KCNK5 in Physiology and Pathophysiology
- Focus on Cell Volume Control

PhD Thesis
Signe Skyum Kirkegaard Petersen

Academic advisors: Professor Else Kay Hoffmann & Dr.med. Steen Gammeltoft
Submitted: September 30th 2013
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PhD Thesis
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Preface and acknowledgements

This PhD thesis will focus on the potassium channel KCNK5 and its role in different cell types i.e. the cell lines Ehrlich Ascites Tumor cells and Ehrlich Lettré Ascites cells and in human primary T cells. More precisely I have studied the involvement of protein tyrosine kinases in swelling-induced activation of the channel, the long-term effects of hypotonicity on channel physiology and expression pattern and last but not least I have looked into its role in T cells.

The thesis is based on the following papers:

**Paper I:**
Activation of the TASK-2 channel after cell swelling is dependent on tyrosine phosphorylation
Signe Skyum Kirkegaard, Ian Henry Lambert, Steen Gammeltoft and Else Kay Hoffmann

**Paper II:**
KCNK5 is functionally down-regulated upon long-term hypotonicity in Ehrlich ascites tumor cells
Signe Skyum Kirkegaard, Tune Wulff, Steen Gammeltoft and Else Kay Hoffmann
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**Paper III:**
The potential role of KCNK5 in activated T cell physiology with specific focus on cell volume control
Signe Skyum Kirkegaard, Pernille Dyhl Strøm, Anker Jon Hansen, Steen Gammeltoft and Else Kay Hoffmann

Through this thesis I will touch upon various themes such as the activation of the KCNK5 channel upon acute cell swelling, the physiological function and expression of the channel upon long-term hypotonicity and on its potential role in activated T cell physiology. I thus used different cells and cell systems in order to investigate these topics - a fact that will be reflected in this thesis, where the introductory part of the thesis will deal with a variety of quite different topics. I will describe how the immune system works, how a cell can regulate its volume and hereunder various sub-topics, what is known about the KCNK5 channel and about potassium channels in general. With relative many large topics to describe some details will not be addressed, thus the reader might find some topics left unmentioned but hopefully this will not cloud the greater overview, issues and facts presented here.

During my time as a PhD student at Section for Cell and Developmental Biology at the University of Copenhagen and at the Department of Clinical Biochemistry, Glostrup Hospital, Denmark I have had the pleasure of guidance from my two supervisors, Else Kay Hoffmann and Steen Gammeltoft. I have had great scientific challenges and have learned a lot about research, project
First of all I would like to thank Professor Else K. Hoffmann for everything! – for giving me a chance in her laboratory, first as a master student and later on as a PhD student, for sharing her extensive expertise on the area of cell volume, ion flux and on physiology in general. On a more personal level I would like to thank her for always having time to talk, listen and give advice. One thing in particular also comes to mind when thinking of the opportunities given to me by Else – I have attended a great deal of meetings and conferences and have met so many interesting scientists (probably more than most other PhD student), which is due to the fact that conference attendance and networking is highly prioritized by Else. You are indeed a very social person Else, and that fact has always had a huge impact on the great working environment in the lab. During the last couple of years we have visited Slovenia, USA, Canada and Japan besides the many meetings in Denmark. I have enjoyed every chance of presenting my work and have found the participation in all the conferences and meetings very beneficial. Thank you so much!

I am also extremely grateful to Dr. Steen Gammeltoft for giving me the opportunity to be a part of his group, first as a master student, since as a research assistant and last but not least as a PhD student. The project might never have been without his financial and scientific support. I would also like to thank Dr. Ian Lambert for always having his door open for me, whenever I needed advise or practical help and for including me in the little group of “Friends of the Coulter counter”.

I would very much like to express my gratitude to Dr. Anker Jon Hansen from Novo Nordisk, for giving me a chance to work in his lab, for giving me the opportunity to see how the industry works and for challenging my knowledge and ability to explain my work and ideas. I have very much appreciated his comments and thoughts.

A special thank should also be given to technicians Birthe Juul Hansen, Pia Birn, Dothe Nielsen and Birte Kofoed for their most appreciated help and friendship.

Last but not least I would like to thank friends and family for their everlasting support and patience, especially I would like to thank my husband Mikkel, for whom without I would not have been able to do this. I would also like to thank my son Oskar for making me learn how to get the most out of, the now more limited time, I could spend at work.

