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DISSERTATION

**Functional expression of thermo-sensitive transient
receptor potential channels in cultivated human corneal
endothelial cells (HCEC-12)**

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Table of content

Abbreviations	1
Abstract	3
Zusammenfassung	4
1. Introduction	6
1.1 The human cornea	6
1.2 The physiology of the human corneal endothelium.....	7
1.3 The pathogenesis of corneal endothelial disease	7
1.4 Corneal transplantation (keratoplasty)	8
1.5 Storage of corneal transplants	11
1.6 Corneal endothelial cell loss	12
1.7 Ion channels and their role for calcium regulation and apoptosis in HCEC.....	14
1.8 Transient receptor potential channels (TRPs).....	15
1.9 Temperature-sensitive TRPs (Thermo-TRPs).....	17
1.9.1 Heat receptor TRPV1	18
1.9.2 Cold receptor TRPM8.....	18
1.9.3 Cold receptor TRPA1	19
1.10 Aim of the thesis.....	20
2. Materials and methods	21
2.1 Chemicals and solutions	21
2.2 Cell culture of human corneal endothelial cells (HCEC).....	22
2.3 Intracellular Ca ²⁺ measurements with fura-2/AM (calcium imaging).....	23
2.4 Electrophysiological measurements (planar patch-clamp recordings)	25
2.5 Data analyses and statistics.....	27
3. Results	28
3.1 Expression of thermo-TRPs in HCEC-12 cells.....	28
3.1.1 Effect of cooling on [Ca ²⁺] _i in HCEC-12 cells.....	28
3.1.2 Response of [Ca ²⁺] _i to repeated cold stimulation.....	30
3.1.3 Effect of moderate cold stimulation on [Ca ²⁺] _i in HCEC-12 cells in the presence of the TRPM8 channel antagonist BCTC.....	31
3.1.4 Icilin increases [Ca ²⁺] _i in HCEC-12 cells through activation of cold receptors.....	32
3.1.5 Icilin activates non-selective cation channel currents in HCEC-12 cells.....	33

3.1.6 The icilin-induced Ca^{2+} entry is suppressed by the TRPM8 channel antagonist BCTC.....	35
3.1.7 Effect of heating on $[\text{Ca}^{2+}]_i$ in HCEC-12 cells.....	36
3.2 Interaction of cold and heat receptors in HCEC-12 cells.....	37
3.2.1 Simultaneous pharmacological activation of TRPM8/TRPA1 and TRPV1.....	37
3.2.2 CAP and icilin increased whole-cell currents.....	39
3.3 Normal HCEC compared to HCEC-12 cells.....	41
3.3.1 Morphology of the cells.....	41
3.3.2 Icilin increased $[\text{Ca}^{2+}]_i$ in normal HCEC.....	42
4. Discussion	43
4.1 Cold receptor activity in HCEC-12 cells	43
4.2 Heat receptor activity in HCEC-12 cells	45
4.3 Interaction between cold and heat receptors?.....	45
4.4 Using normal HCEC from a human donor cornea.....	46
4.5 Cold receptor activity in normal HCEC.....	47
4.6 Clinical relevance.....	47
4.6.1 Thermo-TRPs and apoptosis.....	48
4.6.2 Cold receptors and storage temperature of grafts in the context of lacking donor material	49
4.6.3 TRPM8 – a possible target against HCE cell loss?	51
4.6.4 High sensitive electrophysiological methods for graft evaluation.....	52
4.7 Conclusion	52
References	53
Eidesstattliche Versicherung	62
Curriculum Vitae.....	65
List of publications.....	66
Acknowledgements.....	67

Abbreviations

ACAID	Anterior chamber-associated immune deviation
BCTC	N-(4-tert-butylphenyl)-4-(3-chloropyridin-2-yl)tetrahydropyrazine-1(2H)-carboxamide
BZgA	Federal Office for Health Education, Germany
$[Ca^{2+}]_i$	Intracellular calcium concentration
CAP	Capsaicin
DLEK	Deep lamellar endothelial keratoplasty
DMEK	Descemet membrane endothelial keratoplasty
DMEM/HAM's	Dulbecco's modified eagle medium/ Ham's nutrient mixture
DMSO	Dimethyl sulfoxide
DSEK/DSAEK	Descemet stripping (automated) endothelial keratoplasty
EGF	Epidermal growth factor
FEPS	Familial episodic pain syndrome
Fura-2/AM	Fura-2/acetoxymethyl ester
HCE	Human corneal endothelium
HCEC	Human corneal endothelial cells
HCEC-12	Human corneal endothelial cell line 12
HCEC-B4G12	Human corneal endothelial clonal daughter cell line B4G12
HCEC-H9C1	Human corneal endothelial clonal daughter cell line H9C1
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
IL-6/-8	Interleukin 6/8
PBS	Phosphate buffered saline
PKP	Penetrating keratoplasty
RT-PCR	Reverse transcription polymerase chain reaction
SEM	Standard error mean
SV40	Simian virus 40
THC	Tetrahydrocannabinol
Thermo-TRP	Thermo-sensitive transient receptor potential
Thermo-TRPs	Thermo-sensitive transient receptor potential channels
TRP	Transient receptor potential
TRPs	Transient receptor potential channels

TRPA1	Transient receptor potential channel ankyrin subtype 1 (previously named ANTKM1)
TRPC	Transient receptor potential channel canonical subfamily
TRPM2	Transient receptor potential channel melastatin subtype 2
TRPM7	Transient receptor potential channel melastatin subtype 7
TRPM8	Transient receptor potential channel melastatin subtype 8 (also known as CMR1)
TRPML	Transient receptor potential channel mucolipin subfamily
TRPN	Transient receptor potential channel NO-mechano-potential subfamily
TRPP	Transient receptor potential channel polycystin subfamily
TRPV1	Transient receptor potential channel vanilloid subtype 1 (also known as capsaicin receptor)
TRPV1-4	Transient receptor potential channel vanilloid subtypes 1 to 4

Abstract

The human corneal endothelium (HCE) is indispensable for maintaining corneal transparency and clear vision. There is HCE cell loss during life and after corneal injury or diseases. A limited HCE cell density may cause opacity which can ultimately only be treated by corneal transplantation (keratoplasty). Even after penetrating keratoplasty, HCE cell loss of up to 50 % occurs. During graft storage, about 30 % of the few available corneal grafts have to be discarded due to HCE cell loss. In addition, the durability of corneal grafts depends on the storage temperature. In Western Europe, grafts are stored at ≈ 37 °C for periods of up to four weeks, whereas they can only be stored for about two weeks when stored at cold temperatures (≈ 5 °C) as preferred in North America. The molecular mechanisms leading to HCE cell loss are still unknown, but a connection to apoptosis is assumed. Therefore, this thesis was undertaken to investigate the functional expression of thermo-sensitive transient receptor potential channels (thermo-TRPs) in HCE cells (HCEC) and their relevance regarding HCE cell loss. Since access to corneal grafts is limited, immortalized HCEC (HCEC-12) were used and primary cultivated HCEC were used additionally. The functional expression of thermo-TRPs was investigated using fluorescence calcium imaging (fura-2/AM) and planar patch-clamp recordings under various physical and pharmacological conditions. Cooling from room temperature (≈ 22 °C) to 14 - 18 °C caused an increase of intracellular calcium ($[Ca^{2+}]_i$). In contrast, the cold receptor antagonist BCTC (10 μ M) suppressed this cold-induced Ca^{2+} increase. The super-cooling agent icilin (50 μ M) caused an increase of $[Ca^{2+}]_i$ and also slightly increased whole-cell outwardly rectifying currents (60 μ M icilin). This icilin-induced Ca^{2+} increase could be suppressed by BCTC (10 μ M). Primary cultivated HCEC responded with a similar icilin-induced increase of $[Ca^{2+}]_i$, indicating that normal HCEC also express cold receptors. In contrast, temperature rises over 43 °C as well as the application of capsaicin (20 μ M) increased $[Ca^{2+}]_i$ by activating heat receptors such as the capsaicin receptor TRPV1. These results demonstrate the functional expression of cold receptors (e.g. menthol receptor TRPM8), as well as heat receptors (e.g. TRPV1) in HCEC-12 cells. It is suggested that these thermo-TRPs are important for the function of HCE and that they might be linked to HCE cell loss. Consequently, thermo-TRPs might present targets for pharmacological modulation to prevent HCE cell loss, which is in the interest of eye banks and patients worldwide.

Zusammenfassung

Das humane Hornhautendothel (HCE) ist für die Transparenz der Cornea und damit für die Sehkraft unentbehrlich. Im Laufe des Lebens, nach Hornhautverletzungen oder -krankheiten kommt es zum Verlust von HCE-Zellen. Eine zu geringe Dichte an HCE-Zellen führt zu einer irreversiblen Trübung der Hornhaut, die letztendlich nur mit einer Hornhauttransplantation (Keratoplastik) behandelt werden kann. Auch nach einer penetrierenden Keratoplastik gehen bis zu 50 % der HCE-Zellen des Transplantats zugrunde. Während der Lagerung der Spenderhornhäute führt der HCE-Zellverlust zum Ausschluss von ca. 30 % des ohnehin knappen Spendergewebes. Die Haltbarkeit der Spenderhornhäute ist außerdem von der Lagerungstemperatur abhängig. In Westeuropa werden sie bei $\approx 37\text{ }^{\circ}\text{C}$ gelagert und sind ca. vier Wochen haltbar, bei gekühlter Lagerung ($\approx 5\text{ }^{\circ}\text{C}$), wie beispielsweise in Nordamerika bevorzugt, jedoch nur zwei Wochen. Die molekularen Mechanismen, die zum HCE-Zellverlust führen, sind bisher unbekannt. Ziel der Arbeit war es, die funktionelle Expression von temperatursensitiven Transient-Rezeptor-Potential-Kanälen (thermo-TRPs) im HCE zu untersuchen, da diese im Zusammenhang mit dem HCE-Zellverlust über die Regulation von Calcium und Apoptose stehen könnten. Aus Mangel an Spenderhornhäuten wurde die immortalisierte Zelllinie HCEC-12 für die Experimente verwendet. Eine HCE-Primärzellkultur wurde ebenfalls untersucht und diente als Kontrolle. Die funktionelle Expression der thermo-TRPs wurde mithilfe von Fluoreszenz Calcium Imaging (Fura-2/AM) und planarem Patch-Clamping unter verschiedenen physikalischen und pharmakologischen Bedingungen untersucht.

Die Kühlung beginnend bei Raumtemperatur ($\approx 22\text{ }^{\circ}\text{C}$) auf $14 - 18\text{ }^{\circ}\text{C}$ führte zu einem Anstieg der intrazellulären Calciumkonzentration ($[\text{Ca}^{2+}]_i$). Der Kälterezeptorantagonist BCTC ($10\text{ }\mu\text{M}$) war dagegen in der Lage, den Kälte-induzierten Calciumanstieg zu hemmen. Pharmakologische Kühlung mit dem Kühlwirkstoff Icilin ($50\text{ }\mu\text{M}$) führte ebenfalls zu einem Anstieg der $[\text{Ca}^{2+}]_i$ sowie zu einem leichten Anstieg von nicht-selektiven auswärts gerichteten Ganzzellströmen ($60\text{ }\mu\text{M}$ Icilin). Der Icilin-induzierte Calciumanstieg konnte durch BCTC ($10\text{ }\mu\text{M}$) inhibiert werden. Frisch kultivierte HCE-Primärzellen reagierten mit einem ähnlichen Anstieg des $[\text{Ca}^{2+}]_i$ nach Icilingabe, was auf die Expression von Kälterezeptoren in normalen HCE-Zellen hindeutet. Temperaturen über $43\text{ }^{\circ}\text{C}$ sowie die Zugabe von Capsaicin ($20\text{ }\mu\text{M}$) erhöhten das $[\text{Ca}^{2+}]_i$ durch Aktivierung von Hitzerezeptoren wie dem Capsaicinrezeptor TRPV1.

Diese Ergebnisse zeigen die funktionelle Expression von Kälterezeptoren (z.B. Mentholrezeptor TRPM8) und Hitzerezeptoren wie TRPV1 in HCEC-12 Zellen. Es liegt nahe, dass diese Kanäle für die Funktion der HCE-Zellen von Bedeutung sind und dass es möglicher Weise einen Zusammenhang zwischen ihnen und dem HCE-Zellverlust gibt. Thermo-TRPs könnten daher einen vielversprechenden Angriffspunkt für eine Pharmakotherapie gegen HCE-Zellverlust darstellen, was für Hornhautbanken und Patienten weltweit von Nutzen wäre.

1. Introduction

1.1 The human cornea

The human cornea is a transparent tissue, which covers the front of the eye like a dome-shaped window. It is highly specialized in transmitting and refracting light (the cornea contributes 65 to 75 percent of the eye's total focusing power) (Wilson and Last, 2004). The corneal tissue is structured into five layers (Fig. 1). The outermost non-keratinized stratified squamous epithelium serves as a barrier between the environment and the stroma of the cornea. It protects from external influences such as microorganisms, noxious agents and mechanical damage. Via interaction with the tear film, the epithelium forms a smooth transparent surface, necessary for clear vision. The basal cells are attached to a basement membrane, which adjoins the Bowman's layer. The Bowman's layer (also known as the anterior limiting lamina) contributes to the cornea's stability. Underneath lies the stroma, consisting of collagen fibers. According to the lattice model (Maurice, 1957), these fibers are perfectly arranged, meeting the basic condition for the cornea's transparency. The stroma is followed by the Descemet's membrane (also known as the posterior limiting lamina), the basement membrane for the endothelial layer. The single-layered low cuboidal endothelium borders the anterior chamber of the eye. Its barrier and pump functions are essential for sustaining corneal transparency.

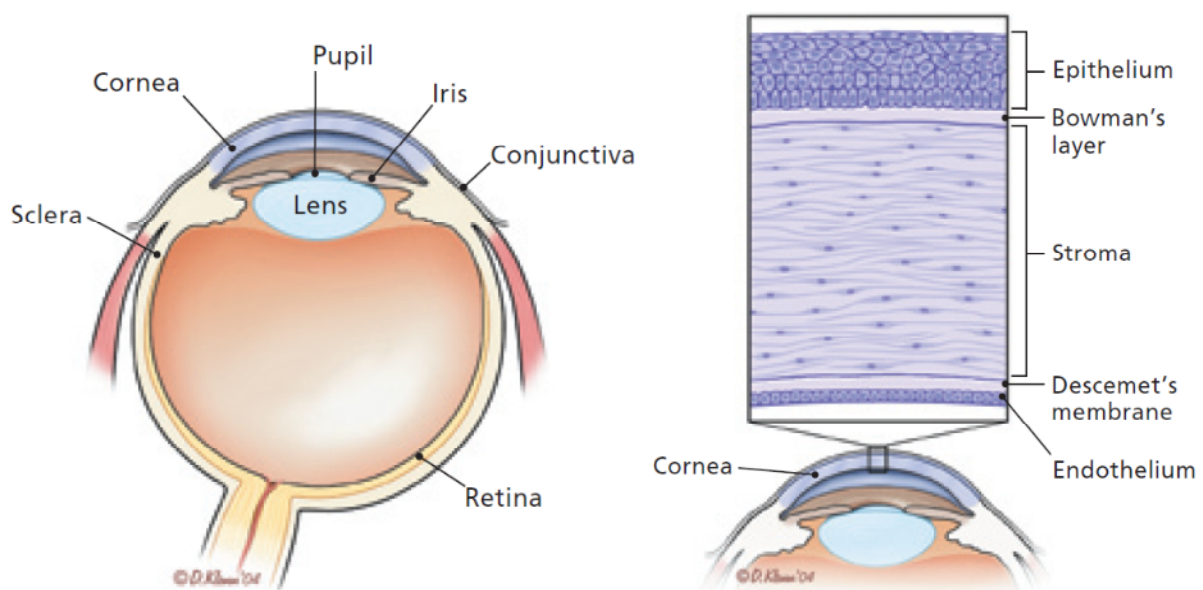


Fig. 1 Left: A diagrammatic representation of the cornea in relationship to the eye. Right: The cornea's histology (from Wilson and Last, 2004).

1.2 The physiology of the human corneal endothelium

The human corneal endothelium (HCE) plays a fundamental role in maintaining the transparency of the cornea. For this purpose, its barrier and pump functions are essential. The endothelial monolayer is 4 - 6 μm thick and consists of polygonal (mostly hexagonal) cells (Schroeter and Rieck, 2009). The cells form a permeable barrier between the hydrophilic corneal stroma and the anterior chamber of the eye. This leaky barrier is formed by intercellular tight junctions and gap junctions, which do not seal the cells completely (Watsky et al., 1990). Due to this permeability, aqueous humor is able to pass into the stroma and thereby it provides the non-vascular cornea with essential nutrients. Other sources of nourishment for the cornea are the tear film, oxygen from the air and the outer capillary network of the conjunctiva (Grehn, 2012). So far, diverse ion transport mechanisms have been identified to be involved in the pump process (Bonanno, 2003). In brief, active pump mechanisms shift Na^+ - and HCO_3^- -ions into the anterior chamber (Hodson, 1974; Hodson and Miller, 1976). This causes a passive movement of fluid from the stroma back to the anterior chamber and guarantees a constant stromal dehydration. The energy for this process is provided via adenosine triphosphatase (Na^+ - K^+ -ATPase) activity (Tervo and Palkama, 1975). Fluid influx and efflux must be balanced otherwise the corneal transparency cannot be maintained. The breakdown of either endothelial barrier or pump function leads to corneal edema (Maurice, 1972).

1.3 The pathogenesis of corneal endothelial disease

In contrast to human corneal endothelial cells (HCEC), corneal epithelial cells are able to heal and renew themselves by proliferation and differentiation of stem cells located in the limbus (Lu et al., 2001). In some animal species such as rats, cats and rabbits, corneal endothelial regeneration has been reported (Tuft et al., 1986; Landshman et al., 1989; Gloor et al., 1986). Unlike human corneal epithelial cells and the corneal endothelial cells of several animal species, the HCEC cannot easily regenerate. The inability of the HCEC to proliferate is due to the fact that these cells are arrested in the G1-phase of the cell cycle and therefore are not mitotically active (Joyce, 2003). For this reason, the population of HCEC *in vivo* decreases with age (Bourne et al., 1997). Newborns possess a cell density of 3,500 - 4,000 cells/ mm^2 , whereas adults only have an average of 1,500 - 2,500 cells/ mm^2 (Schroeter and Rieck, 2009). Apart from aging, HCE cell loss may be caused by eye diseases, such as Fuchs' endothelial dystrophy

(Eghrari and Gottsch, 2010), injury or as a result of eye surgery, for example cataract extraction (Schultz et al., 1986). Up to a certain point, normal endothelial function can be maintained in spite of the cell loss by compensation of the remaining cells. The cells flatten, extend their surface and lose their typical hexagonal shape (Waring et al., 1982). However, if the number of HCEC falls below a limit of approximately 1,000 cells/mm², the compensation mechanisms become insufficient (Landshman et al., 1988). The interspaces between the cells widen out and more fluid from the anterior chamber passes into the stroma. From a certain level, the pumping capacity of the remaining cells is not high enough to work against the incoming fluid. Finally, the cornea becomes thick and milky, which leads to corneal edema and opacity. At present, the only possible therapy to restore visual acuity under these conditions is corneal transplantation (keratoplasty).

1.4 Corneal transplantation (keratoplasty)

Each year, approximately 4,800 corneal transplantations are performed in Germany, which makes the cornea the most frequently transplanted organ in medicine (Seitz et al., 2004). Over 100 years ago, Dr. Eduard Zirm was the first to report the effective grafting of a human cornea (Zirm, 1906). Since then, the technique of the so called “penetrating keratoplasty” (PKP) (Fig. 2A) in which the entire cornea is replaced by a donated graft has practically remained unchanged. However, in the last few years there was considerable progress in the development of more specialized surgical techniques such as the so-called lamellar keratoplasty. The basic idea here is to replace only the actual diseased part of the host’s cornea, while leaving the healthy corneal layers unaffected. In 1998, Melles et al. described an effective technique for transplantation of posterior corneal tissue for treating corneal endothelial disorders, which became known as DLEK (“deep lamellar endothelial keratoplasty”, Fig. 2B) (Melles et al., 1998). Out of this, DSEK/DSAEK (“Descemet stripping [automated] endothelial keratoplasty”, Fig. 2C) emerged (Price and Price, 2006) and was further developed as DMEK (“Descemet membrane endothelial keratoplasty”, Fig. 2D) (Melles et al., 2006). Using DMEK, one can now replace only the innermost layers of the cornea (Descemet’s membrane and the corneal endothelium) by donor tissue.

