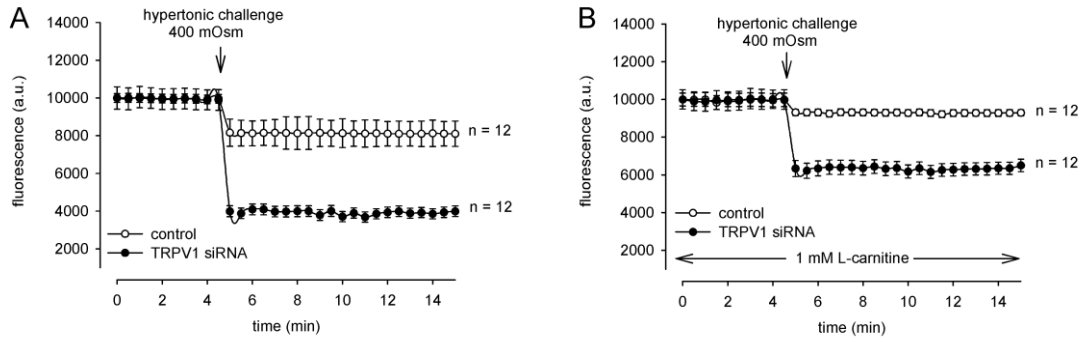

Fig. 2. L**-carnitine effect is associated with TRPV1.** A: Reduction of the cell volume in TRPV1 siRNA treated cells (filled circles) was at larger levels compared to controls (both n = 12; open circles). B: Same experiment as shown in A, but in cells pre-incubated in 1 mM L-carnitine. This clearly reduced the hypertonicity induced reduction in cell volume [7].

6-5- Effect of 3-T1AM on TRPM8-mediated Ca^{2+} regulation and whole-cell current

Similar to icilin, 3-T1AM (1 µM) increased intracellular Ca^{2+} levels. In contrast, preincubation with BCTC (10 µM) abolished this effect. Therefore, it is suggested that 3-T1AM exposure leads to TRPM8 activation. In order to assess if there is a correspondence between the effects of 3-T1AM on the whole-cell currents, the planar patch-clamp technique was again used. 3-T1AM (1 µM) augmented in- and outward currents in whole-cell configuration. This effect was fully blocked during exposure to BCTC (Fig. 3).