Signe Skyum Kirkegaard Petersen
### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>[Ca^{2+}]_i</td>
<td>Intracellular Ca^{2+} concentration</td>
</tr>
<tr>
<td>AA</td>
<td>Arachidonic acid</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen presenting cell</td>
</tr>
<tr>
<td>AVD</td>
<td>Apoptotic volume decrease</td>
</tr>
<tr>
<td>B cell</td>
<td>B lymphocyte (&quot;B&quot; from bursa of Fabricius or bone marrow)</td>
</tr>
<tr>
<td>BCR</td>
<td>B cell receptor</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>CRAC</td>
<td>Ca^{2+} release-activated Ca^{2+} channel</td>
</tr>
<tr>
<td>ChTX</td>
<td>Charybdotoxin</td>
</tr>
<tr>
<td>DAG</td>
<td>Diacylglycerol</td>
</tr>
<tr>
<td>EAT cells</td>
<td>Ehrlich ascites tumor cells</td>
</tr>
<tr>
<td>ELA cells</td>
<td>Ehrlich lettré ascites cells</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>FAK</td>
<td>Focal adhesion kinase</td>
</tr>
<tr>
<td>FLAP</td>
<td>5-LOX activating protein</td>
</tr>
<tr>
<td>fMLP</td>
<td>N-formyl-methionine-leucine-phenylalanine</td>
</tr>
<tr>
<td>I_{Cl,\text{vol}}</td>
<td>Swelling activated Cl^- current</td>
</tr>
<tr>
<td>IK</td>
<td>Intermediate conductance</td>
</tr>
<tr>
<td>I_{K,\text{vol}}</td>
<td>Swelling activated K^+ current</td>
</tr>
<tr>
<td>IP_3</td>
<td>1,4,5-inositol triphosphate</td>
</tr>
<tr>
<td>JAK</td>
<td>Janus kinase</td>
</tr>
<tr>
<td>K_2P</td>
<td>Two-pore domain K^+ channel</td>
</tr>
<tr>
<td>KCa3.1</td>
<td>Ca^{2+}-activated K^+ channel 3.1</td>
</tr>
<tr>
<td>KCNKS</td>
<td>Two-pore domain channel 5.1</td>
</tr>
<tr>
<td>Kv1.3</td>
<td>Voltage-gated K^+ channel 1.3</td>
</tr>
<tr>
<td>LTC_4</td>
<td>Leukotriene C_4</td>
</tr>
<tr>
<td>LTD_4</td>
<td>Leukotriene D_4</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
</tr>
<tr>
<td>NFAT</td>
<td>Nuclear factor of activated T cells</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PIP_2</td>
<td>Phosphatidylinositol 4,5-biphosphate</td>
</tr>
<tr>
<td>PLC_\gamma</td>
<td>Phospholipase C-\gamma</td>
</tr>
<tr>
<td>PTK</td>
<td>Protein tyrosine kinase</td>
</tr>
<tr>
<td>RA</td>
<td>Rheumatoid arthritis</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse transcription polymerase chain reaction</td>
</tr>
<tr>
<td>RVD</td>
<td>Regulatory volume decrease</td>
</tr>
<tr>
<td>RVI</td>
<td>Regulatory volume increase</td>
</tr>
<tr>
<td>STIM</td>
<td>Stromal interacting molecule</td>
</tr>
<tr>
<td>T cell</td>
<td>T lymphocyte (&quot;T&quot; from thymus)</td>
</tr>
<tr>
<td>TALK</td>
<td>TWIK-related alkaline pH activated K^+ channel</td>
</tr>
<tr>
<td>TASK</td>
<td>TWIK-related acid-sensitive K^+ channel</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>TEA</td>
<td>Tetraethylammonium</td>
</tr>
<tr>
<td>THIK</td>
<td>Tandem pore domain halothane-inhibited K^+ channel</td>
</tr>
<tr>
<td>TM</td>
<td>Transmembrane</td>
</tr>
<tr>
<td>TRAAK</td>
<td>TWIK-related arachidonic acid-stimulated K^+ channel</td>
</tr>
<tr>
<td>TREK</td>
<td>TWIK-related K^+ channel</td>
</tr>
<tr>
<td>TWIK</td>
<td>Tandem of p domains in a weak inwardly rectifying K^+ channel</td>
</tr>
<tr>
<td>VRAC</td>
<td>Volume-regulated anion channel</td>
</tr>
<tr>
<td>qPCR</td>
<td>Real-Time quantitative PCR</td>
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</table>
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The purpose of this thesis can be divided into three sub-purposes all centered around the two pore-domain potassium channel KCNK5 (or TASK-2).