Compared to other posterior lamellar keratoplasty methods such as DSEK/DSAEK, DMEK results in a quicker and better functional visual rehabilitation (Cursiefen and Kruse, 2010). For example, within the first three months after surgery, 92 % of patients

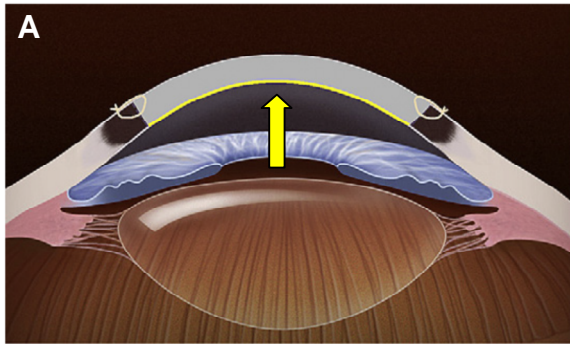
achieve a best-corrected visual acuity of 0.5 or better and 60 % reach 0.8 or better (Ham et al., 2009), whereas it takes 62 % of patients treated with DSEK six months to achieve visual acuity of 0.5 or better (Price and Price, 2005). However, only 43 % of patients achieve a best-corrected visual acuity of 0.5 or better over time after penetrating keratoplasty (Williams et al. 1995). The less satisfactory results of PKP are explainable by post-operative astigmatism, caused by wound healing and sutures (Hoppenreijns et al., 1993). In contrast to PKP, posterior lamellar keratoplasty procedures are performed without sutures, as the graft is inserted through a small limbal incision and attached from the inside by an air bubble (Melles et al., 1998).

In general, rejection of the corneal graft happens rarely compared to transplantation of other human tissues. This is due to the fact that the cornea is avascular. In addition, the cornea enjoys active immune privilege, so called anterior chamber-associated immune deviation (ACAID) (Niederhorn et al., 2010). Nevertheless, the risk of rejection remains, but it increases with the amount of transplanted tissue antigens. Within 2 years after surgery, the risk of rejection after DMEK is 20 times lower than after PKP and 15 times lower than after DSEK (Anshu et al., 2012). Accordingly, it is advantageous to exchange only the actual diseased layers, as the latest techniques allow.

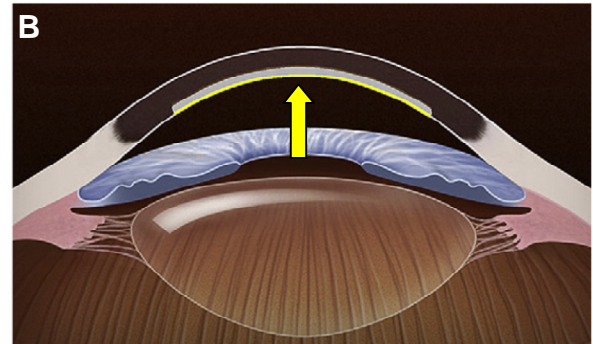
Furthermore, topical corticosteroids are used routinely to prevent graft rejection after keratoplasty (Nguyen et al, 2007). As DMEK shows decreased rejection rates, it allows less use of corticosteroids postoperatively, which consequently reduces the undesirable side effects of topical corticosteroids such as elevation of intraocular pressure or secondary cataract (Anshu et al., 2012).

At present, DSEAK remains the standard procedure for treating corneal endothelial disorders because of its high degree of standardization (Maier et al., 2013). But it seems only a matter of time until DMEK becomes the preferred method, because of its advantages in terms of faster wound healing, better visual rehabilitation and almost no risk of rejection.

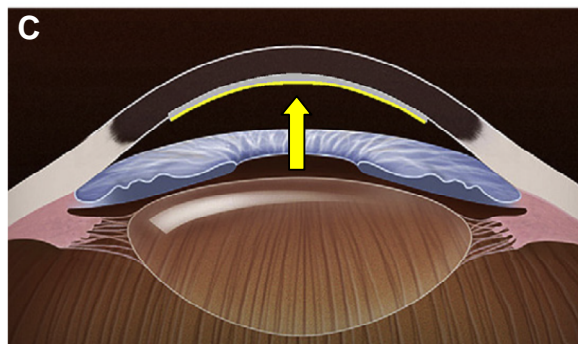
Penetrating keratoplasty (PKP)



Deep lamellar endothelial keratoplasty (DLEK)



Descemet stripping endothelial keratoplasty (DSEK)



Descemet membrane endothelial keratoplasty (DMEK)

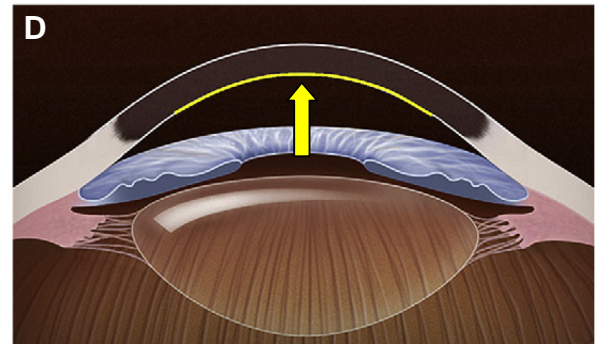


Fig. 2 Different keratoplasty techniques for endothelial diseases (modified and adapted from Patel, 2012). The corneal endothelium is highlighted in yellow and additionally marked by an arrow. **(A)** “Penetrating keratoplasty” (PKP). The complete host cornea is removed and replaced by a donor cornea, which is fixed with a continuous suture. A relatively large stromal wound is produced which may create an astigmatism (Hoppenreijs et al., 1993). **(B)** “Deep lamellar endothelial keratoplasty” (DLEK). Here, the posterior host stroma, Descemet’s membrane and endothelium are replaced by donor tissue through a small limbal incision, while the surface of the cornea remains untouched. No corneal sutures are needed for posterior lamellar keratoplasty methods. Instead, the graft is attached from the inside by an air bubble. **(C)** In “Descemet stripping endothelial keratoplasty” (DSEK), the host Descemet’s membrane and endothelium are stripped. In return, donor tissue consisting of posterior stroma, Descemet’s membrane and endothelium is inserted, adding thickness to the stromal tissue. **(D)** In “Descemet membrane endothelial keratoplasty” (DMEK), only the host Descemet’s membrane and endothelium are removed and replaced by the donor Descemet’s membrane and endothelium (without any stromal tissue).

1.5 Storage of corneal transplants

Worldwide, various methods are used for corneal preservation. They differ mainly in their storage temperature and composition of medium. In the early 1970s, an organ culture technique was established (Summerlin et al., 1973). It is based on the idea of simulating physiological conditions by preserving the cornea in medium kept close to body temperature (30 - 37 °C). This organ culture method is preferably used in Western Europe (Ehlers et al., 2009), as it allows to store the grafts up to four weeks. This improves the availability of corneal tissue for surgery. In contrast, cold storage temperatures (2 - 8 °C) are preferred in North America because of the simpler culture procedure, but grafts can only be kept for about 7 - 14 days (Armitage, 2011). During the preservation period, the grafts are examined microbiologically and evaluated by slit-lamp examination. The endothelial cells are visualized microscopically and counted in a defined area to calculate the cell density per square millimeter. Also, the grafts are stained with trypan blue to detect apoptotic cells (Schroeter and Rieck, 2009). Only flawless corneas are selected for transplantation to prevent graft failure. Exclusion criteria would for example be damage, opacity, signs of pathologies such as cornea guttata (spot-like lesions in the endothelium as a sign of decompensation) or an excessively low density of endothelial cells. The cut-off points for endothelial cell density vary among different eye banks and lie somewhere between 2,000 and 2,500 cells/mm² (Pels and Rijnveld, 2009). Unfortunately, up to 30 % of the corneal grafts have to be discarded by the eye banks because of poor endothelial condition (Fuchsluger et al., 2011).

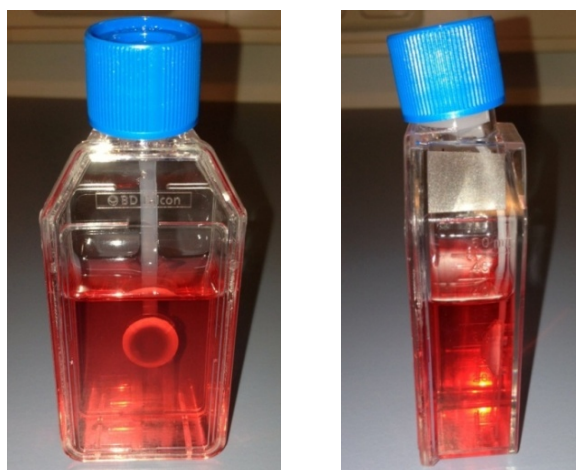


Fig. 3 Storage containers containing a corneal graft. The cornea is excised from the donor's eye together with adjacent sclera. The corneoscleral button is seen hanging in the sterile culture medium. (Photos taken at the Corneabank of the Ophthalmology Department of the Charité University Hospital Berlin.)

1.6 Corneal endothelial cell loss

Grafts stored in organ culture suffer the loss of corneal endothelial cell density over time, which eventually makes them unsuitable for transplantation (Fig. 4 and Fig. 5; Armitage and Easty, 1997). This limitation has severe consequences since the demand for donated corneas is higher than the supply.

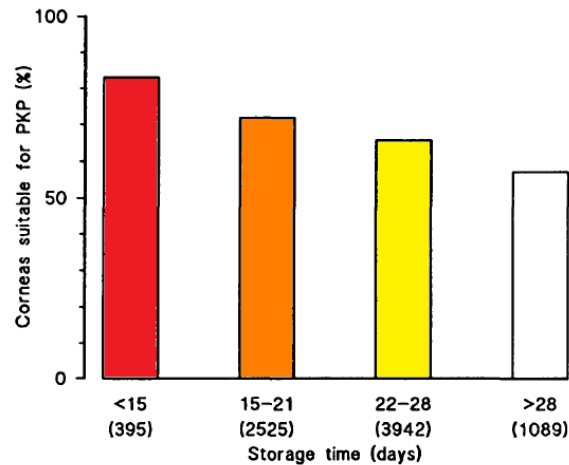


Fig. 4 The storage time of organ cultured corneas has an influence on the suitability of corneas for penetrating keratoplasty (figure adapted and modified from Armitage and Easty, 1997). The shorter the graft storage time, the better the graft is suitable for transplantation. Besides HCE cell loss, microbial contamination endangers the graft's quality over storage time. (Values in parentheses are total numbers of corneas for each storage time.)

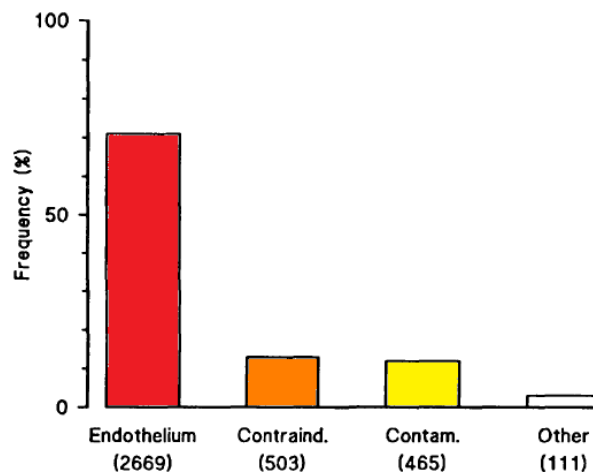


Fig. 5 Reasons for unsuitability of organ cultured corneas for penetrating keratoplasty (figure adapted and modified from Armitage and Easty, 1997). Values in parentheses are total numbers of corneas which could not be used for keratoplasty. The most frequent cause for the graft's unsuitability is endothelial deficiency (indicated as "Endothelium"), which is primarily based on the endothelial cell density. In this study, the cut-off was set at 2200 cells/mm². Additional reasons for unsuitability are medical contraindication ("Contraind."), contamination ("Contam.") and other.

For reasons that are still unknown, the number of HCE cells decreases continuously in the graft after PKP (Fig. 6; Ing et al., 1998). Especially during the first year after surgery, HCE cell loss of up to 48 % has been reported (Obata et al., 1991). Likewise, the newer lamellar keratoplasty methods are also associated with a loss of HCE cells, but rather in the early postoperative phase, where they suffer a loss of about 25 % of the endothelial cell density (Ham et al., 2009). This endothelial cell loss seems to be caused by trauma to the fragile donor tissue during the challenging surgical intervention (Patel, 2012). This may also explain the comparatively high number of primary graft failures which appear after DSEAK (0 - 29 %, after DMEK 0 - 9 % and after PKP 0 - 3 %) (Maier et al., 2013). The short-term success rate of PKP is usually reported to be excellent (about 90%) (Seitz et al., 2004). But in the long-term, for instance after seven years, the success rate is considerably lower at approximately 70 % (Williams et al., 1995). If graft failure occurs, whether caused by rejection, HCE cell loss, or other complications, a re-keratoplasty becomes necessary. In this context, it is understandable that the HCE homeostasis as well as the graft's storage conditions remain a researched topic to improve the availability of corneal grafts and the outcome of keratoplasty.

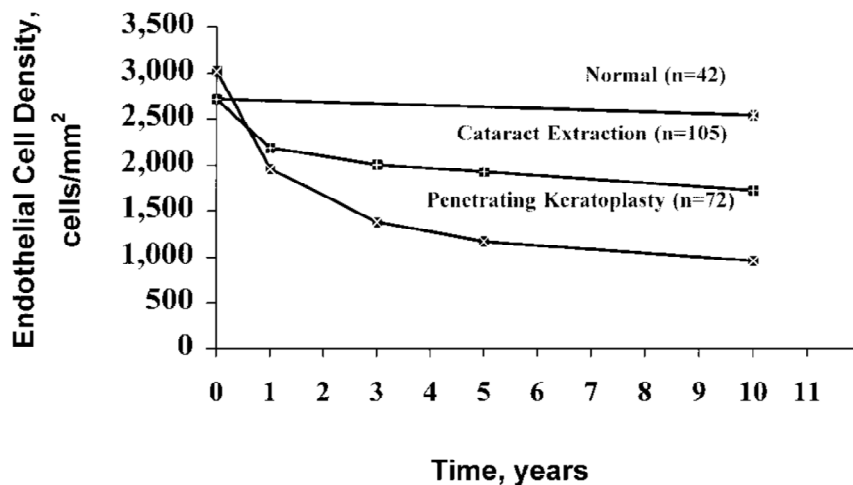


Fig. 6 Endothelial cell density decreases over time (figure adapted from Ing et al., 1998). The annual rate of endothelial cell loss in a normal adult eye is 0.6 % per year measured over a period of 10 years. It is 2.5 % per year in patients who had cataract surgery and 4.2 % per year after penetrating keratoplasty. This indicates that the annual rate of endothelial cell loss is seven times the normal rate from 5 to 10 years after penetrating keratoplasty.

1.7 Ion channels and their role for calcium regulation and apoptosis in HCEC

The molecular mechanisms leading to HCE cell loss have not yet been sufficiently clarified. For this reason, the working group around Mergler et al. at the Ophthalmology Department at Charité Campus Virchow Clinic began to investigate the functional expression of ion channels in HCEC. In 2003, they were the first to discover voltage-operated Ca^{2+} channels (L-types) in a HCE cell line as well as in a primary cell culture. It is of note that they could demonstrate the regulation of these channels by growth factors such as the basic fibroblast growth factor (FGF-2) and the epidermal growth factor (EGF) (Mergler et al., 2003; Mergler et al., 2005).

Ion channels are involved in all basic cellular processes including regulation of the membrane potential, cell growth, proliferation or secretion. In general, ion channels are transmembrane proteins that form pores, which allow ions to flow across the cell membrane following their electrochemical gradient. There are different types of ion channels which can be classified by gating, etc. On the one hand, voltage-gated ion channels can be activated by voltage changes of the membrane potential (e.g. L-type Ca^{2+} channel). On the other hand, ligand-controlled ion channels only open after their specific ligand binds to them. Apart from that, ion channels can also be activated by a mechanical stimulus. In either way, an opened ion channel enables over ten million ions to pass per second through each channel (Clapham, 2003). Since most ion channels are highly ion-selective, one can also classify them by their specificity for certain ions such as sodium-, potassium-, calcium- and chloride-channels.

By mediating rapid electrical signals, ion channels play important roles in many different physiological functions, amongst others in regulating programmed cell death (apoptosis). The mechanisms of apoptosis are highly complex. There are two main known pathways, the extrinsic or death receptor pathway and the intrinsic or mitochondrial pathway (Elmore, 2007). Each is activated by specific signals, which trigger an energy-dependent cascade of molecular events, including the activation of a group of cysteine proteases called caspases that finally lead to the death of the cell. Apoptosis is regulated by many intracellular proteins, such as the Bcl-2 family (they either promote or inhibit cell death). Furthermore, it is known that calcium (Ca^{2+}) is a key element of apoptotic signaling pathways, e.g. by activating enzymes (Orrenius et al., 2003). Calcium is a mineral that is involved in a multitude of cellular functions in the

human body, ranging from growth of teeth and bones over muscle contraction and even to learning. For this purpose, calcium homeostasis is strictly controlled by Ca^{2+} permeable channels and pumps. More precisely, the concentration of intracellular calcium in resting cells is maintained at approximately 100 nM, which is about 10,000 times lower than outside the cell (Carafoli, 2007). Various stimuli lead to an increased intracellular Ca^{2+} level, either by influx of extracellular Ca^{2+} , or by release from intracellular stores (mitochondria and endoplasmic reticulum). This increased intracellular Ca^{2+} is associated with activation and modulation of apoptotic processes (Orrenius et al., 2003).

It is suggested that the mechanisms leading to HCE cell loss are somehow linked to an apoptosis-inducing process, which involves certain Ca^{2+} permeable ion channels. In 2005, a first indication of a certain type of Ca^{2+} channels such as *transient receptor potential channels* (TRPs) in HCEC was found (Mergler et al., 2005). Since TRPs are known to be non-selective cation channels, they are naturally permeable to calcium ions and therefore possibly involved in many cellular processes through regulation of intracellular calcium, including apoptosis. Therefore, TRPs are suggested to be expressed in HCEC and linked to the molecular mechanisms leading to HCE cell loss.

In the context of the latest discovery of TRPs in HCEC, further investigation of TRP channel activities in HCEC might provide important information on HCE cell loss. Naturally, it is in the interest of patients and eye banks worldwide to prevent cell loss in the corneal endothelium and to achieve extended durability of corneal grafts.

1.8 Transient receptor potential channels (TRPs)

TRPs represent a family of non-selective cation channels with polymodal activation properties (Ramsey et al., 2006). They were first discovered in a mutant strain of the fruit fly *Drosophila melanogaster* whose photoreceptors reacted to light stimulus with a transient membrane current, in contrast to the persistent currents observed in the wild type (Consens and Manning, 1969). In 1989, the TRP gene was first cloned and its gene product was identified as a transmembrane protein (Montell and Rubin, 1989). The TRPs form a superfamily which can be divided into seven subfamilies (Montell, 2005). Namely, there are TRPC (canonical), TRPV (vanilloid), TRPM (melastatin),

TRPP (polycystin), TRPML (mucolipin), TRPA (ankyrin) and TRPN (NOMP = NO-mechano-potential, not present in mammals) (Fig. 7; Nilius and Owsianik, 2001). Despite their differences in the way of activation, selectivity for ions and physiological functions, they all have one common property: They are built of six transmembrane domains with a cation-permeable pore between segments five and six and their carboxyl and amino termini are located intracellularly (Wu et al, 2010) (Fig. 8). TRPs are expressed in almost every cell type and play an important role in several physiological processes, with a focus on sensory physiology (Nilius and Owsianik, 2001). Recently, thermo-sensitive TRP channels (thermo-TRPs) were detected in HCEC for the first time by Mergler et al. (Mergler et al., 2010; Mergler et al., 2011). These channels belong to the TRP vanilloid receptors subtype 1-4 (TRPV1-4 channels) and will be described more precisely in the following chapter.