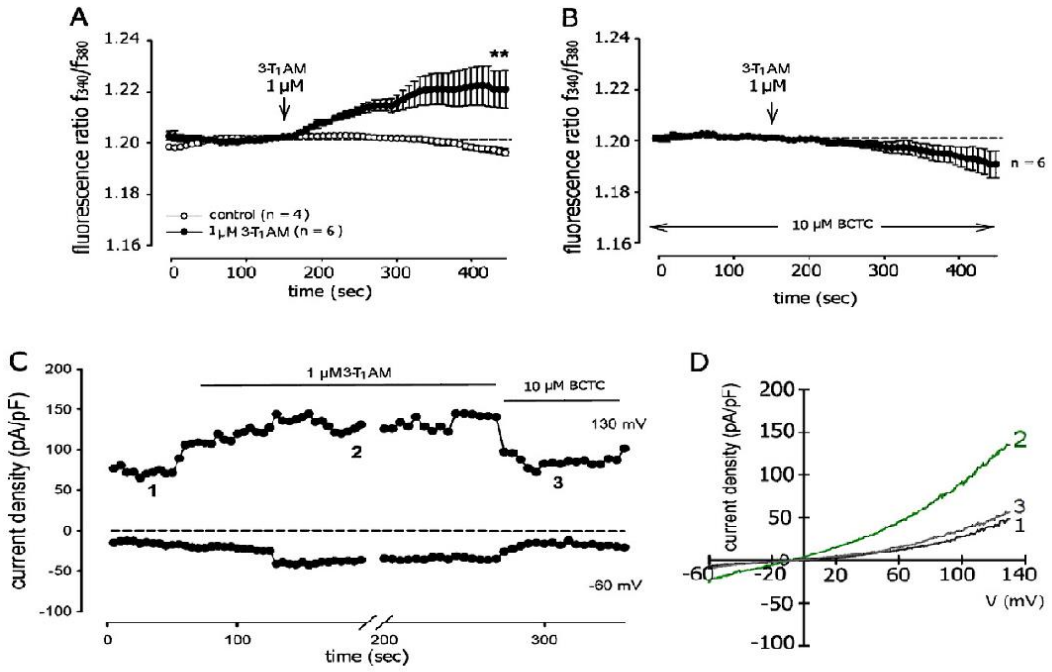

Fig. 3. 3-T1AM increased Ca^{2+} influx and whole-cell currents in IOBA-NHC cells and BCTC blocks this effect. A: 1 µM 3-T1AM induced a Ca^{2+} entry (n = 6; filled circles).Without 3-T1AM application, no changes in Ca^{2+} influx could be observed (n = 4; open circles). B: 10 µM BCTC suppressed the 3-T1AM -induced Ca2+ influx (n=6). C: Time course of whole-cell currents at −60 mV (lower trace) and 130 mV (upper trace) showing the current activation by 1 µM 3-T1AM in IOBA-NHC. The currents were normalized to capacitance to obtain current density (pA/pF). D: Original traces of 3-T1AM activated current responses to voltage ramps from −60 mV up to +130 mV (500 ms, with leak current subtraction) in the whole-cell configuration of the planar patch-clamp technique. Currents are shown before application (labeled as 1) and during application of 1 µM 3-T1AM (labeled as 2) and in the presence of 10 µM BCTC (labeled as 3)[35].

6-6- Icilin and 3-T1AM suppress CAP-induced Ca^{2+} influx

TRPV1 and TRPM8 are co-expressed in IOBA-NHC cells and TRPM8 activation is reported to suppress TRPV1 activation [14]. In this set of experiments, it could be shown that TRPV1 activation by CAP elicited an irreversible and large increase in f_{340}/f_{380}**.** When cells were pre-incubated with icilin (20 µM) or 3-T1AM (1 µM) no further increase in $[Ca^{2+}]_i$ was detected after addition of CAP (20 µM). Therefore, it is suggested that there is a negative relationship between TRPM8 and TRPV1 activation since 3-T1AM or icilin suppressed CAP-induced increases in Ca^{2+}.

6-7- 3-T1AM suppresses TRPV1-induced interleukin-6 (IL-6) release

To investigate the crosstalk between TRPM8 and TRPV1, it was assessed if changes in a physiological response mediated by TRPV1 activation would change by exposure to 3-T1AM. Exposure to CAP (20 µM) elicited a 2.5-fold increase in IL-6 release above its basal value.

Notably, 3-T1AM (1 µM) could blunt TRPV1-induced rises in IL-6 release. Such suppression shows that TRPM8-induced suppression of TRPV1 activation elicits a downstream physiological effect through linked signaling pathways that is reflective of the crosstalk between TRPM8 and TRPV1 (Fig. 4).

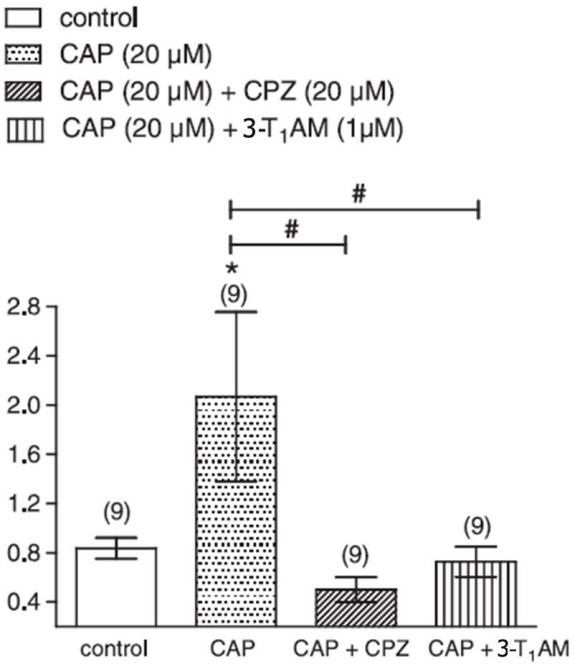

Fig. 4. 3-T1AM suppresses TRPV1 induced IL-6 release. After 24 h incubation, ELISA analysis reveals a clear increase by CAP (20 µM) on IL-6 release. Following pre-incubation either with 20 µM CPZ or 1 µM 3-T1AM for 30 min suppressed the CAP effect on IL-6 release Results were shown in (pg/µg protein). The error bars are representative of SEM [35].

7- Discussion

The results of this thesis show that through the use of novel alternative endogenous TRP channel modulators therapeutic opportunities may be developed to suppress TRPV1-mediated inflammation in DES. Furthermore, the development of a possible adjuvant therapy can be supported by this approach. Both TRPV1 and TRPM8 are important channels, co-expressed in HCjE cells and mediate opposite effects in DES. Therefore, the interplay between these receptors and our suggested modulators, L-carnitine and 3-T1AM probably opens a promising perspective for future dry eye therapy. In this thesis it was also confirmed that the co-expression and interaction of TRPM8 and TRPV1 is detectable in tumor eye cells and this approach can provide also a new therapeutic option to suppress inflammation in uveal melanoma.