1) To study the potential role of protein tyrosine kinases in the swelling-mediated KCNK5 activation in Ehrlich Ascites Tumor (EAT) and Ehrlich Lettré Ascites (ELA) cells

2) To study the long-term effect of hypotonicity on KCNK5 physiology and expression in EAT and ELA cells

3) To study the expression pattern and potential role of KCNK5 in activated human T cell physiology
Abstract

The KCNK5 potassium channel (also known as TASK-2 or K_{2p,5.1}) belongs to the latest discovered potassium channel family - the two-pore domain potassium channels. KCNK5 was first described in 1998 by Lazdunski and co-workers and has since been given a role in e.g. volume regulation, and recent been proposed to play a role in T cell activation and in multiple sclerosis.

When a cell experiences swelling due to osmotic changes in the extracellular or intracellular environment most mammalian cells will regulate their volume back towards the starting point – an important feature in keeping cellular homeostasis. This swelling-induced response is called Regulatory Volume Decrease (RVD) and involves the efflux of KCl and organic osmolytes through specific volume sensitive channels, with a concomitant water efflux and shrinkage back towards starting volume as a result. KCNK5 has been shown to be an important player in RVD in different cell types and tissue e.g. in Ehrlich cells. It has been speculated that tyrosine phosphorylation is necessary for the swelling-activation of the channel and in “Paper I” we show how acute hypotonic swelling induces a time-dependent tyrosine phosphorylation upon the channel itself.

Acute cell swelling and its regulatory mechanisms is by far the most studied part of the cells response to hypotonic stress and little is known about the consequences of long-term hypotonic stimuli on cells, thus we induced a long-term hypotonic stimulation on EAT cells and ELA cells to study the effect on physiology and channel expression patterns (Paper II). We found that 48 h of hypotonic stimulation reduced the maximum current through the channel and likewise decreased the cells ability to perform RVD. This impairment was found to be likely due to lowered KCNK5 protein expression.

In 2010 it was suggested that KCNK5 play a pivotal role in the autoimmune disease multiple sclerosis more precise in activated T cell. Numerous studies on ion channels in T cell activation have been published and potassium channels Kv1.3 and KCa3.1 are thought to be the two main channels involved. A potential role for KCNK5 made us shift cell type and thus look closer into the channel in human T cells. We found a time-dependent massive up-regulation of KCNK5 on mRNA and protein levels in activated human T cells, but KCNK5 up-regulation did not facilitate an increased RVD, instead we found RVD in activated T cells to be impaired in comparison with non-activated control cells. This impairment, despite of up-regulation of KCNK5, we show to be due to a decreased volume activated Cl⁻ flux, thus making the volume regulated anion channel (VRAC) the rate-limiting factor in RVD in activated human T cells (Paper III).
Resumé

Kaliumkanalen KCNK5 (også kaldet TASK-2 eller K_{2p}5.1) tilhører den senest opdagede familie af K⁺ kanaler og tilhører således gruppen af to-pore-domæne K⁺ kanaler. KCNK5 blev første gang beskrevet af Lazdunski et al. i 1998. KCNK5 er vist at være involveret i volumenregulering i en række celletyper og er relativt for nylig foreslået at være af vigtig betydning i T celle aktivering og i den autoimmune sygdom multipel sklerose.