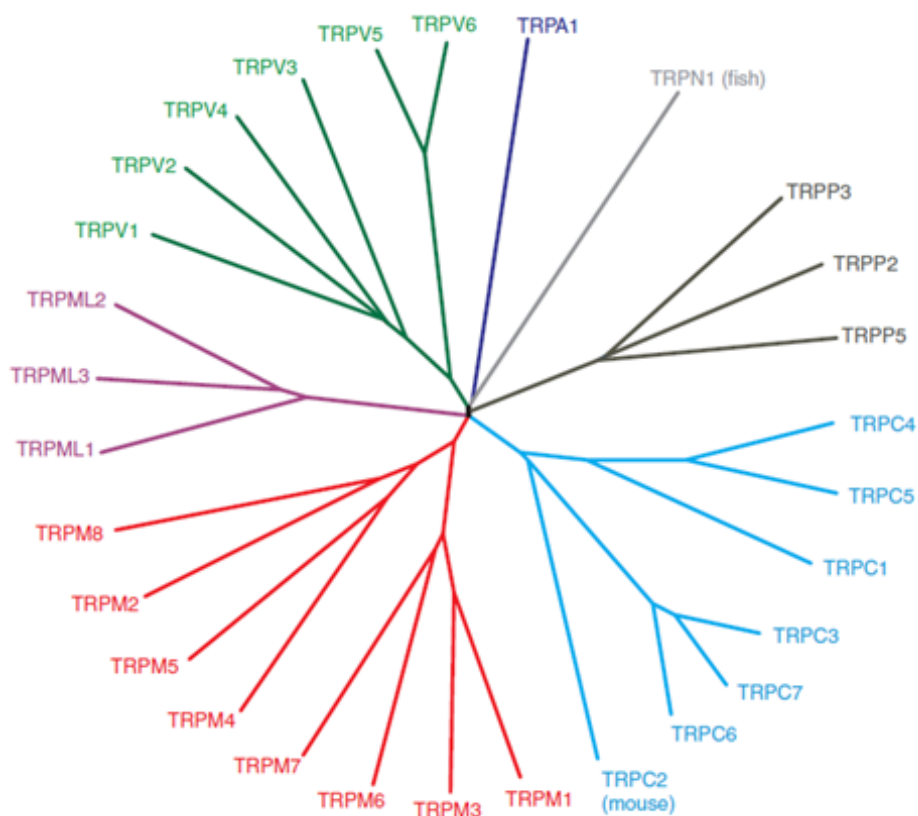


Fig. 7 The TRP superfamily adapted from Nilius et al. (Nilius and Owsianik, 2001). The seven TRP subfamilies are illustrated by different colors. With the exception of TRPN1 which is present in fish, these TRPs can all be found in humans.

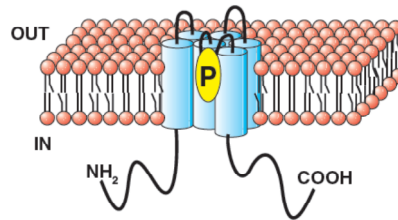


Fig. 8 The general structure of TRPs (Nilius and Owsianik, 2001). The channels are composed of six transmembrane segments and a pore region. The intracellular carboxyl and amino termini are variable in length, depending on each TRP.

1.9 Temperature-sensitive TRPs (Thermo-TRPs)

Until today, six thermo-TRPs have been described to be expressed in primary sensory nerves, covering all temperature sensations from cold to hot (Vay et al., 2012). TRPV1-4 are activated by temperatures from moderate warmth to extreme heat, whereas TRPM8 and TRPA1 respond to cold temperatures, shown pictorially in Fig. 9.

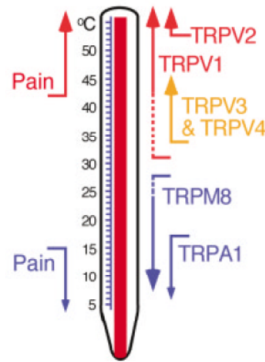


Fig. 9 The six thermo-TRPs and their temperature thresholds for activation (Tominaga and Caterina, 2004). Temperatures above ≈ 43 °C and below ≈ 15 °C cause pain.

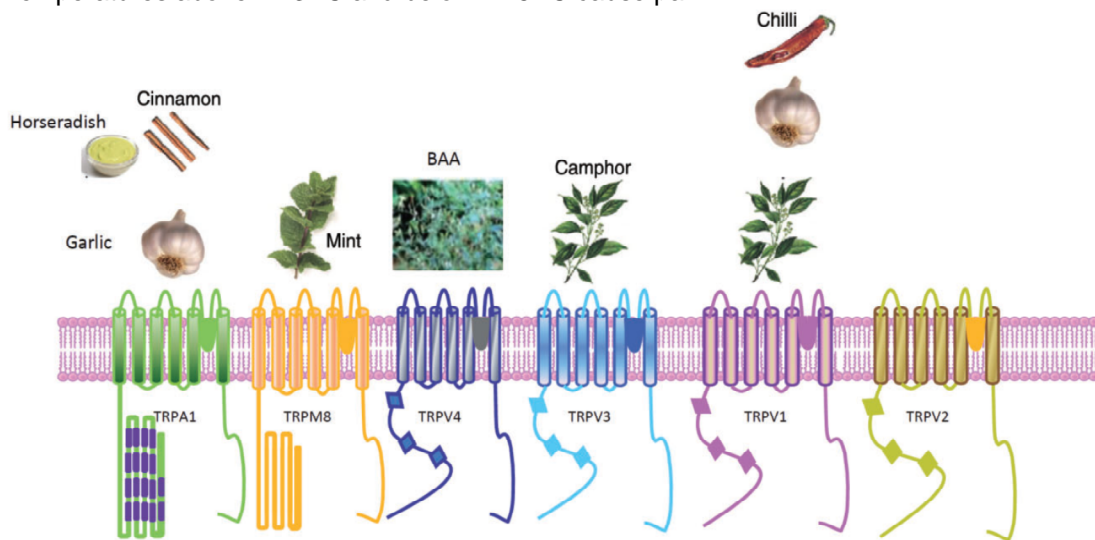


Fig. 10 Overview of the naturally occurring agonists of thermo-TRPs (figure adapted and modified from Vay et al., 2012). (BAA = bisandrographolide A, ingredient of the Chinese herbal plant *Andrographis paniculata*)

1.9.1 Heat receptor TRPV1

The most-investigated thermo-TRP channel is the TRP vanilloid receptor 1 (TRPV1) which is a Ca^{2+} permeable channel and sensitive to heat over 43 °C (Caterina et al., 1997). Its first detected ligand was capsaicin (CAP), the ingredient of chili peppers that mediates the sense “hot” in nociceptors. Since CAP is a vanilloid, the channel was therefore named TRPV1. Apart from CAP and heat, it can also be activated by protons ($\text{pH} < 5.9$) (Tominaga et al., 1998) and other endogenous substances such as arachidonic acid metabolites (Hwang et al., 2000).

Due to the fact that TRPV1 is involved in the process of pain perception, it is not surprising that this channel is primarily expressed in nociceptive neurons of the peripheral nervous system (Tominaga and Caterina, 2004). But it was also detected in non-neuronal cells, for example in epithelial cells of the urinary bladder of mice (Birder et al., 2001). In 2007, TRPV1 was first discovered in ocular tissue, more precisely in rabbit and human corneal epithelial cells lines (Zhang et al., 2007; Mergler et al., 2011). A few years later, the channel was also identified in the human corneal endothelial cell line HCEC-12, where it could be shown that TRPVs are involved in maintaining corneal endothelium function by regulating cytosolic Ca^{2+} levels (Mergler et al., 2010). These recent discoveries show that thermo-TRPs seem to play a role beyond the sensation of temperature and pain. Some human diseases could even be traced back on mutations in several TRPs (Nilius et al., 2007). The question arises as to which function thermo-TRPs fulfill in corneal tissue and whether they might be involved in pathophysiology relating to HCE cell loss.

1.9.2 Cold receptor TRPM8

Other thermo-TRPs such as the cold receptor of the melastatin subtype 8 (TRPM8) may also play a role in HCE physiology. In particular, TRPM8 is suggested to be expressed in HCEC because the super cooling agent icilin (Rawls et al., 2007) induced Ca^{2+} transients in these cells (Mergler et al., 2005). This was the first indication of functional co-expression of heat and cold receptors in these cells.

More specifically, TRPM8 is also known as the cold and menthol receptor CMR1 and is activated by moderate cooling below 28 °C (McKemy et al., 2002). Pharmacologically, TRPM8 can be activated by the cooling agents menthol, eucalyptol and icilin, whereas BCTC and capsazepine antagonize its effects (McKemy, 2005; Peier et al., 2002;

Behrendt et al., 2004; Feng, 2011). Its encoding gene, named *trp-p8*, was first discovered to be up-regulated in prostate cancer and other malignancies such as breast adenocarcinoma, melanoma and colorectal adenocarcinoma (Tsavaler et al., 2001). TRPM8 was also detected in healthy tissues, mostly in the male genital tract (e.g. prostate and testicles) and in the bladder (Stein et al., 2004). So far, its physiological function has only been clarified in sensory neuronal cells where it senses moderate cold temperatures under a threshold of 25 – 28 °C (McKemy et al., 2002; Peier et al., 2002; Bautista et al., 2007; numbers vary depending upon source). The role of TRPM8 in cancer cells remains unclear. It continues to be a key subject of research, as the channel might be a promising target for pharmacological cancer therapy and perhaps a diagnostic and prognostic marker for prostate cancer or other tumor diseases (Zhang and Barritt, 2006; Valero et al., 2010; Mergler et al., 2007). The possible physiologic and pathophysiologic aspects of TRPM8 in HCE are mentioned in the discussion chapter.

1.9.3 Cold receptor TRPA1

TRPA1 belongs to the ankyrin TRP subtype family and was previously named ANKTM1. TRPA1 was first reported in human fibroblasts (Jaquemar et al., 1999). In contrast to TRPM8, TRPA1 is activated by stronger cooling (temperature threshold \approx 17 °C) (Story et al., 2003). Apart from a variety of pungent compounds such as in garlic (allicin) (Macpherson et al., 2005), mustard oil (Jordt et al., 2004) and cinnamon oil (Bandell et al., 2004), TRPA1 can also be activated by cannabinoids (THC) (Jordt et al., 2004) and bradykinin, an inflammatory peptide (Bandell et al., 2004). The cooling agent icilin also activates TRPA1, but less effectively than TRPM8 (McKemy et al., 2002; Story et al., 2003). The channel can be found in nociceptive sensory neurons, where it is coexpressed with the heat receptor TRPV1, and in hair cells (Story et al., 2003; Corey et al., 2004). It must be noted that the effects of TRPA1 regarding its cold and mechanical sensation are discussed controversially (reviewed by McKemy, 2005). Whereas Story et al. were able to detect activation of TRPA1 by cold temperatures, other groups such as Jordt et al. failed to confirm this discovery (Story et al., 2003; Jordt et al., 2004). Notably, TRPA1 gene expression could also be very recently detected in HCEC (Valtink et al. 2013, unpublished observation). Overall, the functional expression of thermo-TRPs remains an intensively researched topic and will also be considered in this thesis.

1.10 Aim of the thesis

Based on the results of previous studies cited above, the three following hypotheses were proposed:

- 1) Expression of thermo-TRPs such as the cold receptors TRPM8 and TRPA1, as well as heat receptors (e.g. TRPV1) exists in HCEC.
- 2) There is interaction between cold and heat receptors.
- 3) The immortalized cell line HCEC-12 is an appropriate model to study HCEC.

The investigation of the functional expression of thermo-TRPs in HCEC could reveal important information about HCE pathophysiology, in particular in context with cell loss. Notably, a specific characterization of these TRPs may contribute to developing endogenous TRP channel modulators which may positively influence cell apoptotic processes. Also, it might help understanding the role of thermo-TRPs concerning the storage temperature of cornea grafts. As a consequence, this may extend the durability of corneas before transplantation and also improve the outcome of keratoplasty. Thereby, the balance of supply and demand of donor material might be changed for the better.

To confirm the hypotheses, two methods were used:

- a) The calcium imaging method (fura-2/AM)
- b) The planar patch-clamp recording technique

Both are highly sensitive methods suitable for characterization of thermo-TRPs in HCEC. Currently, cornea banks use conventional methods such as counting and staining the cells to evaluate the endothelium. In the future, highly sensitive electrophysiological methods such as those used in this thesis might better support the detection of flawless corneal grafts for transplantation.

2. Materials and methods

2.1 Chemicals and solutions

Medium for cultivation of the investigated cells:

500 ml DMEM/HAM's F12 culture medium with L-glutamine (Life Technologies Invitrogen, Karlsruhe, Germany and Biochrom AG, Berlin, Germany), 2 ml penicillin/streptomycin (100 µg/ml) (Gibco Invitrogen, Darmstadt, Germany), 50 ml fetal calf serum (10 % final concentration) (PAA GmbH, Pasching, Germany) and PBS for washing (cell culture): 137 mM NaCl, 2.7 mM KCl, 12 mM PO₄³⁻.

Accutase solution:

500 µl accutase (PAA GmbH, Pasching, Germany) was used in a 25 ml flask to detach cells for dilution (cell passage) or for preparing cell suspensions (patch-clamp).

Composition of the solution (mM) for fluorescence calcium imaging:

Ringer-like solution: 150 mM NaCl, 6 mM CsCl, 1 mM MgCl₂, 1.5 mM CaCl₂, 10 mM glucose, and 10 mM HEPES (pH adjusted to 7.4). K⁺ was replaced by Cs⁺ to block putative potassium conductance.

Composition of the internal solution (mM) for whole-cell measurements:

50 mM CsCl, 10 mM NaCl, 2 mM MgCl₂, 60 mM CsF, 20 mM EGTA and 10 mM HEPES, pH adjusted to 7.2 with KOH (osmolarity: 288 mOsmol). Cs⁺ in the solution blocks outward potassium channel currents, and the hydrate-covering of fluoride can block possible anion chloride channels. This solution was created by the Nanion Company to specifically detect non-selective cation channel currents (Nanion Technologies, Munich, Germany).

Composition of the external solution (mM) for whole-cell measurements:

140 mM NaCl, 4 mM KCl, 1 mM MgCl₂, 2 mM CaCl₂, 5 mM D-glucose monohydrate and 10 mM HEPES, pH adjusted to 7.4 with NaOH (osmolarity: 298 mOsmol). This solution was also created by the Nanion Company to specifically detect non-selective cation channel currents (Nanion Technologies, Munich, Germany).

Chemicals:

Icilin was purchased from Cayman Chemical Company (Ann Arbor, Michigan, USA), BCTC and fura-2/AM from TOCRIS Bioscience (Bristol, United Kingdom). All other reagents were purchased from Sigma-Aldrich (Deisenhofen, Germany). All reagents were dissolved in dimethyl sulfoxide (DMSO). DMSO vehicle effects were not encountered and its concentration did not exceed 0.1 %.

2.2 Cell culture of human corneal endothelial cells (HCEC)

Since the supply of corneal grafts is limited, established cell lines are commonly used for investigating HCEC. In this thesis, the immortalized HCEC-12 cell line was kindly provided by Dr. Monika Valtink (Institute of Anatomy, TU Dresden, Germany). Originally, this cell line was established according to the method by Bednarz et al. In particular, electroporation was used for the transfection of HCEC with a plasmid containing the early region of simian virus 40 (SV40), causing immortality of the cells (Bednarz et al., 2000). The advantage of working with this HCE cell line, which is now available commercially (DSMZ, Braunschweig, Germany), is that it permanently provides homogenous cells which express properties similar to those of primary cultivated HCEC (Valtink et al., 2008). Therefore, it is possible to achieve highly reproducible results, independent from HCE tissue availability.

A culture of normal HCEC was used as a control, which was also kindly provided by Dr. Monika Valtink (Institute of Anatomy, TU Dresden, Germany). These cells were freshly isolated from a donor cornea that was rated unsuitable for transplantation (Engelmann et al., 1988).

The cells were cultivated as already described by several working groups (Valtink et al., 2008; Mergler et al., 2010). In brief, cells were grown on coverslips in a humidified atmosphere containing 5 % CO₂ at 37 °C. The medium contained 10 % fetal calf serum and antibiotics (100 IU/ml penicillin/streptomycin). The cells were subcultured with accutase, whose chemical reaction was stopped by rinsing with serum-supplemented growth medium. Then, the cells were centrifuged at 800 rcf for 5 minutes and subsequently seeded at a split ratio of 1:10 onto T25 flasks. The medium was changed three times a week. At the earliest, experiments were started from 24 h after seeding.

2.3 Intracellular Ca²⁺ measurements with fura-2/AM (calcium imaging)

Calcium imaging is a highly sensitive calcium detecting method to visualize and measure the intracellular calcium concentration ($[Ca^{2+}]_i$) of a cell (Barreto-Chang and Dolmetsch, 2009). In order to perform calcium imaging, a Ca²⁺ sensitive fluorescent dye has to be applied to the cells. After diffusing across the cell membrane, the dye forms chelate complexes together with ions. This causes a conformational modification in the dye molecule, which leads to a change of its spectral properties. In this study, HCEC-12 cells were pre-incubated with culture medium containing 1 μ M of the fluorescent dye fura-2/AM for 20 - 40 minutes at 37 °C in a dark incubator (5 mM stock solution of fura-2/AM used). After incubation, the coverslips containing fura-2/AM loaded cells were rinsed with the aforementioned Ringer-like solution (see chapter 2.1) (pH adjusted to 7.4). This procedure stopped the staining process and cleaned off any existing cell debris. In some experimental settings, the cells were initially pre-incubated with a drug for 30 - 60 minutes (e.g. 20 μ M icilin or 10 μ M BCTC). For this purpose, the drug was added to the culture medium before the fura-2/AM loading process and also to the Ringer-like solution (approximately 15 minutes pre-incubation time).

The coverslips were then transferred into a chamber containing Ringer-like solution on the stage of an inverted microscope (Olympus BW50WI, Olympus Europa Holding GmbH, Hamburg, Germany). A few minutes later, the cells were adapted to room temperature (\approx 22 °C). Using the microscope, a group of cells with high fluorescence level was selected (fura-2/AM loaded cells are shown in Fig. 23B in chapter 3.3.1).

Fura-2/AM has two absorption wavelengths maxima, namely at 340 nm and 380 nm, and its emission maximum is at 510 nm (Grynkiewicz et al., 1985). The ratio (f_{340}/f_{380}) directly correlates to the concentration of intracellular free calcium ($[Ca^{2+}]_i$) (Grynkiewicz et al., 1985). The digital photometry imaging system (T.I.L.L. Photonics, Munich, Germany) was used to record the fura-2/AM specific fluorescence at these excitation wavelengths. In addition, a photomultiplier, a camera amplifier (both Hamamatsu, Japan) and the software TIDA (HEKA, Lamprecht, Germany) were necessary to perform the measurements.

The single recordings lasted 10 minutes, in which the first 3 minutes were used for detecting the $[Ca^{2+}]_i$ baseline level (set as control). Depending on the experimental design, either some pharmacological TRP channel modulators (e.g. 20 - 50 μ M icilin, 10 μ M BCTC or 20 μ M capsaicin) were added or Ringer-like solution of different temperatures (from 8 °C to 43 °C) was manually applied with a pipette. For the cooling

experiments, a thermometer was used to measure the temperature inside of the chamber and a pump drained the added 3 - 4 ml cold Ringer-like solution parallel to the application to avoid spilling. Simultaneously, the fluorescence responses were recorded by the software.