7-1- Evidence for functional TRPV1 and TRPM8 expression

In this thesis, a non-transfected, spontaneously immortalized human conjunctival IOBA-NHC cell line as a relevant model of ocular surface cell biology was used. These cells mimic close cell morphology of normal HCjE cells [26]. In order to characterize TRP channels co-expression and interaction also in tumour cells, established immortalized UM 92.1 cells were used as an ideal model of uveal melanoma cancer cells [27]. The result of this thesis provided convincing evidence that TRPV1 and TRPM8 are functionally expressed in the IOBA-NHC as well as UM 92.1 cells. This conclusion is warranted since along with TRPV1 and TRPM8 gene and protein expression, Ca^{2+} transients and underlying currents were identified indicative of their activation by documented agonists and antagonists as well as changes in temperature or osmoalrity. These TRPV1- and TRPM8 mediated Ca^{2+} responses correspond to those described in various cell types including tumor cells [32, 36].

7-2- Possible endogenous modulators of TRPV1 and TRPM8

Based on the experimental results, two TRPs modulators such as L-carnitine and 3-T1AM are suggested since both have an inhibitory effect on TRPV1 channel activity. However, the Ca^{2+} signalling and the following downstream signalling mechanisms between these modulators are completely different. It has been suggested that L-carnitine suppresses cell shrinkage thorough blocking osmosensitive TRPV1 channel and by increasing its intracellular uptake. While 3-T1AM has inhibitory effect on IL-6 secretion thorough TRPV1 suppression and by activation of TRPM8.

Independently, the investigation of their characteristics and properties can help to develop an alternative therapeutic approach for DES. At least, this may be interesting to develop an adjuvant therapy for DES patients.

The results of this thesis are novel, since previously no interaction was reported between TRPV1 and L-carnitine as an osmoprotectant in HCjE cells although the essential role of TRPV1 in osmosensation was reported in numerous tissues [9, 37]. Here, it could be demonstrated that L-carnitine suppressed CAP and hypertonic challenge-induced Ca^{2+} increases and cell shrinkage. Notably, the decline in $[Ca^{2+}]_i$ and cell shrinkage in the presence of L-carnitine was comparable to that of CPZ. This reversal effect by L-carnitine was

associated with declines in TRPV1 function since hypertonic-induced shrinkage was less pronounced in TRPV1 gene silenced cells than in their non-transfected counterpart (fig. 2). Nevertheless, relative cell volume was nearly invariant in non-transfected cells during exposure to either L-carnitine or CPZ. Furthermore, it could be shown that L-carnitine blocked shrinkage induced by such a challenge through a presumable increase in its intracellular uptake by a described L-carnitine Na^+ coupled co-transporter. This suggestion is consistent with our finding that in a Na^+-free medium L-carnitine had less of an effect on hypertonic-induced relative cell volume shrinkage.

Previous studies showed in evaporative dry eye mouse models that L-carnitine supplementation of artificial tears markedly improves ocular surface health by reducing inflammation and neovascularisation [36]. Therefore, L-carnitine is a relevant osmoprotectant with an inhibitory effect. Indeed, the results of this thesis revealed that TRPV1 by acting as a hypertonic osmosensor appears to elicit increases in L-carnitine uptake to reduce shrinkage. Therefore, it is suggested that L-carnitine may be possible modulator in a clinical setting to reduce hyperosmotic-induced ocular surface damage by suppressing TRPV1 activation.

Since there is functional TRPM8 expression in IOBA-NHC, it was suggested that 3-T1AM may interact with TRPM8 (fig. 3). Notably, the systemic temperature lowering effect of 3-T1AM lies within the range adequate for eliciting TRPM8 activation. As 3-T1AM induces responses similar to those mediated by icilin, it also acts as a cooling agent by interacting with this channel at a ligand binding site rather than a temperature sensitive site. 3-T1AM activates cognate G-protein coupled receptors (GPCRs) such as TAAR1 [38, 39]. In addition, 3-T1AM activates GPCRs such as beta adrenergic receptors (ADRBs) (unpublished data). This would point to a “MULTI-Target” property of 3-T1AM, which mimics what has been described for other ligands like dopamine interacting with a GPCR. 3-T1AM is also reported to be a substrate for a series of transporters [38, 40]. GPCR activation can lead to hypothermia through unknown downstream signaling pathway(s).