Der har været utallige studier af ionkanaler i T celle aktivering og K⁺-kanalerne Kv1.3 og KCa3.1 menes, at være de to vigtigste kanaler i denne proces. Da det er blevet foreslået, at KCNK5 spiller en vigtig rolle i sygdommen multipel sklerose og også er involveret i T celle aktivering besluttede vi, at kigge nærmere på en potentiell rolle for KCNK5 i aktivering af humane T celler. Vi fandt, at KCNK5 blev kraftigt opreguleret i aktiverede T celler både på mRNA- og proteinniveau. Denne opregulering medførte dog ikke et øget RVD, tværtimod fandt vi at RVD i aktiverede T celler var hæmnet i forhold til RVD i ustimulerede kontrolceller. Vi har evidens for, at denne hæmning af RVD skyldes en nedsat volumenaktiveret Cl⁻ flux, hvilket gør VRAC (volume regulated anion channel) til den begrænsende faktor for KCl efflux under RVD i aktiverede T celler (Paper III).
1. Introduction

Potassium channels are ubiquitously distributed in all living organisms and are thus found in cellular membranes in such different organisms as e.g. bacteria, plants and mammals. To this day more than 75 known mammalian potassium channel subunit coding genes are known, which makes this family of ion channels the most diverse of all ion channel families. They are found in almost all cell types and hold a variety of cellular functions. This thesis will focus on the potassium channel KCNK5 and its activation in response to acute hypotonicity, its physiology when experiencing hypotonicity over a longer period of time and its role in T cell physiology. The following sections will thus (in broad terms) deal with potassium channels in general, KCNK5, volume regulation and T cell physiology.

1.2 K⁺ channels in general

K⁺ channels are protein complexes made of subunits that allow the passive transport of K⁺ across the membrane which, if not for ion channels and transporters, would be functional impermeable to ions. The passive transport follows the electrochemical gradient. K⁺ serves a wide range of functions including setting the membrane potential, controlling the action potential in excitable cells and being involved in cell proliferation, cell volume control, muscle contraction and secretion of various hormones and transmitters. As potassium channels are implicated in such various physiological functions, many diseases or pathological conditions can be contributed to an altered K⁺ channel function. Of diseases and pathological conditions implicating K⁺ channels can be mentioned arrhythmia, diabetes, neuronal diseases and renal diseases, for further information see e.g. (60; 140). Several K⁺ channels are also found to be up-regulated in cancer cells (see (125)), though one way of cancer cells to survive is to avoid apoptosis, which can be obtained by the down-regulation of K⁺ channels involved in apoptotic volume decrease (see section 1.4.2.1).

As stated above, potassium channels hold a range of different functions and so they differ both in topology and regulation. The K⁺ current running through the channel can be activated in different ways reflecting the physiology of the cell or tissue in mind. There are different ways of grouping the potassium channels e.g. by activation and type of current or by topology (see section 1.2.1). There are four main types of potassium channels based on activation and current; i) voltage-gated K⁺ channels which are sensitive to changes in membrane potentials and opens or closes accordingly, ii) Ca²⁺-activated K⁺ channels which, as the name implies, has a Ca²⁺ dependent activation, iii) Inwardly-rectifying K⁺ channels that favors K⁺ fluxes in an inward...
direction and last but not least iii) two-pore domain K⁺ channels which are constitutively active and carries currents designated leak-currents see (110).

The potassium channels consist of various transmembrane (TM) spanning regions (or TM domains) together with a highly conserved pore-forming domain, that can transport between 10⁵ to 10⁸ K⁺ ions across the membrane pr. sec, while at the same time rejecting other cations (110; 140). The pore region of the channel is a feature shared by all potassium channels and thus, all potassium channel subtypes poses the very conserved amino acid sequence threonine, valine, glycine, tyrosine and glycine (TVGYG). This amino acid sequence make up the selective filter of the pore (60) and is called the signature sequence of potassium channels (48). To make a functional K⁺ pore, four pore-domains have to come together.

Scientists are always on the look for new discoveries including new ion channel families, thus the pore sequence was used to search DNA sequence databases for new potassium channels. This approach resulted in the discovery of the two-pore domain potassium channel family further described in the following sections (83).

1.2.1 Potassium channel grouping by topology
One way to group the many different K⁺ channels is by activation pattern and another is by topology and thus the number of TM segments. Dividing the potassium channels by topology gives three main groups, the 2TM family, the 4TM family and the 6TM family which also include a 7TM spanning member (the BK family of KᵦᵦCa potassium channels (21; 60)) (see Figure 1).