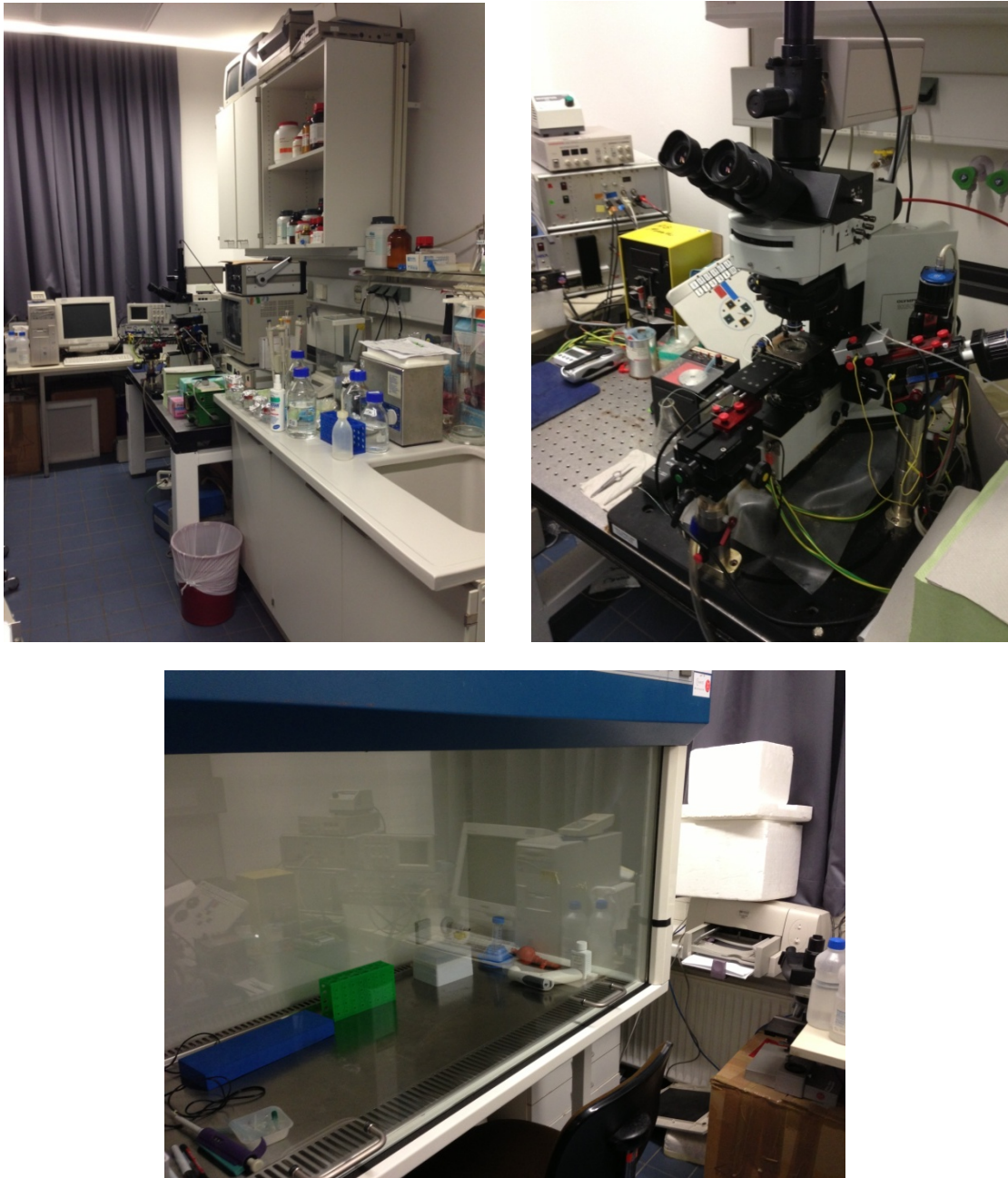


Fig. 11 The calcium imaging lab provided by the Department of Ophthalmology and Gastroenterology at the Charité University, Campus Virchow Clinic (upper left picture). This lab was used for the calcium imaging experiments in this thesis. The upper right picture shows the photometry setup including a microscope, photomultiplier and camera. The picture below shows the workbench for cell cultivation and preparation of experiments. It is noteworthy that the measurements had to be performed in darkness to avoid bleaching effects during the measurements. Only a little flashlight could be used for pipetting.

2.4 Electrophysiological measurements (planar patch-clamp recordings)

In general, the patch-clamp technique enables the study of single ion channels located on the cell membrane (Hamil et al., 1981; Brüggemann et al., 2006). Erwin Neher and Bert Sakmann invented this groundbreaking technique and were awarded the Nobel Prize for Physiology or Medicine in 1991 (Neher and Sakmann, 1976).

In this thesis, the electrophysiological recordings of whole-cell currents were performed with a novel high throughput planar patch-clamp system (*Port-a-Patch*®, Nanion, Munich, Germany, shown in Fig. 12). Notably, this is the smallest patch-clamp setup world-wide (according to the manufacturer).

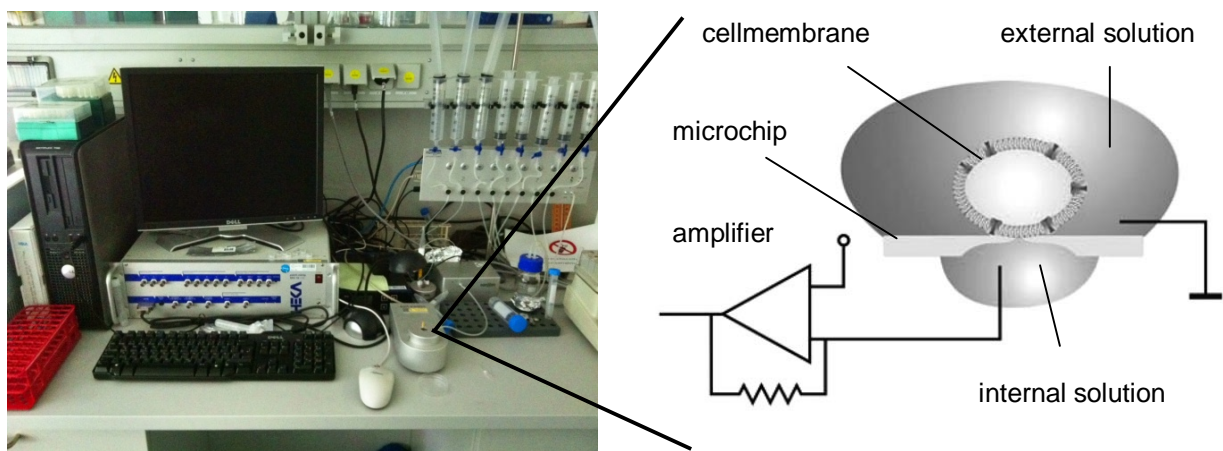


Fig. 12 Planar patch-clamp setup provided by the Department of Ophthalmology at the Charité University, Campus Virchow Clinic (partially supported by Berliner Sonnenfeld-Stiftung; S. Mergler) (left panel). This setup was used for the patch-clamp recordings in this thesis. The planar patch-clamp setup (*Port-a-Patch*®, Nanion, Munich, Germany) consists of a microchip unit, a pump, an amplifier and a PC. A simplified scheme of the microchip unit is shown in the right panel (modified after Brüggemann et al., 2006).

For the experiments, an HCEC-12 cell suspension had to be prepared. At first, the cells were incubated with 500 μl accutase in a 25 ml culture flask at 37 °C to gently detach the cells. After 3 - 5 minutes, medium containing fetal calf serum was added to break the enzymatic reaction. To separate the cells, the suspension was centrifuged at 800 rcf for 2 minutes. The supernatant had to be removed very carefully before re-suspending the cells in 200 - 500 μl extracellular measuring solution depending on cell density (ingredients described in chapter 2.1). It was aspired to achieve a cell density of approximately $1 \times 10^6/\text{ml}$. Fig. 13 shows a HCEC-12 cell suspension under the microscope after this preparation.

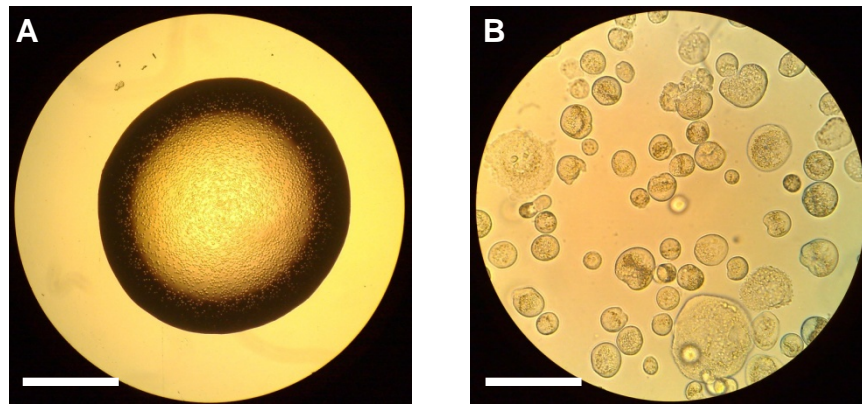


Fig. 13 HCEC-12 cell suspension micrographs. A high quality of the cell suspension is important for successful patch-clamp recordings. The left picture (**A**) shows a drop of HCEC-12 cell suspension prepared for the patch-clamp measurements (scale bar ≈ 0.5 mm). Single cells of different sizes are visible under the microscope using a higher magnification (**B**) (scale bar ≈ 30 μ m).

Before the patch-clamp recordings, the intracellular side of the microchip (resistance of $\approx 2 - 3.5$ M Ω) was filled with the internal solution (see chapter 2.1) (osmolarity: 288 mOsmol). The microchip (Fig. 12, right panel) was then screwed down on the patch-clamp unit. After that, the external solution (see chapter 2.1) (osmolarity: 298 mOsmol) was added to the extracellular side of the chip. Finally, the HCEC-12 cell suspension was pipetted onto the microchip while the software protocol was started. Specifically, the pump induced a negative pressure by the software to attach a single cell onto the aperture of the microchip (cell attached configuration). After sealing, further suction pulses were used to break into the whole-cell configuration, making it possible to measure whole-cell currents. These membrane currents were recorded using an EPC 10 amplifier in connection with the Patchmaster version 2.5 software for Windows (HEKA, Lambrecht, Germany). Membrane capacitance and access resistance of the HCEC-12 cells were calculated with this software and compensated by the patch-clamp amplifier. A mean access resistance (R_s) of 32 ± 5 M Ω ($n = 10$) and a mean membrane capacitance (C_m) of 9.2 ± 0.3 pF ($n = 10$) were registered. All experiments were performed at room temperature (≈ 22 $^{\circ}$ C) in an air-conditioned room. The holding potential was set to 0 mV to avoid any voltage-dependent Na $^{+}$ and Ca $^{2+}$ channel activity. In this configuration, cells were electrophysiologically measured by applying voltage stimulation and drugs such as the cooling agent icilin (20 and 60 μ M) and capsaicin (20 μ M) to study the behaviour of cold and heat receptors. Whole-cell currents were recorded for 400 ms using voltage steps ranging between -60 and

+130 mV (10 mV increments). In addition, current-voltage relations were obtained from voltage ramps from -60 to +130 mV (500 ms duration). The resulting currents were normalized by dividing the current amplitude (pA) through the cell membrane capacitance (pF) to obtain current density ([pA/pF]).

2.5 Data analyses and statistics

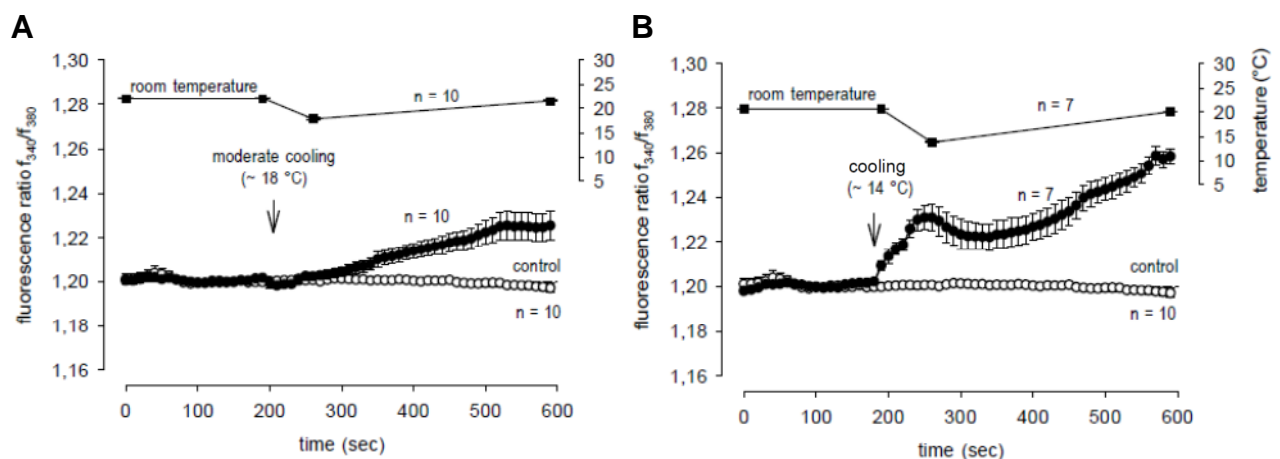
In each experiment, the number of replicate tests is stated in brackets, as well as close to the graphs or bars. All values are reported as means \pm SEM. If the values were normally distributed according to Gaussian distribution, statistical significance was determined using Student's *t*-test. The paired Student's *t*-test was used to compare paired observations on the same subject (for example, measuring the intracellular Ca²⁺ level before and after changing the temperature). For unpaired observations, the unpaired Student's *t*-test was carried out to compare two different sets of experiments (for instance, analyzing the intracellular Ca²⁺ level after adding one drug versus another drug at a certain time point). If the numbers did not show a normal distribution, the non-parametric Mann-Whitney test was used to calculate a possible significant difference between two samples of independent measurements. P values < 0.05 were considered to be significant. The statistical analyses were performed with SigmaPlot software version 12.3 (Systat Software, San Jose, California, USA) and GraphPad Prism version 5 (GraphPad Software, California, USA). All plots and bar charts were also generated with this software.

3. Results

3.1 Expression of thermo-TRPs in HCEC-12 cells

3.1.1 Effect of cooling on $[Ca^{2+}]_i$ in HCEC-12 cells

In the following experiments, fluorescence calcium imaging (fura-2/AM) was used for detecting changes of intracellular Ca^{2+} in HCEC-12 cells after cold stimulation. Fig. 14A illustrates the effect of moderate cooling from room temperature ($\approx 22^\circ C$) to about $18^\circ C$ on the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$). The f_{340}/f_{380} fluorescence ratio rose from 1.200 ± 0.002 to 1.225 ± 0.006 at 600 sec (\pm SEM; $n = 10$; $p < 0.01$), which is proportional to an increase of intracellular calcium (Grynkiewicz et al, 1985). Lower cooling from room temperature to about $14^\circ C$ caused a higher increase of $[Ca^{2+}]_i$ (Fig. 14B). At this cold stimulation, the f_{340}/f_{380} fluorescence ratio increased from 1.200 ± 0.002 to 1.233 ± 0.017 at 350 sec (\pm SEM; $n = 7$; $p < 0.01$). Strong cooling from room temperature below $10^\circ C$ resulted in heterogeneous Ca^{2+} responses as shown in Fig. 14C. Due to inhomogeneous data, the Ca^{2+} changes did not reach any statistical significance. Fig. 14D summarizes these cooling experiments with cold stimulation and shows the statistical analysis. Taken together, moderate cold stimulation induced Ca^{2+} influxes, whereas stronger cooling induced heterogeneous Ca^{2+} responses. These temperature-induced effects are reflective of selective increases in thermo-TRP channel activation.



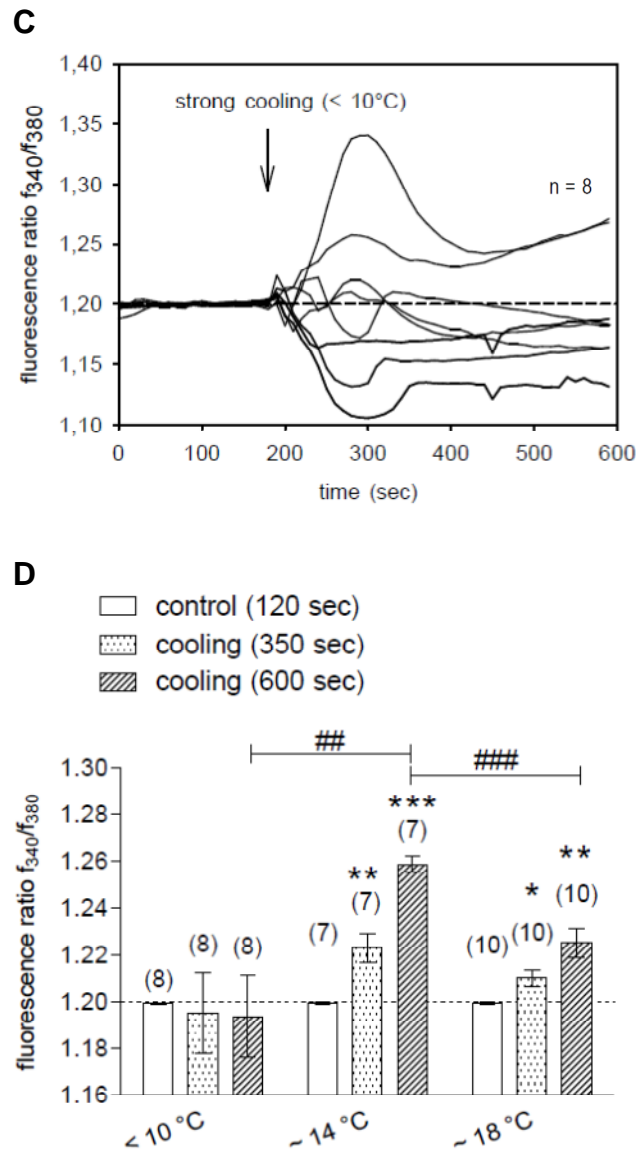


Fig. 14 Various intracellular Ca^{2+} responses of HECE-12 cells after cold stimulation. The temperature was lowered at the time indicated by arrows. Data are mean \pm SEM of 7 - 10 experiments. **(A)** The traces show mean fluorescence ratios which are proportional to the intracellular Ca^{2+} concentration. Several neighboring cells were measured after moderate cooling (filled circles, $n = 10$) and without cooling (open circles, $n = 10$). The corresponding mean temperature trace is shown above the Ca^{2+} traces ($n = 10$). Temperature lowering from $\approx 22^{\circ}\text{C}$ to $\approx 18^{\circ}\text{C}$ led to an increase of intracellular Ca^{2+} . **(B)** Same experimental design as shown in **(A)**. However, the temperature was lowered to $\approx 14^{\circ}\text{C}$ ($n = 7$). **(C)** Single traces show the variety of intracellular Ca^{2+} responses measured after strong cooling below 10°C ($n = 8$). **(D)** Summary of experiments with cold stimulation. The asterisks (*) mark significant differences (at the minimum $p < 0.05$; paired Student's t -test) between control (Ca^{2+} base level at 120 sec) and cooling (at 350 and 600 sec). The hashes (#) signal a significant difference (at the minimum $p < 0.01$; unpaired Student's t -test) between the Ca^{2+} level increases at different temperatures.

3.1.2 Response of $[Ca^{2+}]_i$ to repeated cold stimulation

In this set of experiments, the change of intracellular Ca^{2+} after strong cooling was recorded in HCEC-12 cells. The cold stimulation was repeated after recovery. As shown in Fig. 15, a repeated strong temperature lowering ($< 10\text{ }^{\circ}C$) always caused reproducible decreases in the f_{340}/f_{380} fluorescence ratios. Specifically, it decreased from 1.202 ± 0.0005 to 1.145 ± 0.022 (\pm SEM; $n = 7$; $p < 0.05$) after the first strong cold stimulation. Similarly, it decreased from 1.183 ± 0.008 to 1.128 ± 0.014 after the second cooling (\pm SEM; $n = 7$; $p < 0.05$). Notably, the cold-induced decrease of $[Ca^{2+}]_i$ almost returned to baseline after the first cooling and had the same tendency after the second cooling.