7-3- TRPM8 and TRPV1 co-expression and interaction

Co-expression of TRPM8 and TRPV1 has been documented for many different cell types including rat hippocampal neurons, intralobar pulmonary arteries, aorta [41, 42],

neuroendocrine tumor cells, retinoblastoma cells and corneal nerve fibers [13, 43]. There is a recent report in a mouse model of human inflammatory colitis showing that TRPM8-induced suppression of TRPV1 activation accounts for declines in inflammation during the course of this disease. These data can be explained by the fact that there is a desensitization of CAP/TRPV1 binding which has been previously described [14].

This putative suppression was also investigated in this thesis. Indeed, such an interaction also exists in IOBA-NHC cells because during exposure to icilin and 3-T1AM, CAP-induced increases in Ca^{2+} influx were blocked.

Previously, it has been revealed in HCEC that hyperosmolarity-induced TRPV1 channel activation followed by increases in plasma membrane Ca^{2+} influx leading to global MAPK stimulation and increases in IL-6 and IL-8 release [9]. This increase is associated with tissue inflammation in DES [9]. This thesis similarly showed that CAP-induced Ca^{2+} influx leads to increases in IL-6 release in IOBA-NHC cells. The suppressing effect of 3-T1AM on CAP-induced increases in IL-6 release mirror the effects of blocking TRPV1-induced signalling with 3-T1AM (fig. 4). Therefore, 3-T1AM is not only suppressing TRPV1-induced Ca^{2+} signalling in HCjEC, but also IL-6 secretion which is associated with inflammatory processes.

Inflammation is also a common feature of human cancers, including those that develop in immunoprivileged sites, such as the eye [44]. Previous observation demonstrated in some types of cancers there is an association between dysregulated cell cycle progression and dysfunctional TRPV1 and TRPM8 channel activity [45, 46]. Therefore, in this thesis we elucidated a co-expression of TRPM8 and TRPV1 in uveal melanoma cells. It is demonstrated TRPM8 expression is absent in the human healthy uveal tissues while the high expression was detected in UM 92.1 cells. Therefore, it is suggested TRPM8 expression may be reflective of the tumorous condition because it was reported to be expressed in other tumor cells such as prostate cancer [47] or breast and bladder carcinoma [48, 49]. In this thesis also the reversible Ca^{2+} rise occurred above 43 °C in UM 92.1 cells indicating functional TRPV1 expression and corresponding to its activity in retinoblastoma cells [13] and other cancer cells [50-52].

7-4- Summary and conclusion

There is an increasing awareness that TRP functional expression in a host of tissues mediates responses that are essential for tissue homeostasis. This realization has prompted studies regarding how TRP activity is modulated and also how this channel superfamily transduces such control through different cell signaling networks activated by transient increases in intracellular Ca^{2+} activity. Regarding possible therapeutic opportunities for dry eye disease and uveal melanoma, some principle findings of this thesis may provide a potential novel target strategy, at least to develop an adjuvant therapy. The realization that a variety of different thermosensitive TRP isotypes and a thermoinsensitive counterpart are expressed on the corneal nerves and the cells of the corneal, conjunctival epithelium as well as tumor cells has prompted interest in developing drugs that selectively modulate their functional.

For example, inhibition of TRPV1 can reduce inflammation that is symptomatic in some DES and uveal melanoma patients. It has been demonstrated in a mouse corneal wound healing model, restoration of corneal transparency was markedly improved in either $TRPV1^{-/-}$ knockout mice or in wild type mice treated with TRPV1 antagonists subsequent to an alkali burn [53, 54]. Another study demonstrated heat induced TRPV1 activation leads to rises in VEGF-A secretion and increases in proinflammatory cytokines release by cultured UM tissue [55].

Patients who are suffering from DES may benefit in the future from the development of TRPM8 agonists whose activation by temperature lowering can be promoted to enhance basal tear flow and contribute to the maintenance of ocular surface hydration. At this point, 3-T1AM may be a potential substance in addition to L-carnitine for eye drops since at least L-carnitine has already been described to possibly have a protective effect on eye cells [7, 36, 56]. Additionally, this interaction might serve as valid explanation for increased effectiveness of moderately cold eye drops.

8- References

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9- Affidavit

I, Noushafarin Khajavi certify under penalty of perjury by my own signature that I have submitted the thesis on the topic [**Functional expression of thermo-sensitive transient receptor potential channels in cultivated human conjunctival epithelial and human uveal melanoma cells**] I wrote this thesis independently and without assistance from third parties, I used no other aids than the listed sources and resources.