1.2.2 The 2TM and 6TM families
In the 2TM family we find the Kᵦᵢᵦ channels which are inward rectifying channels that include the ATP-sensitive channels as well as G-protein coupled channels (fig. 1). Kᵦᵢᵦ channels are found in e.g. skeletal and heart muscle and in neurons see e.g. (21). The family contains 7 members and is found as a tetramer in the membrane, since the generation of a functional pore requires four pore domains to come together and in the 2TM family each subunit holds one pore-forming domain.

The voltage-gated, the Na⁺-activated and the Ca²⁺-activated K⁺ channels all belong to the 6TM family which in spite of the name also include a 7TM channel namely the large (big) conductance (BK) Ca²⁺-activated potassium channel (60) (see fig.1). The functional channel is, as for the 2TM family found as a tetramer with four pore domains generating the pore.
1.2.3 The 4TM family – the two pore-domain potassium channels

The 4TM family comprises the latest discovered family of $K^+$ channels, namely the two-pore domain channels in which we find the channel of special interest for this thesis – the KCNK5 $K^+$ channel (also known as TASK-2 or $K_{2.5.1}$). The 4TM family has an interesting structure that varies from the other two families since members of the 4TM family have four TM segments and two pore-forming domains (43; 82). Because of the two pore domains in each subunit the functional channel is found as a dimer and not as a tetramer as seen in the other two families (84) see also (83).

The KCNK5 channel belongs to the TALK (TWIK-related alkaline pH activated $K^+$ channel) subfamily and besides TALK the 4TM family consists of five other subfamilies namely the TWIK (tandem of p domains in a weak inwardly rectifying $K^+$ channel) subfamily, the THIK (tandem pore domain halothane-inhibited $K^+$ channel) subfamily, the TREK (TWIK-related $K^+$ channel) subfamily, the TRAAK (TWIK-related arachidonic acid-stimulated $K^+$ channel) subfamily and the TASK (TWIK-related acid-sensitive $K^+$ channel) subfamily (9) (fig. 2).
The idea of potassium leak-currents in the resting membrane was proposed a long time before it was possible to measure ion currents across the membrane and before cloning techniques made channel identity possible. Thus Bernstein had in 1912 proposed that the resting membrane was more permeable to potassium than other ions, which was described and further analyzed by Hodgkin and Huxley (49; 50). Though the idea of leak-currents came to light long ago, it was not until 1995 that the identity of a background channel was known by the cloning of the TOK1 channel in *Saccharomyces cerevisiae* (66). The TOK1 channel differs from the mammalian two pore-domain channels since it has eight TM domains, whereas all other two pore-domain K+ has the characteristic four TM segments and it further more differs in functionality. The first mammalian two pore-domain potassium channel with the characteristic morphology of two pore-domains and four TM domains to be cloned, was TWIK-1 which was cloned in 1996 (82).

Despite a relatively poor sequence similarity between some of the members of this relatively newly described family, they share not only the distinct topology, but are also similar in regard to electrophysiological properties. They are (almost) voltage independent, constitutively active at resting membrane potentials and have “open rectification”. For more on two-pore domain potassium channels see e.g. (32).

The background channels or potassium leak channels all share the same morphology with four membrane spanning domains, two of them being pore forming and they are special in the way that they are constitutively active at resting membrane potential (41). The potassium channel signature sequence is conserved in the first pore-forming loop, but is slightly different in the second pore-forming loop, where GYG is substituted by GFG or GLG (140). Since this group of K+ channels posses two pore domains, only two subunits are required to make a functional channel. Thus the channel is formed by the dimerization of two subunits (83; 84).

The potassium leak-channels are defined by the lack of voltage and time-dependencies, which means that they are open even at resting membrane potentials leaking K+ ions, and thus
bringing the plasma membrane potential towards the equilibrium potential for K⁺. So they serve an important function in maintaining the negative resting membrane potential by constitutively leaking K⁺ ions down the concentration gradient, which in turn helps maintaining the Na⁺/K⁺ pump function see ref. (42).

To this day there are 15 known members of the family of human two-pore domain potassium channels see refs. (20; 41). For more about two-pore domain potassium channels see e.g. refs. (8; 32; 41; 42; 66; 115).