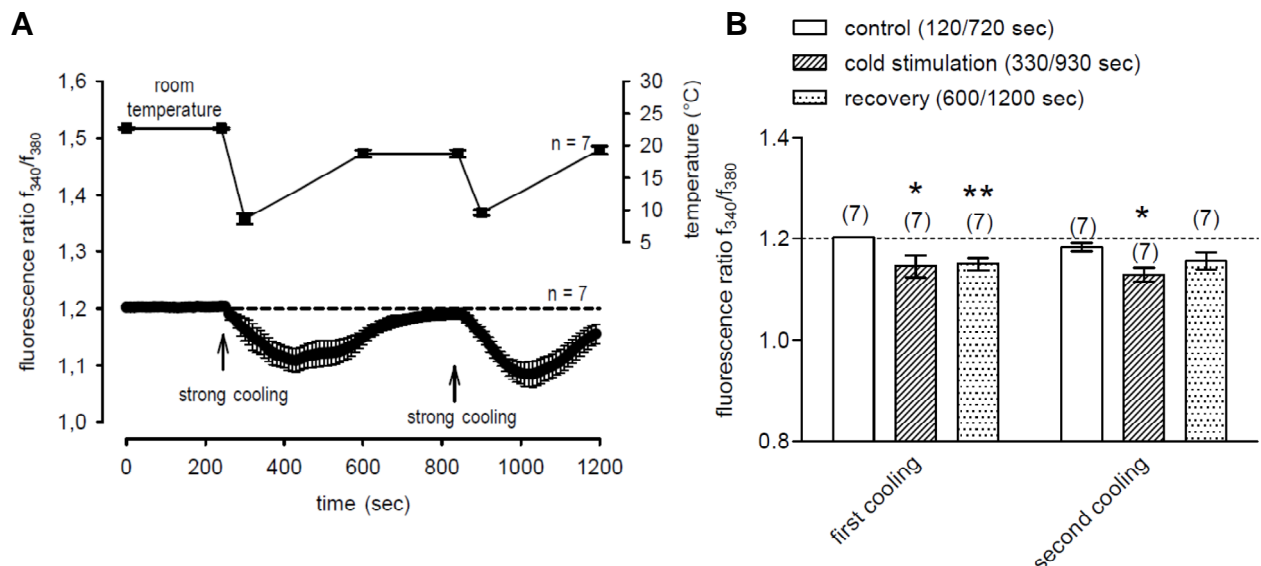


Fig. 15 Change in intracellular Ca^{2+} concentration in HCEC-12 cells caused by repeated strong cooling. The temperature changes were carried out at the time indicated by the arrows. Data are mean \pm SEM of 7 experiments. **(A)** The graph shows $[Ca^{2+}]_i$ of several neighboring cells measured after cooling ($n = 7$). The corresponding temperature course is shown above the Ca^{2+} trace ($n = 7$). First and second lowering of bath solution temperature from 22 to below 10 $^{\circ}C$ resulted in Ca^{2+} level decrease. **(B)** Summary of the experiments with thermal stimuli in HCEC-12 cells. The asterisks (*) indicate a statistically significant difference (at the minimum $p < 0.05$) between controls (Ca^{2+} base levels) and first cooling at 330 sec, second cooling at 930 sec, and recovery at 600 and 1200 sec, respectively. The paired Student's t -test was used.

3.1.3 Effect of moderate cold stimulation on $[Ca^{2+}]_i$ in HECE-12 cells in the presence of the TRPM8 channel antagonist BCTC

To directly delineate thermo-sensitive TRPM8 activity, the temperature was lowered to 18 °C in the presence and absence of the TRPM8 antagonist BCTC (Fig. 16) (Behrendt et al., 2004). To block TRPM8, the cells were pre-incubated with 10 μ M BCTC for 30 - 60 minutes. As shown in Fig. 16A, the Ca^{2+} increases (n = 6) induced by moderate cooling were significantly suppressed in the presence of 10 μ M BCTC at 350 sec compared to those without BCTC (data shown in Fig. 14A). Whereas the f_{340}/f_{380} ratio at 350 sec was 1.210 ± 0.011 (n = 10), the level was significantly reduced to 1.200 ± 0.005 (n = 6; $p < 0.05$) in the presence of BCTC. Overall, specific moderate cooling to 18 °C and the utilization of BCTC reflect TRPM8 channel activity.

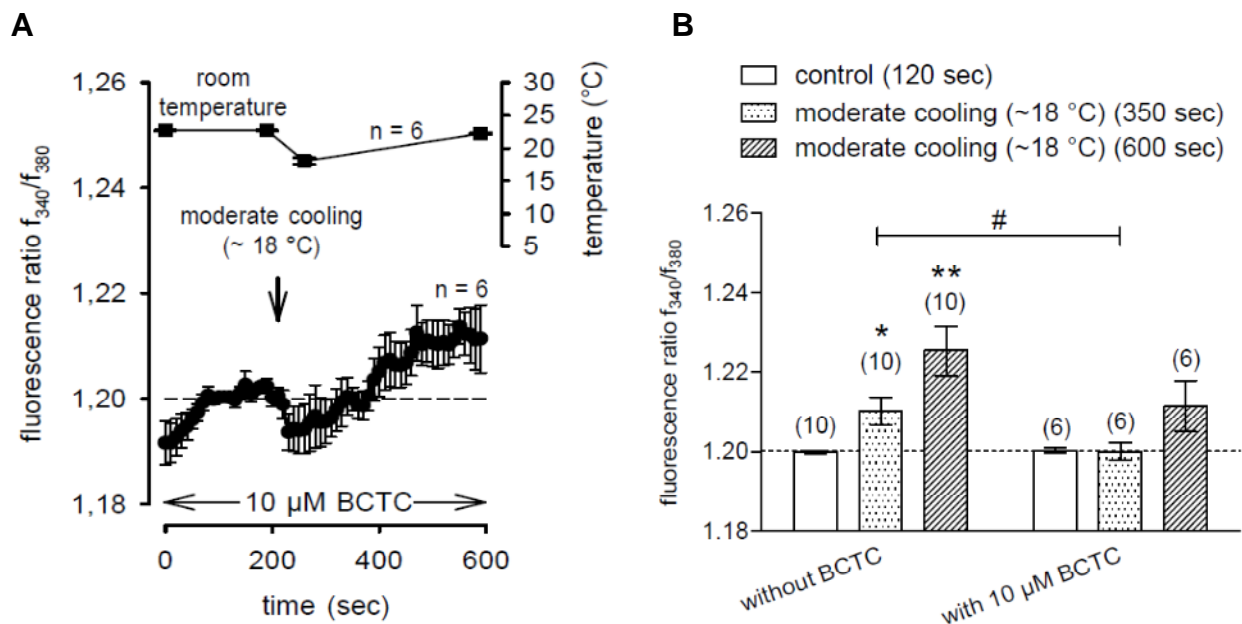


Fig. 16 Effect of the TRPM8 channel antagonist BCTC on the Ca^{2+} entry induced by moderate cooling. The cooling was performed at the time indicated by the arrow. The temperature graph is shown above the Ca^{2+} trace (n = 6). HCEC-12 cells were incubated with 10 μ M BCTC before and during the experiment. Data are mean \pm SEM of 6 - 10 experiments. **(A)** Temperature lowering from ≈ 22 °C to ≈ 18 °C led to a small increase in intracellular Ca^{2+} while BCTC was present. **(B)** Comparison of two moderate cooling experiments (Fig. 14A and Fig. 16A). The asterisks (*) show significant differences (at the minimum $p < 0.05$; paired Student's *t*-test) between control (Ca^{2+} level at 120 sec) and moderate cooling (at 350 and 600 sec). The hash (#) indicates the significance ($p < 0.05$; unpaired Student's *t*-test) between the cold-induced Ca^{2+} increase with and without 10 μ M BCTC.

3.1.4 Icilin increases $[Ca^{2+}]_i$ in HCEC-12 cells through activation of cold receptors

To test for functional cold receptor expression in HCEC-12 cells, the changes in intracellular Ca^{2+} were recorded after adding the TRPM8/TRPA1 agonist icilin (McKemy et al., 2002). Application of 50 μ M icilin caused a fast increase in the f_{340}/f_{380} fluorescence ratio (Fig. 17). In particular, f_{340}/f_{380} increased from 1.199 ± 0.001 to 1.210 ± 0.003 at 300 sec (\pm SEM; $n = 6$; $p < 0.05$). The icilin-induced $[Ca^{2+}]_i$ increase was irreversible (no decrease after washout). The results suggest a possible expression of thermo-TRPs such as TRPM8 and/or TRPA1.

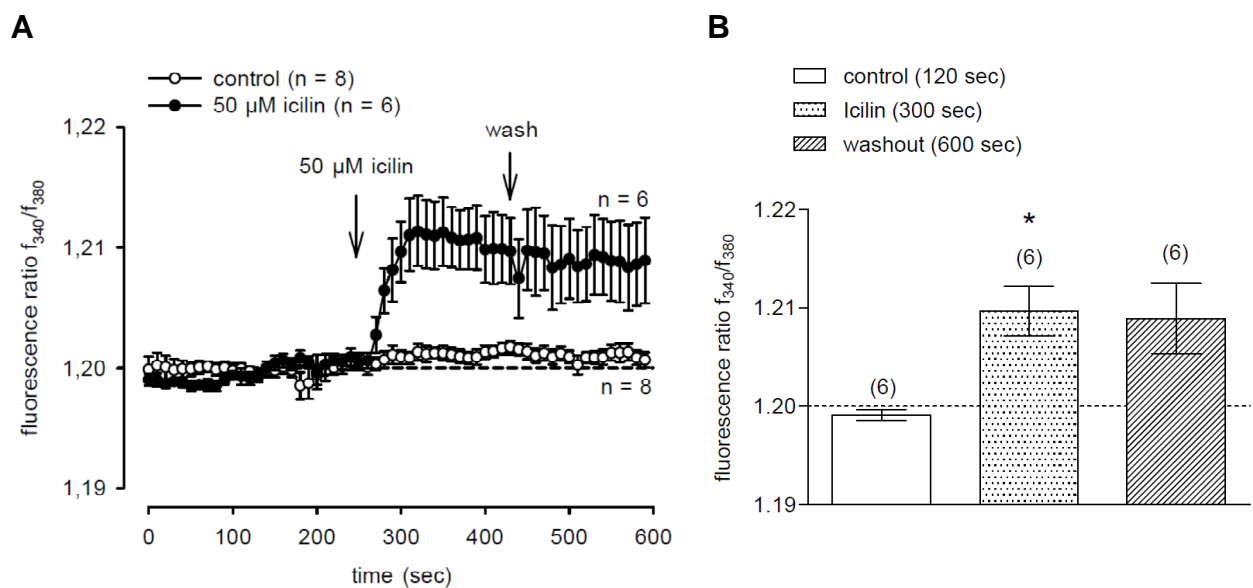
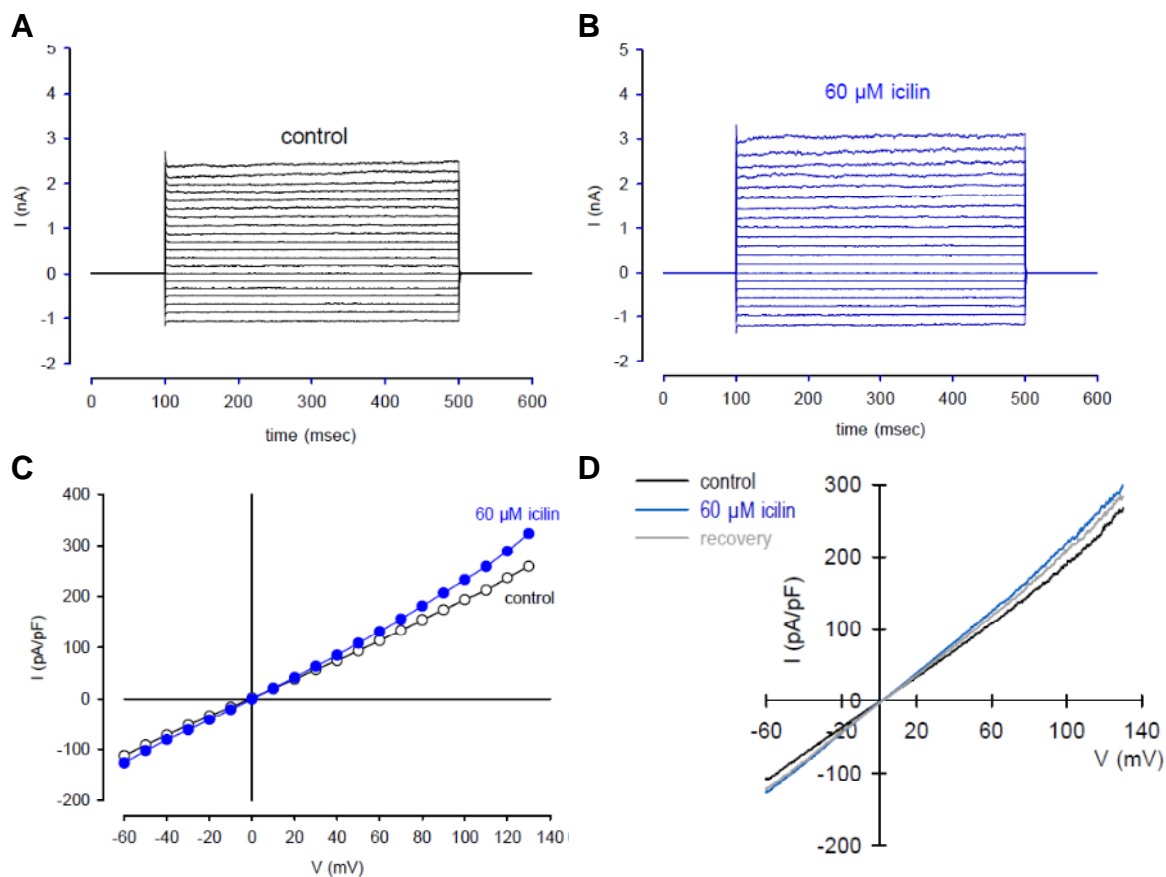


Fig. 17 Effect of the cooling agent icilin on HCEC-12 cells. Arrows indicate the time at which the reagent was added and washed out. Data are mean \pm SEM of 6 - 8 experiments. **(A)** Extracellular application of 50 μ M icilin induced an increase of Ca^{2+} influx. Traces show intracellular Ca^{2+} of several neighboring cells measured with icilin (filled circles) ($n = 6$) and without icilin (open circles) ($n = 8$). **(B)** Summary of the experiments with 50 μ M icilin in HCEC-12 cells. The asterisk (*) indicates a statistically significant difference between the controls (Ca^{2+} base levels) and icilin at 300 sec using paired Student's t -test ($n = 6$; $p < 0.05$).

3.1.5 Icilin activates non-selective cation channel currents in HCEC-12 cells

For investigating the ionic currents underlying thermo-sensitive variations in Ca^{2+} influx, the whole-cell mode of the planar patch-clamp technique was used. This highly sensitive method allows validating the underlying ionic currents of the aforementioned thermo-TRPs at the cell membrane. At first, cells were stimulated according to a specific voltage protocol. Fig. 18A exemplarily shows the corresponding whole-cell current responses. Notably, most of them are non-ionic currents (so-called leak currents) which were not subtracted in this set of experiments. After application of $60 \mu\text{M}$ icilin, a slight increase of the currents could be detected (Fig. 18B). Fig. 18C shows the corresponding current-voltage relationship at which the voltages are plotted against the currents. These currents were normalized against the cell surface (pA/pF) so that whole-cell currents from cells of different sizes were comparable. A slightly icilin-induced outward current could be detected, which increased from $260 \pm 10 \text{ pA/pF}$ to $308 \pm 10 \text{ pA/pF}$ ($\pm \text{SEM}$; $n = 3$; $p < 0.05$). Similar results were obtained with voltage ramp protocols (-60 to $+130 \text{ mV}$ for 500 ms) in which a slight increase of inward currents could be additionally detected (Fig. 18D). Overall, these results further suggest that there is functional expression of cold receptors such as TRPM8/TRPA1 in HCEC-12 cells.



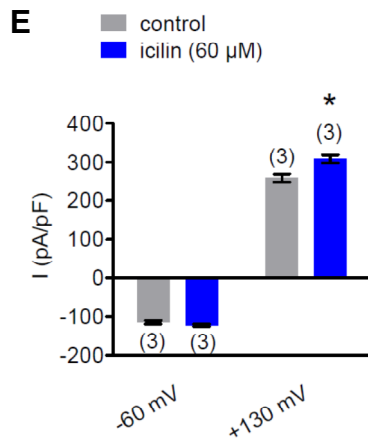


Fig. 18 Increase of whole-cell channel currents by icilin in HCEC-12 cells. Data are mean \pm SEM of 3 experiments. **(A)** Whole-cell channel currents induced by depolarization from -60 mV to +130 mV in 10 mV steps after establishing the whole-cell configuration. Holding potential was set to 0 mV to avoid activation of voltage-operated Ca^{2+} channels. **(B)** Application of 60 μM icilin increased whole-cell channel currents. **(C)** Effect of icilin summarized in a current/voltage plot (I-V plot) (data from A and B). The currents were normalized to capacitance to obtain current density (pA/pF). The upper trace (blue filled circles) was obtained after application of 60 μM icilin and the lower trace (open circles) under control conditions. Icilin-induced increases in outward currents were discernable at potentials above approximately +60 mV. **(D)** Original traces of icilin-induced TRPM8/TRPA1 channel responses to voltage ramps from -60 mV up to +130 mV. The traces show currents before application of 60 μM icilin (black), during the stimulation by application of 60 μM icilin (blue), and after washout (grey). The currents were normalized to capacitance to obtain current density (pA/pF). **(E)** Comparison of inward and outward current densities in the absence (grey bar) and presence (blue bar) of 60 μM icilin in HCEC-12 cells at -60 mV and +130 mV. The asterisk (*) denotes a statistically significant increase by application of icilin using paired Student's *t*-test (\pm SEM; $n = 3$; $p < 0.05$).

3.1.6 The icilin-induced Ca^{2+} entry is suppressed by the TRPM8 channel antagonist BCTC

To further investigate putative functional TRPM8 activity, the TRPM8 channel blocker BCTC was applied (Behrendt et al., 2004). While 20 μM icilin increased the f_{340}/f_{380} fluorescence ratio from 1.200 ± 0.002 to 1.220 ± 0.014 at 400 sec (\pm SEM; $n = 18$; $p < 0.0001$, Fig. 19B), the application of 20 μM BCTC led to a significant suppression of Ca^{2+} influx to 1.205 ± 0.009 at 400 sec (\pm SEM; $n = 18$; $p < 0.0001$, Fig. 19C). This shows that the effect of icilin is clearly suppressed by BCTC. In summary, the results are indicative of TRPM8 activity.