All points based literally or in spirit on publications or presentations of other authors are, as such, in proper citations (see "uniform requirements for manuscripts (URM)" the ICMJE www.icmje.org) indicated. The sections on methodology (in particular practical work, laboratory requirements, statistical processing) and results (in particular images, graphics and tables) correspond to the URM (s.o) and are answered by me. My contributions in the selected publications for this dissertation correspond to those that are specified in the following joint declaration with the responsible person and supervisor. All publications resulting from this thesis and which I am author of correspond to the URM (see above) and I am solely responsible.

The importance of this affidavit and the criminal consequences of a false affidavit (section 156,161 of the Criminal Code) are known to me and I understand the rights and responsibilities stated therein.

_____

Date Signature

10- Declaration of any eventual publications

Noushafarin Khajavi had the following share in the following publications:

PUBLIKATION 1

Calcium regulation by temperature-sensitive transient receptor potential channels in human uveal melanoma cells. Mergler S, Derckx R, Reinach PS, Garreis F, Böhm A, Schmelzer L, Skosyrski S, Ramesh N, Abdelmessih S, Plat OK, Khajavi N, Riechardt A. Cell Signal, 2014 Jan; 26(1):56-69

Anteil 10 %

Beitrag im Einzelnen: participation in analysing the data and performing the calcium measurements.

PUBLIKATION 2

Khajavi N, Reinach PS, Skrzypski M, Lude A, Mergler S. L-Carnitine Reduces in Human Conjunctival Epithelial Cells Hypertonic-Induced Shrinkage through Interacting with TRPV1 Channels. Cell Physiol Biochem, 2014; 34(3):790-803.

Impact Factor: 3.55

Anteil 70 %

Beitrag im Einzelnen: participation in designing the study, analysing the data and writing and the manuscript. Performing PCR analysis, immunohistochemistry and cell volume measurement, calcium measurements, planar patch-clamp recordings and plot analyses.

PUBLIKATION 3

Khajavi N, Reinach PS, Slavi N, Skrzypski M, Lucius A, Strauß O, Köhrle J, Mergler S. Thyronamine induces TRPM8 channel activation in human conjunctival epithelial cells. Cell Signal, 2015 Feb; 27(2):315-25

Anteil 80 %

Beitrag im Einzelnen: participation in designing the study, analysing the data and writing and the manuscript. Performing PCR analysis, immunohistochemistry, ELISA, calcium measurements, planar patch-clamp recordings and plot analyses.

Signature, date and stamp of the supervising University teacher

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Signature of the doctoral candidate

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11- List of selected publications

11-1- Stefan Mergler et al. (2014)

Mergler S, Derckx R, Reinach PS, Garreis F, Böhm A, Schmelzer L, Skosyrski S, Ramesh N, Abdelmessih S, Plat OK, Khajavi N, Riechardt A. Calcium regulation by temperature-sensitive transient receptor potential channels in human uveal melanoma cells. Cell Signal, 2014 Jan; 26(1):56-69

Impact Factor: 4.47

http://www.ncbi.nlm.nih.gov/pubmed/24084605

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11-2- Noushafarin Khajavi et al. (2014)

Khajavi N, Reinach PS, Skrzypski M, Lude A, Mergler S. L-Carnitine Reduces in Human Conjunctival Epithelial Cells Hypertonic-Induced Shrinkage through Interacting with TRPV1 Channels. Cell Physiol Biochem, 2014; 34(3):790-803.

Impact Factor: 3.55

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11-3- Noushafarin Khajavi et al. (2015)

Khajavi N, Reinach PS, Slavi N, Skrzypski M, Lucius A, Strauß O, Köhrle J, Mergler S. Thyronamine induces TRPM8 channel activation in human conjunctival epithelial cells. Cell Signal, 2015 Feb; 27(2):315-25

Impact Factor: 4.47

http://www.ncbi.nlm.nih.gov/pubmed/25460045

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12- Curriculum vitae

My Curriculum vitae does not appear in the electronic version of my paper for reasons of data protection.

13- Complete list of publication

1. **Khajavi N**, Reinach PS, Slavi N, Skrzypski M, Lucius A, Strauß O, Köhrle J, Mergler S. Thyronamine induces TRPM8 channel activation in human conjunctival epithelial cells. Cell Signal, 2015 Feb;27 (2):315-25.

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3. **Khajavi N**, Reinach PS, Skrzypski M, Lude A, Mergler S. L-Carnitine Reduces in Human Conjunctival Epithelial Cells Hypertonic-Induced Shrinkage through Interacting with TRPV1 Channels. Cell Physiol Biochem 2014; 34:790-803.

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