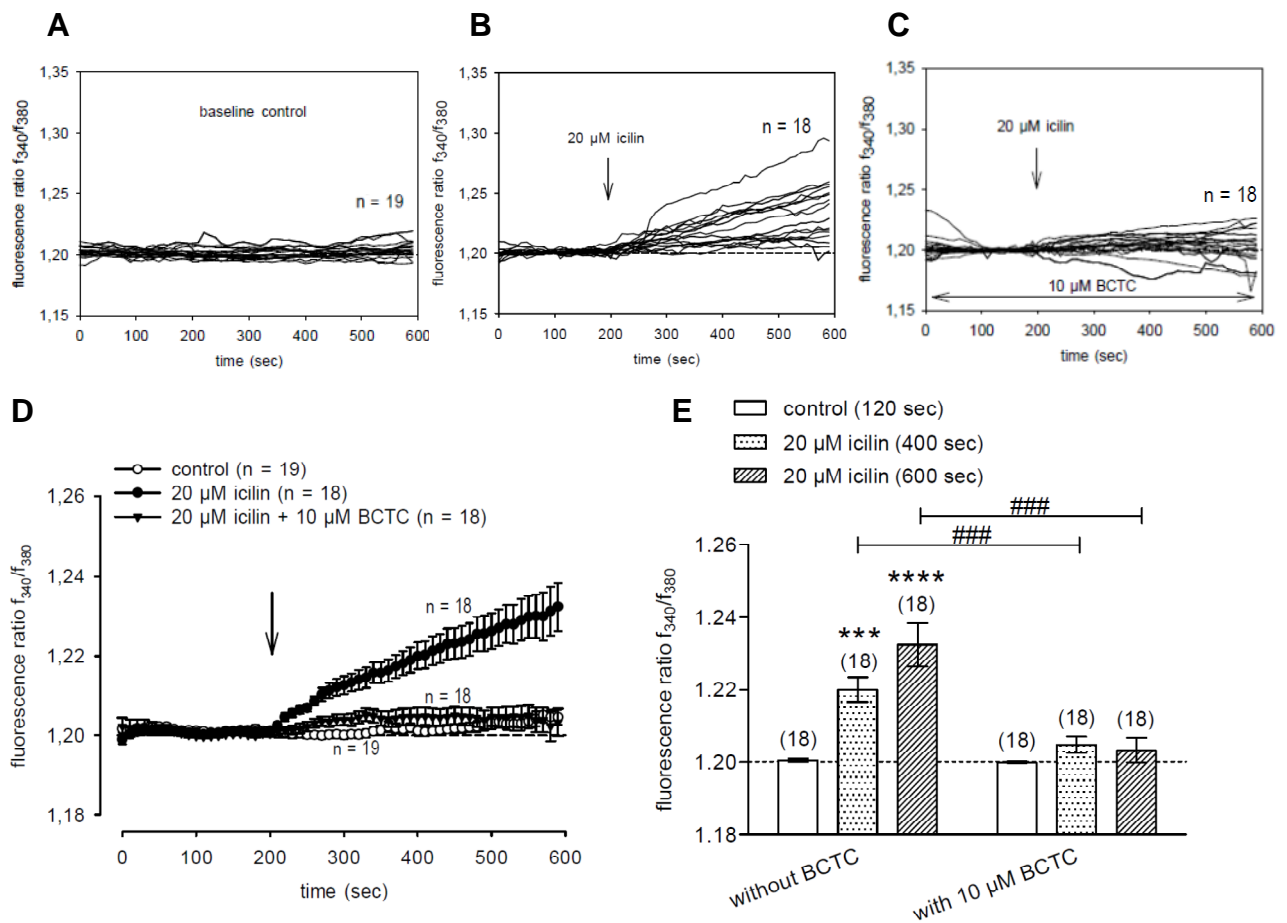


Fig. 19 The TRPM8 channel antagonist BCTC suppressed the icilin-induced Ca^{2+} entry in HCEC-12 cells. Reagents were added at the time indicated by arrows. Data are means \pm SEM of 18 - 19 experiments. **(A)** Ca^{2+} baselines were recorded as controls ($n = 19$). **(B)** The application of 20 μM icilin caused a Ca^{2+} influx ($n = 18$). **(C)** By adding 10 μM of the TRPM8 blocker BCTC, the icilin-induced Ca^{2+} increase was clearly suppressed. **(D)** and **(E)** Summary of the experiments with icilin and BCTC. The asterisks (*) indicate a significant difference ($p < 0.005$; paired Student's t -test) before icilin application (control at 120 sec) and after application of icilin at 400 and 600 sec. The hashes (#) mark a significant difference ($p < 0.005$; unpaired Student's t -test) between the icilin-induced Ca^{2+} increase with and without the TRPM8 channel blocker BCTC.

3.1.7 Effect of heating on $[Ca^{2+}]_i$ in HCEC-12 cells

Since the various aforementioned cold-induced effects are reflective of selective increases in thermo-TRP channel activation, other thermo-TRPs may play a role in this connection. Recently, heat receptors such as TRPV1 could be detected in HCEC-12 cells and in normal HCEC (Mergler et al., 2010). Heat stimulation experiments as shown in Fig. 20 confirmed this observation. Specifically, it could be shown that the f_{340}/f_{380} fluorescence ratio clearly increased from 1.203 ± 0.002 to 1.318 ± 0.030 at 300 sec (\pm SEM; $n = 8$; $p < 0.01$) after heating from room temperature ($\approx 22^\circ\text{C}$) to over 43°C . This heat-induced $[Ca^{2+}]_i$ increase was irreversible. Since the temperature threshold for activation of TRPV1 was exceeded ($> 43^\circ\text{C}$, Tominaga and Caterina, 2004), these results reinforce the expression of TRPV1 in HCEC-12 cells. Therefore, further experiments were carried out to investigate a possible interaction between heat and cold receptors as shown in the following chapter.

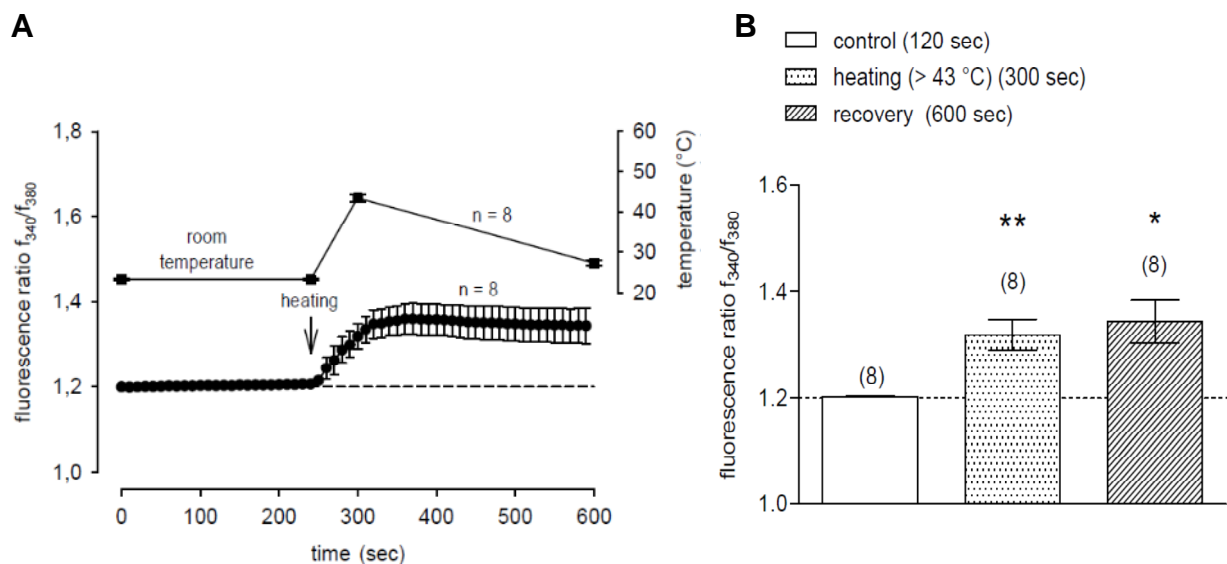
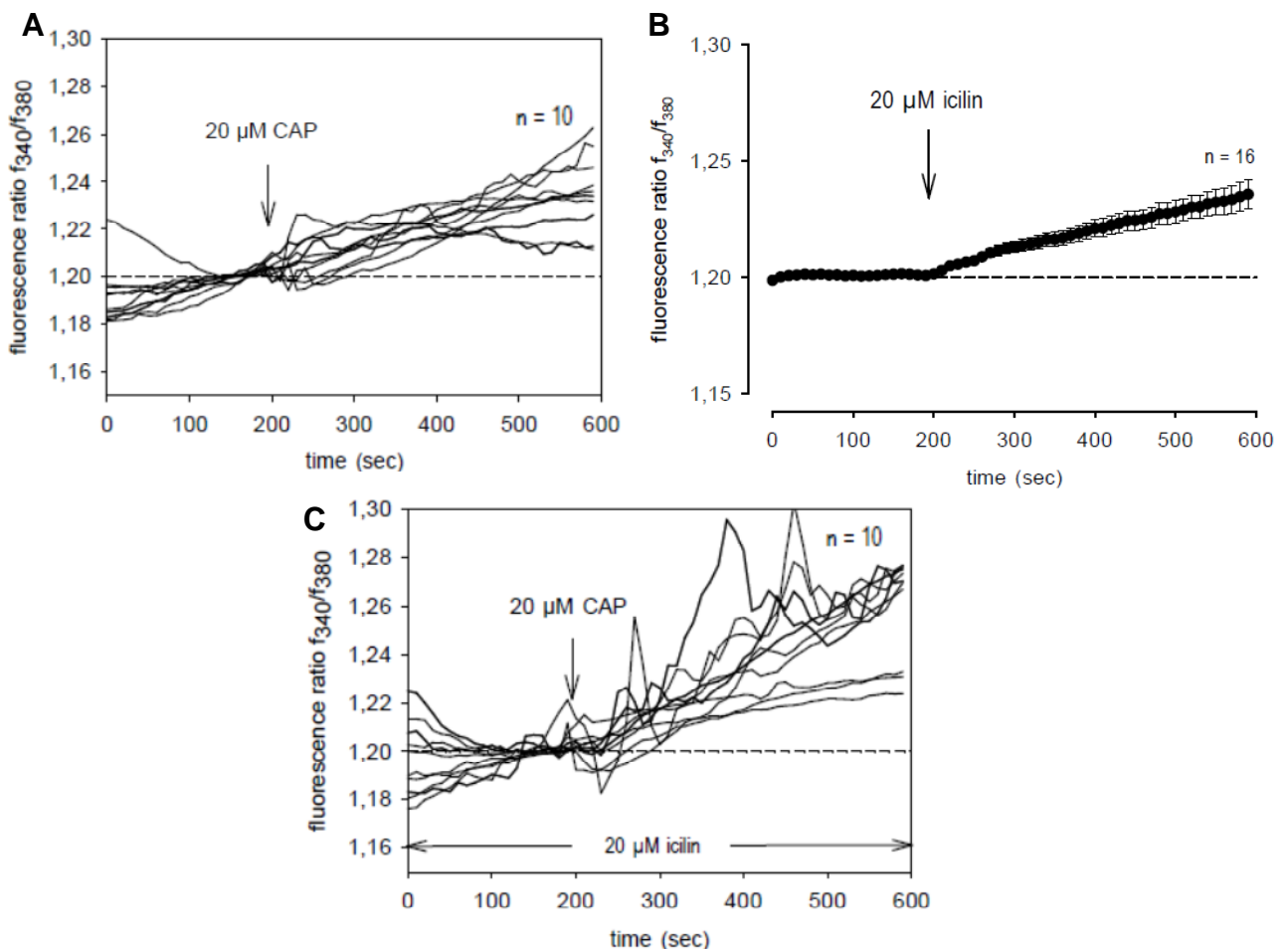


Fig. 20 Increase of the intracellular Ca^{2+} level caused by heat exposure. The temperature change was carried out at the time indicated by the arrow. Data are mean \pm SEM of 8 experiments. **(A)** The graph shows the increase of intracellular Ca^{2+} after heat stimulation from $\approx 22^\circ\text{C}$ to over 43°C ($n = 8$). The corresponding temperature course is shown above the Ca^{2+} trace ($n = 8$). **(B)** Summary of the heating experiments. The asterisks (*) indicate a statistically significant difference (at the minimum $p < 0.05$) between controls (Ca^{2+} base levels) and heat at 300 and 600 sec using paired Student's t -test.

3.2 Interaction of cold and heat receptors in HCEC-12 cells

3.2.1 Simultaneous pharmacological activation of TRPM8/TRPA1 and TRPV1

To elucidate a possible interaction between heat and cold receptors in HCEC-12, the TRPV1 selective agonist capsaicin (CAP) and the TRPM8/TRPA1 agonist icilin were both applied in the following series of experiments. As expected, CAP increased the intracellular Ca^{2+} level (Fig. 21A). The f_{340}/f_{380} fluorescence ratio significantly increased from 1.199 ± 0.002 (control) to 1.222 ± 0.006 at 400 sec in the presence of 20 μM CAP (\pm SEM; $n = 10$; $p < 0.005$). Similar results were obtained using 20 μM icilin (Fig. 21B). In this case, the f_{340}/f_{380} fluorescence ratio rose from 1.201 ± 0.002 to 1.221 ± 0.015 at 400 sec (\pm SEM; $n = 16$; $p < 0.005$). Finally, the HCEC-12 cells were pre-incubated with 20 μM icilin for 30 minutes before applying 20 μM CAP (Fig. 21C). Notably, both icilin and CAP induced a higher Ca^{2+} increase than one drug alone. With both drugs, the f_{340}/f_{380} fluorescence ratio even increased from 1.201 ± 0.002 to 1.238 ± 0.020 at 400 sec (\pm SEM; $n = 10$; $p < 0.005$). Fig. 21D summarizes these experiments and reveals that there seems to be an additional effect of these two agonists. This indicates a possible interactivity between heat and cold receptors.



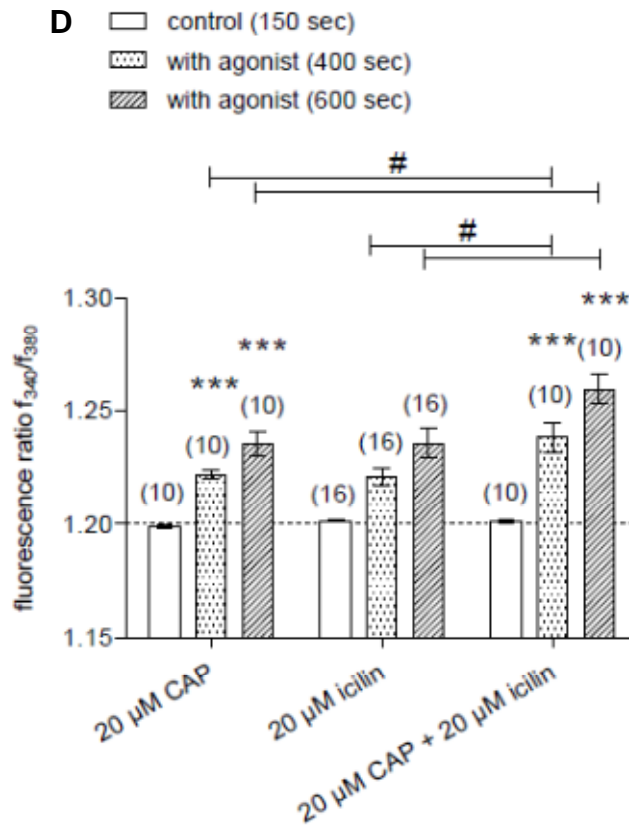
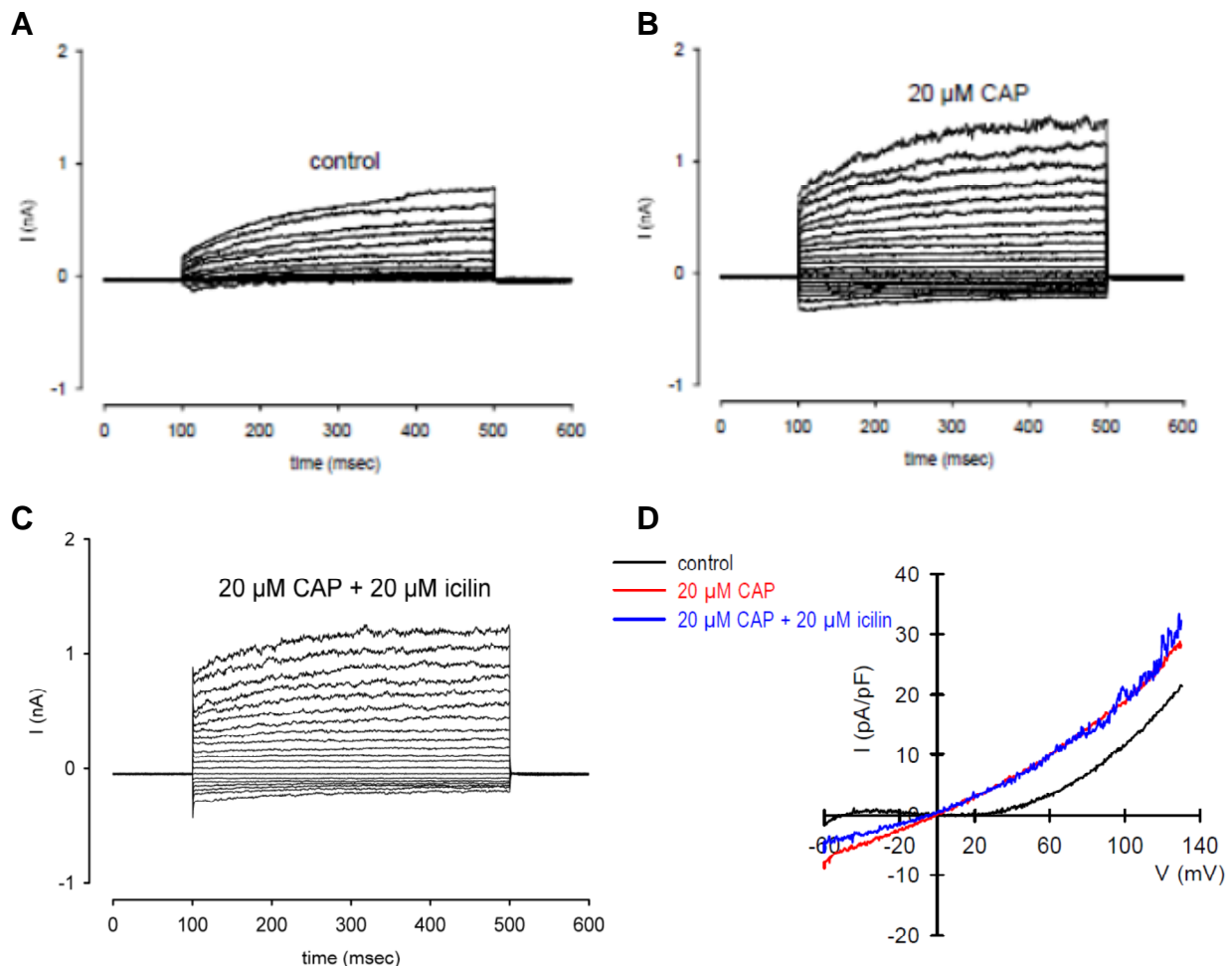


Fig. 21 The Ca^{2+} influx in HCEC-12 cells caused by the TRPV1 channel agonist CAP is modified by the TRPM8/TRPA1 channel agonist icilin. The agonists were added at the time indicated by arrows. Data are means \pm SEM of 10 - 16 experiments. (A) The traces show the rising intracellular Ca^{2+} levels while TRPV1 channels are selectively activated by 20 μM CAP ($n = 10$). (B) This graph illustrates the Ca^{2+} influx caused by TRPM8/TRPA1 after application of 20 μM icilin ($n = 16$). (C) In HCEC-12 cells which were incubated with 20 μM icilin before and during the experiment, the CAP-induced Ca^{2+} influx was at higher levels than without icilin ($n = 10$). (D) Summary of the experiments with 20 μM CAP and 20 μM icilin in HCEC-12 cells. The asterisks (*) mark significant differences ($p < 0.005$; paired Student's t -test) between the control (Ca^{2+} level at 150 sec) and the agonist at 400 and 600 sec. The hashes (#) indicate the significance ($p < 0.05$; unpaired Student's t -test) between the agonist at 400 sec and both agonists at 400 sec, as well as the significance between the agonist at 600 sec and both agonists at 600 sec.

3.2.2 CAP and icilin increased whole-cell currents

To further investigate the interaction of heat and cold receptors in HCEC-12 cells, whole-cell currents were measured. However, in this set of experiments leak currents were subtracted to better identify putative inward currents. Notably, Fig. 22B demonstrates increased inward currents carried by Ca^{2+} ions that were activated by 20 μM CAP. This confirms previous calcium imaging experiments in which TRPV1 activation led to an increase of $[\text{Ca}^{2+}]_i$. Furthermore, outwardly rectifying currents could be detected. Specifically, at -60 mV the inward currents rose from -7 ± 1 pA/pF to -18 ± 4 pA/pF (\pm SEM; $n = 6 - 7$; $p < 0.05$), whereas the outward currents rose from 30 ± 8 pA/pF to 96 ± 33 pA/pF (\pm SEM; $n = 6 - 7$; $p < 0.01$). A very similar effect could be detected while using 20 μM icilin and 20 μM CAP simultaneously (Fig. 22C). Under these conditions, the inward currents increased from -7 ± 1 pA/pF to -16 ± 7 pA/pF at -60 mV (\pm SEM; $n = 5$; $p < 0.01$) and the outward currents increased from 30 ± 8 pA/pF to 88 ± 15 pA/pF at 130 mV (\pm SEM; $n = 5$; $p < 0.01$). The voltage ramp protocol (Fig. 22D) confirms these results. Fig. 22E summarizes the experiments with CAP and icilin and demonstrates the significant increase of whole-cell currents.



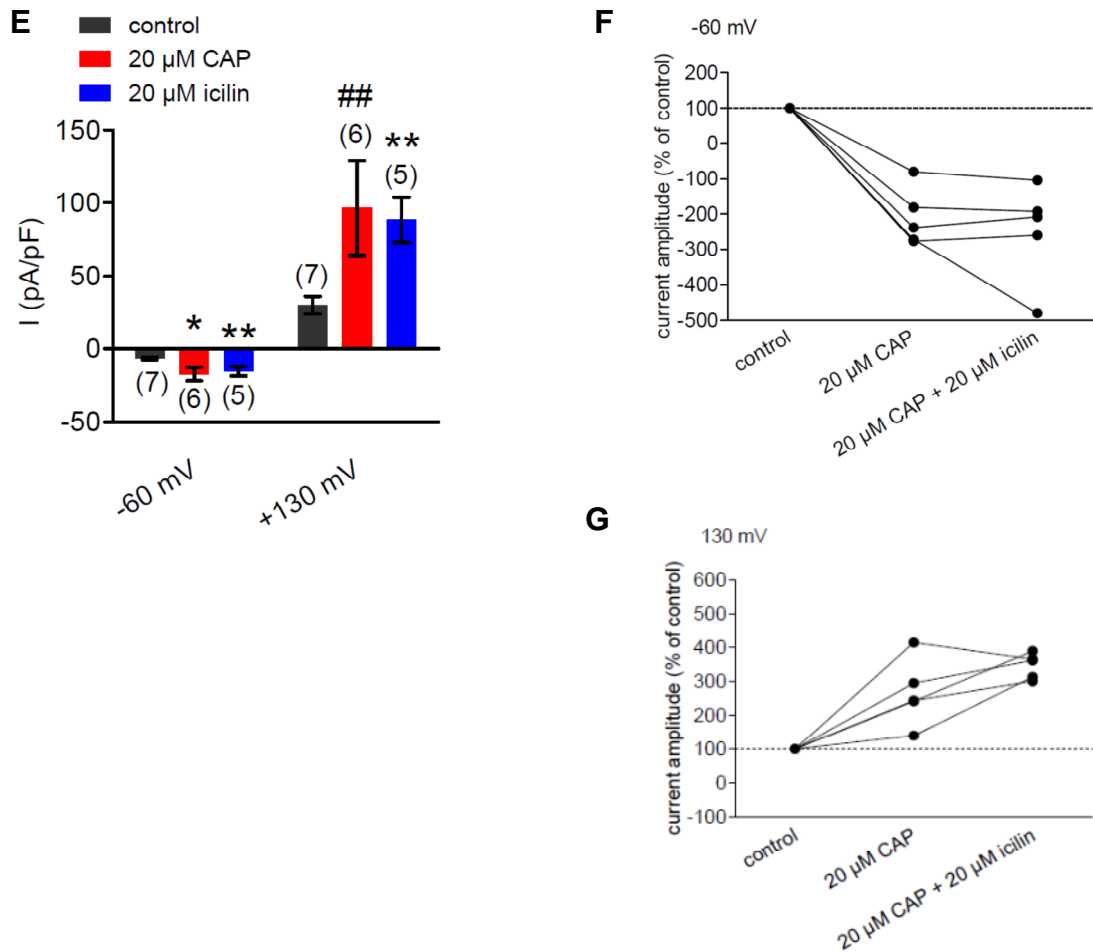


Fig. 22 Activation of whole-cell cation channel currents by CAP and icilin in HCEC-12 cells. Currents were induced by depolarization from -60 mV to 130 mV in 10 mV steps (400 ms). Leak currents were subtracted. Data are mean \pm SEM of 5 - 7 experiments. **(A)** Whole-cell currents under control conditions using the same voltage protocol as shown in Fig. 18. **(B)** The TRPV1 agonist CAP (20 μ M) increased both inward and outward cation channel currents. **(C)** After washing out CAP and adding 20 μ M icilin, the current response did not change. **(D)** The effects of CAP and icilin are summarized in a current/voltage plot (I-V plot; data from A, B and C). The currents were normalized to capacitance to obtain current density (pA/pF). The red trace was obtained with 20 μ M CAP and the black trace without CAP. Adding 20 μ M icilin made no difference in the CAP-induced current increases (blue trace). **(E)** Summary of the patch-clamp experiments with CAP and icilin in HCEC-12 cells. The asterisks (*) represent the significance of the inward currents with and without CAP or icilin ($n = 5 - 7$; $p < 0.05$; unpaired Student's t -test). The hashes (#) indicate a significant difference between the outward currents with and without CAP ($n = 6$; $p < 0.01$; Mann-Whitney test). **(F)** Maximal negative current amplitudes induced by a voltage step from 0 to -60 mV are expressed as percentage of control values before applying 20 μ M CAP. The CAP-induced inward currents were not influenced by 20 μ M icilin (after CAP washout). **(G)** Maximal positive current amplitudes induced by a voltage step from 0 to 130 mV are expressed as percentage of control values before application of 20 μ M CAP. In this case, the CAP-induced outward currents were also not influenced after washout of CAP and application of 20 μ M icilin.

3.3 Normal HCEC compared to HCEC-12 cells

3.3.1 Morphology of the cells

HCEC appear polygonal with a diameter of $\approx 22 \mu\text{m}$ and an area of $\approx 250 \mu\text{m}^2$ *in vivo* (Schroeter and Rieck, 2009). Fig. 23A shows HCEC with their typical hexagonal shape viewed through a microscope at 40 x magnification. In contrast, the cultivated HCEC-12 cells are smaller and have irregular shapes. Fig. 23B shows cultivated HCEC-12 cells (one day after seeding) loaded with the fluorescent dye fura-2/AM. Especially after a short cultivation time (experiments performed 24 h after seeding), some cells seemed to stretch out as if trying to contact each other. At this point, mostly triangular shaped cells were visible. When cells were cultivated longer after seeding (48 - 72 h), the cell density increased and the cell shapes were to some extent comparable to the cells *in vivo* (Fig. 23C). A few spherical shaped cells with a higher fluorescence level could be detected with the fluorescence microscope (Fig. 23B and 23C; arrows). It is most likely that these are dead cells which have detached from the surface area. They were not considered for the measurements. As a control, a culture of normal HCEC from a human donor cornea was used. These cells grew less dense but showed extremely large surfaces (Fig. 23D).

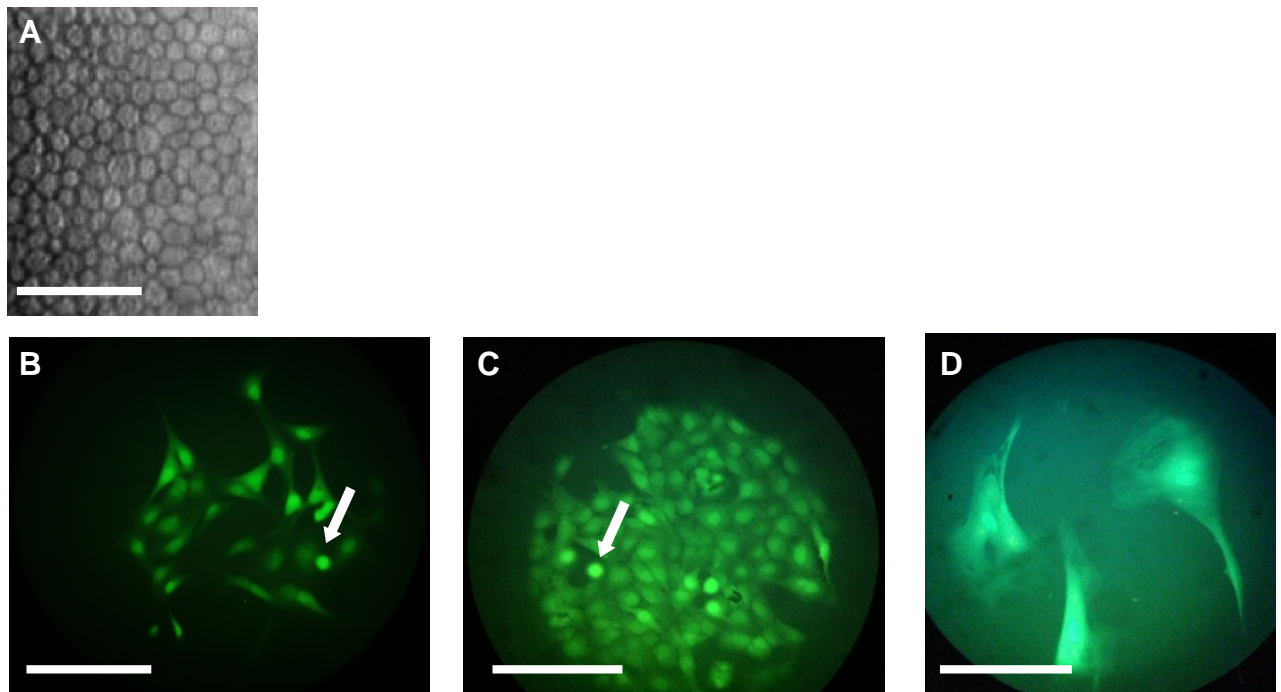


Fig. 23 HCEC micrograph. **(A)** Typical hexagonal shaped HCEC *in vivo* viewed through a microscope at 40 x magnification (picture taken from <http://www.fuhlendorf.info/images/stories/fuhlendorfjpeg/optik/messgeraete/Endothelkamera/endothel.gif>). The scale bar is $\approx 100 \mu\text{m}$. **(B)** Cultivated HCEC-12 cells (one day after seeding) loaded with $1 \mu\text{M}$ fura-2/AM and viewed through a microscope at 510 nm

fluorescence emission. At that stage, HCEC-12 cells expressed a low cell density. (C) HCEC-12 cells cultivated for longer periods (> 2 days after seeding) with higher cell density. The arrows show cells which have detached from the surface area. (D) Primary cell culture of normal HCEC from a human donor cornea. These cells express a large cell surface compared to immortalized HCEC-12 cultures.

3.3.2 Icilin increased $[Ca^{2+}]_i$ in normal HCEC

A primary cell culture of HCEC from a normal human cornea was used as a control to validate the functional expression of cold receptors in primary cultivated cells. The bath temperature was above 28 °C to simulate nearly physiological conditions. As in the case of HCEC-12 cells, application of 60 μ M icilin caused an increase in the f_{340}/f_{380} fluorescence ratio from 1.200 ± 0.0001 to 1.207 ± 0.0021 at 600 sec (\pm SEM; $n = 7$; $p < 0.05$) (Fig. 24). These results indicate a functional expression of cold receptors such as TRPM8 and/or TRPA1 in normal HCEC, confirming the data with HCEC-12 cells.

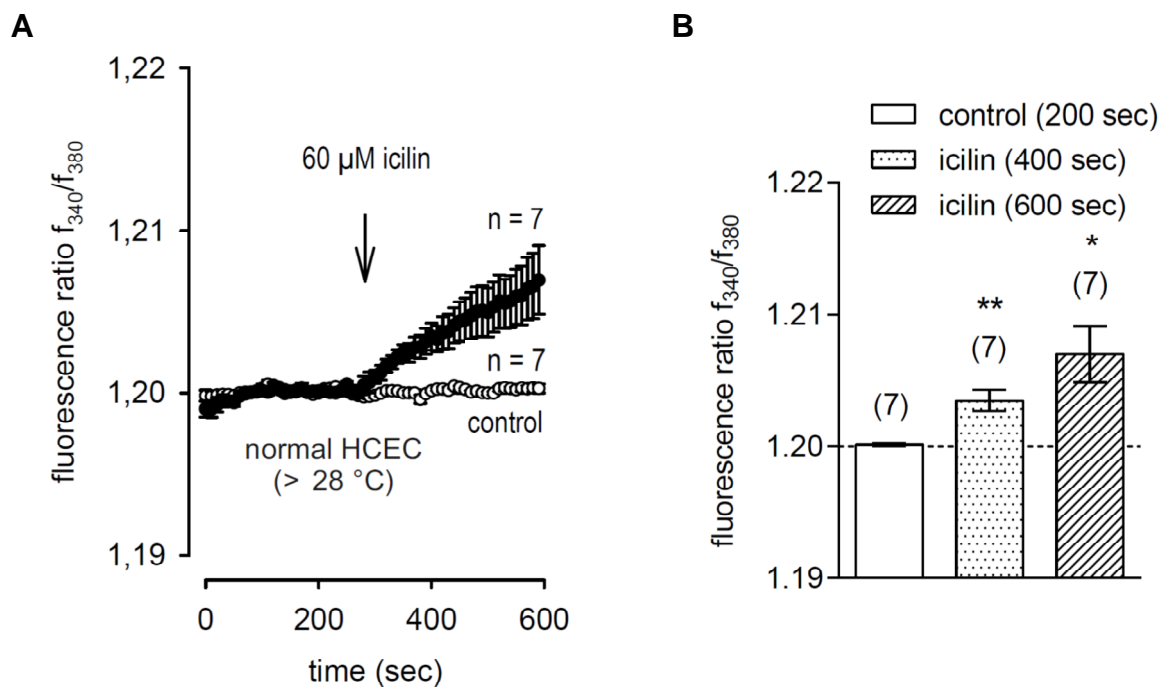


Fig. 24 Effect of the cooling agent icilin on normal HCEC from a human donor cornea under nearly physiological conditions (> 28 °C). Arrows indicate the time at which the reagent was added. Data are mean \pm SEM of 7 experiments. (A) Application of 60 μ M icilin induced an increase of Ca^{2+} influx. Traces show intracellular Ca^{2+} of several neighboring cells measured with icilin (filled circles) ($n = 7$) and without icilin (open circles) ($n = 7$). (B) Summary of the experiments with 60 μ M icilin in normal HCEC. The asterisks (*) show significant differences (at the minimum $p < 0.05$; paired Student's t -test) between control (Ca^{2+} level at 200 sec) and moderate cooling (at 400 and 600 sec).

4. Discussion

The results of this thesis provide convincing evidence that functional thermo-TRP channel expression occurs in the HCE as indicated in the literature (Mergler et al., 2003, 2010, 2012, 2013). This conclusion is supported by experimental data gained with different high sensitive methods such as calcium imaging and the patch-clamp technique using normal HCEC and permanent HCEC-12 cells. This immortalized HCEC-12 cell line is widely accepted and has been successfully used as a model for corneal endothelium in several studies (Bednarz et al., 2000; Valtink et al., 2008; Mergler et al., 2003, 2005, 2010). In this thesis, Ca^{2+} transients and underlying currents could be detected using HCEC-12 cells. They are indicative of thermo-TRP channel activity by changes in temperature and by the utilization of established agonists and antagonists. The responses caused by TRPM8 activation in HCEC-12 cells also occurred in normal HCEC from a human donor cornea under approximately physiological conditions. These results are relevant to the *in vivo* conditions as they demonstrate that the electrophysiological properties of normal HCEC are comparable to those in HCEC-12 cells.

4.1 Cold receptor activity in HCEC-12 cells

Initially, the effect of physical cooling was examined using calcium imaging to investigate the functional expression of putative cold receptors in HCEC-12 cells (Fig. 14). Lowering the bath temperature from room temperature ($\approx 22\text{ }^{\circ}\text{C}$) to below $10\text{ }^{\circ}\text{C}$ was assumed to activate the cold receptors TRPM8 ($\leq 28\text{ }^{\circ}\text{C}$) and TRPA1 ($\leq 17\text{ }^{\circ}\text{C}$) as shown in the literature (McKemy et al., 2002; Story et al., 2003). While moderate cooling (temperatures between $14\text{ }^{\circ}\text{C}$ - $18\text{ }^{\circ}\text{C}$) induced Ca^{2+} influx as expected, stronger cooling (below $10\text{ }^{\circ}\text{C}$) led to a heterogeneous Ca^{2+} response pattern. Surprisingly, repeated strong cooling ($< 10\text{ }^{\circ}\text{C}$) even caused a decrease of intracellular Ca^{2+} in HCEC-12 cells (Fig. 15). This phenomenon has not yet been described.

An explanation for those observations might be found in the unspecific effect of cooling. A decrease in temperature leads to a decreased membrane fluidity, which causes reduced rates of diffusion and transport across the cell membrane (Murata and Los, 1997). Also, cold exposure reduces enzymatic reactions, protein syntheses and cell proliferation in mammalian cells (Fujita, 1999). This implies that the cell's homeostasis

is substantially affected by hypothermia. Therefore, it seems probable that not only thermo-TRP channels are involved in the observed heterogeneous Ca^{2+} responses. As physical cooling appeared too unspecific, it seemed reasonable to activate cold receptors pharmacologically.

In order to validate the functional expression of TRPM8, the TRPM8 antagonist BCTC (Fig. 25A) was used for another set of cooling experiments (Fig. 16) (Behrendt et al., 2004). As expected, the cold-induced Ca^{2+} increase could be suppressed by BCTC. This clearly demonstrates TRPM8 channel activity in HCEC-12 cells. However, the cold-induced Ca^{2+} increase was not inhibited completely, which implies additional cold receptor activity at moderately cold temperatures ($\sim 18\text{ }^{\circ}\text{C}$), for instance TRPA1 with its activation threshold at $\leq 17\text{ }^{\circ}\text{C}$ (Story et al., 2003).

Furthermore, the super cooling agent icilin (Fig. 25B), which is an agonist for TRPM8, was used to confirm TRPM8 expression (McKemy et al., 2002; Rawls et al., 2007). Incidentally, icilin is also known to be an agonist for TRPA1, but with lesser potency than for TRPM8 (McKemy et al., 2002; Story et al., 2003). After application of icilin, an irreversible increase of Ca^{2+} influx was recorded in HCEC-12 cells (Fig. 17). These icilin-induced Ca^{2+} response patterns correspond with those described in studies with other cell types such as human synoviocytes, tumor cells (Kochukov et al., 2006; Mergler et al. 2007) and with those previously reported in the same cell type (Mergler et al. 2005). The irreversibility of the Ca^{2+} responses could be explained by a large Ca^{2+} influx in the presence of unphysiological icilin concentrations in HCEC-12 cells, which the cells are not able to compensate. In addition, an icilin-induced increase of whole-cell currents could also be detected (Fig. 18). Although there was only a weak effect due to a large amount of leak currents, an icilin-increased current could be detected with a reversal potential of $\approx 0\text{ mV}$ which is consistent with its behavior as a nonselective cation channel.

To further validate TRPM8 expression in HCEC-12 cells, additional Ca^{2+} imaging experiments were performed. When HCEC-12 cells were pre-incubated with the TRPM8 antagonist BCTC before application of icilin (Behrendt et al., 2004), it turned out that this TRPM8 blocker was indeed able to suppress the icilin-induced Ca^{2+} influx (Fig. 19). This

again confirms functional TRPM8 expression in HCEC-12 cells as indicated in current studies by Mergler et al. (Mergler et al., 2013).

Taken together, these findings demonstrate the functional expression of the cold receptor TRPM8 in HCEC-12 cells.

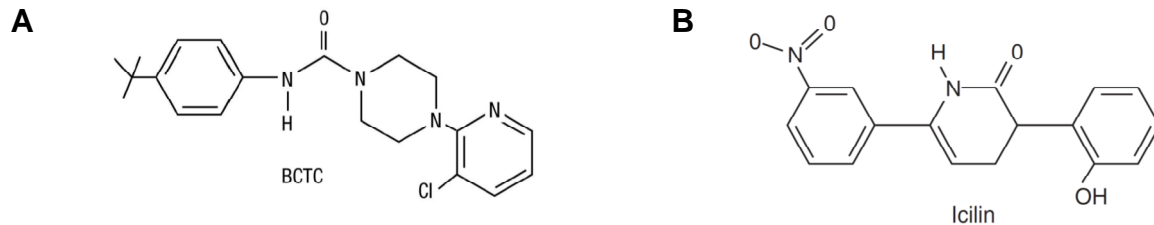


Fig. 25 The chemical structure the TRPM8 antagonist BCTC (**A**) and the TRPM8/TRPA1 agonist icilin (**B**) (Zang and Barritt 2006).

4.2 Heat receptor activity in HCEC-12 cells

The results of the heating experiments confirm the functional expression of heat receptors in HCEC-12 cells. The increase in intracellular Ca^{2+} resulting from heat stimulation (Fig. 20) indicates the activity of thermo-TRPs of the vanilloid receptor subtypes. The heat stimulation used covers the heat-induced activation of TRPV1 (≥ 43 °C), TRPV3 ($\approx 34 - 38$ °C) and TRPV4 (≈ 24 °C - 28 °C) (Tominaga and Caterina, 2004). This observation corresponds to recent studies by Mergler et al., who intensively studied TRPV channels in HCEC-12 cells and freshly isolated HCEC. They specifically demonstrated the expression of heat receptors such as TRPV1 in HCEC-12 cells (Mergler et al. 2010). Also, they succeeded in proving evidence for TRPV1-3 gene expression in HCEC-12 cells and its cloned daughter cell lines HCEC-B4G12 and HCEC-H9C1 using RT-PCR analysis and functional assays such as Ca^{2+} imaging and patch-clamping (Mergler et al., 2010). The results of this thesis are consistent with their observations of functional expression of heat receptors in HCEC-12 cells.

4.3 Interaction between cold and heat receptors?

TRPV1 and TRPM8 gene expression is prominent in HCEC-12 cells and in normal HCEC (Mergler et al., 2010, 2013). Interaction between TRPM8 and TRPV1 has already been described in neuronal cells (Chuang et al. 2004; Crawford et al., 2009). In HCEC-12 cells, there also is indication of possible interaction between TRPM8 and

TRPV1, since icilin-induced Ca^{2+} increases could be increased if cells were treated simultaneously with the selective TRPV1 agonist capsaicin (CAP, Fig. 21 & 26) (Caterina et al., 1997; Megumi et al., 2012). However, the Ca^{2+} imaging data were not congruent with icilin- and CAP-induced increases of whole-cell currents (Fig. 22). Therefore, it is suggested that the activation of cold receptors via icilin does not influence the effect of TRPV1 activation via CAP (Fig. 21C).

As TRPM8 is activated from $\leq 28\text{ }^{\circ}\text{C}$ and TRPV1 from $\geq 43\text{ }^{\circ}\text{C}$ (McKemy et al., 2002; Tominaga and Caterina, 2004), it seems very unlikely that these channels are activated simultaneously under physiological conditions. This might explain the incongruity of the patch-clamp recordings. Based on these results, the question of interaction cannot be answered satisfactorily and will require further investigations.

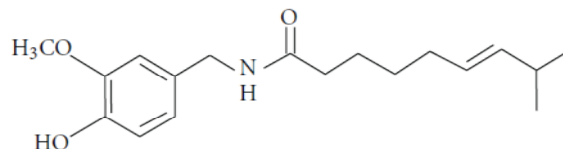


Fig. 26 The chemical structure of the selective TRPV1 agonist capsaicin (CAP) (Megumi et al., 2012).

4.4 Using normal HCEC from a human donor cornea

Even though the immortalized HCEC-12 cell line is widely accepted and has been successfully used as a model for corneal endothelium in several studies (Bednarz et al., 2000; Valtink et al., 2008; Mergler et al., 2003, 2005, 2010), it seemed reasonable to use freshly isolated HCEC as a control to validate cold receptor activity in normal HCE. In general, the research effort with normal HCE is considerably greater than with a commercially available cell line. For ethical reasons, the organ donor or their relatives have to give their consent to the use of the material for research. Due to the lack of donors, very little material is available and it is of course primarily used for keratoplasty. Only if a graft has reduced viability it is discarded and can be used for research. Unfortunately, the cultivation of the primary cells can only be of temporary help in this matter, since these cells start to dedifferentiate after 5 cell passages. In addition, the primary cultivated cells show reduced cell proliferation which limits the choice of cells for measurements (see Fig. 23D). Interestingly, the normal HCE cells appear much larger than the immortalized HCEC-12 cells. This might be explained by their natural behavior

to extend their surface to maintain a leak-proof barrier when the total number of cells is reduced (Waring et al., 1982). Overall, the use of an established immortalized cell line such as HCEC-12 is much easier in terms of purchasing, cultivating and measuring.

4.5 Cold receptor activity in normal HCEC

Regarding TRPM8, the data in this thesis revealed that there is no artifact in the immortalized cell line, since the freshly isolated normal HCEC reacted with a similar increase in intracellular Ca^{2+} , when the cooling agent icilin was added (Fig. 24). For this setup, a slightly higher concentration of icilin (60 μM) was used to ensure clear results. Also, the bath temperature was raised $> 28\text{ }^{\circ}\text{C}$ to be slightly closer to physiological conditions but still comparable to the other measurements with HCEC-12 cells at room temperature. In summary, it could be confirmed that the HCEC-12 cell line is a reasonable cell model for HCEC.

Recently, Mergler et al. were able to demonstrate TRPM8 gene expression in the immortalized cell line HCEC-12 and its cloned daughter cell lines HCEC-B4G12 and HCEC-H9C1 (Valtink et al., 2008), as well as in normal HCEC using conventional RT-PCR analysis (Mergler et al., 2013). These findings reassure that the results of this thesis can be attributed to TRPM8 as it is expressed in HCEC-12 cells and in normal HCEC.

4.6 Clinical relevance

The knowledge of heat- and cold-sensitive TRP channel expression in HCEC-12 cells as well as in normal HCEC may provide an insight into improved management of donor cornea storage or into improving the outcome of keratoplasty. So far, nothing is known about the *in vivo* physiological function of thermo-TRPs in HCEC. Since thermo-TRPs are known to be temperature sensors in primary sensory nerves, it is conceivable that these channels are also involved in mediating responses to temperature changes in the corneal endothelium. But thermo-TRPs can be activated by several stimuli and conditions besides temperature (see chapter 1.9 to 1.12). TRPV1 has been detected to be located at the apical membrane of the corneal endothelium facing the aqueous humor of the anterior chamber of the eye (Mergler et al., 2010). Due to its location, it would be imaginable that TRPV1 interacts with components of the aqueous humor, for

instance growth factors (Chowdhury et al., 2010). Further research might provide information on the location of TRPM8 in the corneal endothelium and if thermo-TRPs can be influenced by growth factors.

In isolated corneal epithelium, it was shown that TRPV1 activation increased the release of the inflammatory mediators IL-6 and IL-8 (Zhang et al., 2007). It can therefore be supposed that thermo-TRPs might play a role in mediation of inflammatory mediator secretion and subsequent hyperalgesia in other corneal tissues. Furthermore, there seems to be a connection between diseases and thermo-TRPs in some non-ocular tissues (Nilius et al., 2007). Both TRPM8 and TRPV1 have been detected in different vascular endothelium cells where they are supposed to transform external stimuli into changes in intracellular calcium, which are eventually coupled to various vascular responses, for instance the vascular tone (Yao and Garland, 2005). Yao and Garland even propose TRPs as possible targets to treat cardiovascular diseases (Yao and Garland, 2005). In prostate cancer, TRPM8 is known to be up-regulated and the channel is suggested as a potential diagnostic tumour marker and is a source of hope for pharmacological cancer therapy (Tsavaler et al., 2001). So far, a successfully targeted TRP channel is TRPV1 via its agonist capsaicin for the treatment of neuropathic pain (Sindrup and Jensen, 1999).

In addition, disturbed thermo-TRPs could directly cause diseases. For example, a point mutation in the gene encoding for the S4 transmembrane segment of TRPA1 was recently identified, which can be linked to an autosomal-dominant familial episodic pain syndrome, short FEPS (Kremeyer et al., 2010). Such defects of ion channels are called channelopathies. Unfortunately, our knowledge regarding thermo-TRP channelopathies is still very limited and further research will be needed.

In summary, the discovery of TRPV1 and TRPM8 channel expression justifies further studies in HCEC for instance to evaluate their possible use as modulators to improve the storage of donor corneas.

4.6.1 Thermo-TRPs and apoptosis

Since thermo-TRPs are amongst other cations permeable by calcium ions, these channels are predominantly involved in calcium homeostasis. If the calcium homeostasis is unstable, it has severe consequences for a cell because increased calcium levels can induce apoptosis (Orrenius et al., 2003). Two family members of the

TRPM family, TRPM2 and TRPM7, have already been shown to be involved in cell death processes (McNulty and Fonfria, 2005). Recently, Li et al. demonstrated that the cooling agent menthol increases intracellular calcium and decreases cell viability via TRPM8 in a human bladder cancer cell line (Li et al., 2009). In addition, they discovered that menthol induces mitochondrial membrane depolarization via TRPM8 which resulted in cell death of the human bladder cancer cells (Li et al., 2009). Yamamura et al. made similar discoveries: Menthol depressed the viability of human melanoma cells via the function of TRPM8 (Yamamura et al., 2008). Furthermore, the TRPV1 agonist capsaicin induces intracellular calcium levels in glioma cells which reduces cell viability and can be connected to apoptosis (Amantini et al. 2007).

The findings of these studies show a clear connection between the pharmacological activation of TRPM8 or TRPV1 and apoptosis in cancer cells. It is not too far-fetched to say that this model can be transferred to healthy tissue as well and that also temperature changes can lead to apoptosis via the specific thermo-TRP.

It now seems increasingly likely that thermo-TRPs can be linked to apoptosis via increasing intracellular calcium levels. This suggests that it could be possible for thermo-TRPs to be involved in the mechanisms leading to HCE cell loss, for instance during cornea storage or after keratoplasty.

4.6.2 Cold receptors and storage temperature of grafts in the context of lacking donor material

A possible role of thermo-TRPs regarding the storage temperature of corneas has not yet been explored. It is recognized that corneal endothelium is more adversely affected when the cornea is preserved under hypothermic conditions as compared to organ culture at higher temperatures. This is one possible explanation for the different time periods of cornea storage. Specifically, grafts can only be stored for up to 2 weeks when kept in cold environment but last up to 4 weeks under organ culture conditions (see chapter 1.5). In this context, the identification of the cold receptor TRPM8 (and very likely also TRPA1) in HCEC-12 cells may be highly relevant. As shown in chapter 3.2, strong cooling leads to a heterogeneous Ca^{2+} response which could be an indication of an overexertion of these cells. It appears that thermo-TRPs might be involved in the mechanisms responsible for the sensitivity of corneal endothelial cells to temperature. During storage, an irreversible HCE cell loss occurs, which makes up to 30 % of corneal

grafts unsuitable for transplantation (Fuchsluger et al., 2011). This issue is aggravated by the lack of donor corneas and the decreased willingness to donate organs in Germany. The total number of organ transplantations in 2012 has decreased by 12.8 % compared to 2011 and has reached its lowest level since 2002 (Deutsche Stiftung Organtransplantation, 2013). This dramatic development is caused by the feeling of insecurity of the population after organ scandals came to light. Several transplantation clinics were accused of manipulating data to help their patients receive a required organ faster. Furthermore, in Germany only people who give explicit consent can become donors, for instance by carrying an organ donation card. This strategy is called “opt-in” and stands in contrast to the “opt-out” system, where anyone who has not refused is automatically a donor (practiced for example in Austria, Italy and Spain). Interestingly, countries with the “opt-out” system have higher donation rates (Eurotransplant International Foundation, Annual Report 2012), perhaps because people do not make the effort to register their unwillingness to donate.

Due to the lack of donor material it is obviously of interest to optimize the available grafts. In the case of the cornea, it could be an attempt to improve storage conditions as well as promoting tissue engineering. As a logical consequence regarding the effect of temperature, the organ culture preservation method should be preferred worldwide and not only in Europe. Apparently, more promotion of this method is necessary to enhance its acceptance. However, if organ culture does not prevail, one could at least try to increase corneal endothelial durability in cold storage by influencing thermo-TRPs. It would be very interesting to investigate for example how a cold receptor antagonist influences the integrity of the HCE cells under hypothermic storage conditions. Possibly, the storage period could be extended as a result – perhaps even beyond that possible at higher temperatures. The development of agents targeting thermo-TRPs could surely also benefit the organ culture method. Without any doubt, further investigations regarding this issue are needed.

4.6.3 TRPM8 – a possible target against HCE cell loss?

The loss of HCE cells is of great importance in the outcome of keratoplasty. If the endothelial cell density decreases too much, the endothelial pump function is not sufficient and the transplant loses its transparency. This leads to dissatisfaction of both the patient and the surgeon. Therefore, this cell loss should somehow be prevented to avoid re-keratoplasty, particularly in view of the lack of donor material.

One approach should be to minimize trauma of the fragile donor tissue during surgery. In this context, current studies are investigating the preparation technique of lamellar grafts for DMEK and the storage of pre-cut grafts regarding potential endothelial cell loss (Bayyoud et al., 2012). Keratoplasty is a challenging intervention, which requires excellently trained surgeons. Especially the new lamellar keratoplasty methods are performed only by a few specialists who will gain more experience over time. They will surely continue to improve the surgical procedures and together with the technical progress we will eventually get even better keratoplasty results.

Also, the rinse solution for intraocular surgery could potentially be optimized. Rinse solutions are generally used at intraocular surgeries to maintain physiological conditions. Its temperature, pH value and salt content are assumed to influence the viability of the corneal endothelial cells. Since thermo-TRPs such as TRPV1 and TRPM8 were found to be functionally expressed in these cells, we should reconsider the utilized temperature of the rinse solution. At present, it is used at room temperature. As we know from the storage of the grafts, warmer physiological temperatures appear to have a better effect on the integrity of the corneal endothelium as their life span is up to 4 weeks long (Ehlers et al., 2009). In addition, the physiological temperature of the cornea is estimated to reach the maximum of 36.5 °C to 37 °C, depending on body and ambient temperature (Kessel et al. 2010). Since the activation threshold of the cold receptor TRPM8 is < 28 °C (McKemy et al., 2002), it is very likely to be activated by the rinse solution. The “cold sensation” through TRPM8 might then trigger a stress reaction within the corneal endothelial cell and cause apoptosis. According to this, it would be worth an attempt to raise the rinse solution temperature up to 37 °C and observe whether the endothelial cells survive better during the first few months after surgery.

If we assume that cold receptors are actually involved in cell loss, TRPM8 for instance would be an imaginable drug target. Antagonists such as BCTC could block an intracellular calcium increase which could potentially cause apoptosis. Injected into the anterior chamber during surgery, this agent might have a positive effect and protect the

corneal endothelial cells from death. Of course, the stability of the agent as well as its side effects would have to be considered. This could be a promising starting point for pharmacological research in ophthalmology.

4.6.4 High sensitive electrophysiological methods for graft evaluation

The experiments for this thesis were performed using calcium imaging and planar patch-clamp recordings. Both methods are highly sensitive and established for electrophysiological research (Barreto-Chang and Dolmetsch, 2009; Hamil et al., 1981; Brüggemann et al., 2006). Calcium imaging allows one to measure the intracellular calcium concentration which gives information on the calcium homeostasis of a cell, whereas planar patch-clamping allows studying ion channels located on the cellular membrane. These electrophysiological procedures open up new technical opportunities to evaluate corneal grafts as they are more sensitive than conventional staining and cell counting. Of course, one would first have to investigate the exact correlation between for instance TRPM8 and corneal endothelial cell viability in order to draw a conclusion. Then, this ion channel could be a promising new marker for the cell viability of the corneal endothelium, which might help to detect the most suitable grafts for keratoplasty.

4.7 Conclusion

This thesis has demonstrated that thermo-TRPs such as the cold receptors TRPM8 and TRPA1 occur in HCEC-12 cells and in normal HCEC. There is evidence for a complex regulation of intracellular Ca^{2+} levels via these thermo-TRPs. Further investigations in this direction may have the potential of helping to improve the storage of human donor corneas and the outcome of keratoplasty – topic of high interest of eye banks and patients worldwide.

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Eidesstattliche Versicherung

„Ich, Charlotte Nora Mertens, versichere an Eides statt durch meine eigenhändige Unterschrift, dass ich die vorgelegte Dissertation mit dem Thema: „Functional expression of thermo-sensitive transient receptor potential channels in cultivated human corneal endothelial cells (HCEC-12)“ selbstständig und ohne nicht offengelegte Hilfe Dritter verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel genutzt habe.

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Meine Anteile an etwaigen Publikationen zu dieser Dissertation entsprechen denen, die in der untenstehenden gemeinsamen Erklärung mit dem Betreuer, angegeben sind. Sämtliche Publikationen, die aus dieser Dissertation hervorgegangen sind und bei denen ich Autor bin, entsprechen den URM (s.o.) und werden von mir verantwortet.

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Anteilserklärung an etwaigen erfolgten Publikationen

Charlotte Nora Mertens hatte folgenden Anteil an den folgenden Publikationen:

Publikation:

Mergler S, Mertens C, Valtink M, et al. Functional significance of thermosensitive transient receptor potential melastatin channel 8 (TRPM8) expression in immortalized human corneal endothelial cells. Exp Eye Res. 2013;116:337-349.

Beitrag im Einzelnen: Durchführung von Calcium Imaging und Patch-Clamp Experimenten mit HCEC-12 Zellen (physikalische Kühlung und Anwendung der Pharmaka Icilin, BCTC und Capsaicin) sowie die Erstellung von Diagrammen und Statistiken.

Poster 1:

Mergler S, Castelo V, Mertens C, Valtink M, Engelmann K and Pleyer U. Expression, Funktion und endogene Modulatoren der transient receptor potential (TRP) Kanäle TRPM8 und TRPA1 in humane Hornhautendothelzellen. DOG Berlin, Germany. Sept 20th, 2012.

Beitrag im Einzelnen: Durchführung von Calcium Imaging Experimenten mit HCEC-12 Zellen (physikalische Kühlung und Anwendung der Pharmaka Icilin und BCTC) sowie die Erstellung von Diagrammen und Statistiken.

Poster 2:

Mergler S, Valtink M, Castelo V, et al. Transient receptor potential melastatin 8 (TRPM8) and ankyrin 1 (TRPA1) channels mediate complex calcium responses in human corneal endothelial cells. ARVO Orlando, Florida, USA. May 10th, 2012.

Beitrag im Einzelnen: Durchführung von Calcium Imaging Experimenten mit HCEC-12 (Anwendung des Pharmakons Icilin und physikalische Kühlung) sowie die Erstellung von Diagrammen und Statistiken.

Hausarbeit:

Mertens C. Calcium Imaging and patch-clamp investigations of Ca^{2+} regulation by thermo-sensitive transient receptor potential (TRP) channels in cultivated corneal endothelial cells (HCEC-12). Charité University Berlin, Department of Ophthalmology. Oct 10th, 2011.

Unterschrift, Datum und Stempel des betreuenden Hochschullehrers

Unterschrift der Doktorandin

“Mein Lebenslauf wird aus datenschutzrechtlichen Gründen in der elektronischen Version meiner Arbeit nicht veröffentlicht.“

List of publications

Publication:

- Mergler S, Mertens C, Valtink M, et al. Functional significance of thermosensitive transient receptor potential melastatin channel 8 (TRPM8) expression in immortalized human corneal endothelial cells. *Exp Eye Res.* 2013;116:337-349.

Posters:

- Mergler S, Castelo V, Mertens C, Valtink M, Engelmann K and Pleyer U. Expression, Funktion und endogene Modulatoren der transient receptor potential (TRP) Kanäle TRPM8 und TRPA1 in humane Hornhautendothelzellen. DOG Berlin, Germany. Sept 20th, 2012.
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Assignment:

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