1.3 KCNK5 – a two-pore domain potassium channel

In the 4TM family one of the subfamilies was named TASK (TWIK-related acid-sensitive K⁺ channel) and started out containing the channels TASK-1 (which was the first TASK channel and the third K₂p channel to be cloned (31)), TASK-2, TASK-3, TASK-4 and TASK-5, but as seen from fig. 2 the TASK-2 channel or KCNK5 together with TASK-4 (also known as TALK-2) are now found in the TALK subfamily (fig. 2). This subfamily re-arrangement was partly due to the poor molecular relation between TASK-2 and TASK-4/TALK-2 against TASK-1, thus the sequence similarity between TASK-1 and TASK-2 is only about 30% (110). In 2001 TALK-1 and TALK-2 were described (26; 39) and they were named as TWIK-related alkaline pH-activated K⁺ channels due to the high extracellular pH needed for activation. TASK-2 and TASK-4 were now regarded as TALK channels due to their alike alkaline pH sensitivity and because of their molecular relations mentioned above. Furthermore TASK-channels are gated only by extracellular pH (32) and thus not affected by intracellular pH as seen in the TALK channels KCNK5 and TALK-2, which are indeed sensitive to intracellular pH changes (105). Since TASK-2 is now regarded as a TALK channel and though the name TASK-2 is still in use, the TASK-2 channel will be designated KCNK5 throughout the rest of this thesis. I should though be noted that the channel is called TASK-2 in “Paper I” (69) and KCNK5 in “Paper II” and “Paper III”.

Figure 3: KCNK5 pH sensitivity
Current-voltage relationship recorded from a TASK-expressing oozyte (31).
1.3.1 KCNK5 history and tissue distribution

KCNK5 was as the first member of the TALK subfamily to be cloned, which was done from human kidney in 1998 by Reyes and co-workers (128). KCNK5 Northern blot analysis have shown KCNK5 to be present in human kidney, pancreas, liver, placenta, lung and small intestine (97; 128) whereas RT-PCR from on mouse tissue showed abundant KCNK5 presence in liver, kidney, small intestine, lung and uterus (97; 128). Some studies including the KCNK5 channel is strictly expression studies and do not deal with physiological importance and between the various studies there is some species variation in regard to KCNK5 expression (see table 1). In that regard some tissue expression is up for discussion, while there is a broad agreement on the expression and importance in kidney cells where many of the functional studies on the channel has been conducted.

Since the initial studies on KCNK5 it has (besides being described as a K⁺ leak-channel) been reported to be implicated in RVD in several cell types including Ehrlich Ascites Tumor (EAT) cells (69; 104), mouse proximal tubules (7), human and murine spermatozoa (4; 5) and murine T lymphocytes (12) but has also been reported to hold other functions in different cell types (see table 1).

1.3.2 KCNK5 characteristics

KCNK5 has been studied in a variety of cell types and it has been found to be highly sensitive to external pH, thus open probabilities increases with alkalinization and decreases with acidification (31; 64; 104) (see fig. 3) with a 90% maximum current measured at pH 8.8 and only 10% at pH 6.5 (31; 128). At pH 6.0 the KCNK5 activity is almost non-existing (107). The pH sensing mechanism has been proposed to be located in the extracellular loop between TM1 and channel pore domain one (99), but in 2007 Niemeyer and co-workers showed how an arginine residue (R224) located near the second channel pore domain acts as the pH sensor and that it functions by applying an electrostatic effect on the pore domain (107).

Figure 4: KCNK5 characteristics

See the text for further details. Own figure.
Table 1

<table>
<thead>
<tr>
<th>Species</th>
<th>Tissue</th>
<th>Function</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse</td>
<td>Ehrlich ascites tumor cells</td>
<td>Volume sensitive</td>
<td>(69; 104)</td>
</tr>
<tr>
<td>Mouse</td>
<td>Proximal tubules</td>
<td>Volume sensitive</td>
<td>(7)</td>
</tr>
<tr>
<td>Human</td>
<td>Spermatozoa</td>
<td>Volume sensitive</td>
<td>(4)</td>
</tr>
<tr>
<td>Mouse</td>
<td>Spermatozoa</td>
<td>Volume sensitive</td>
<td>(5)</td>
</tr>
<tr>
<td>Mouse</td>
<td>T lymphocytes</td>
<td>Volume sensitive</td>
<td>(12)</td>
</tr>
<tr>
<td>Mouse</td>
<td>Proximal kidney cells</td>
<td>AVD</td>
<td>(73)</td>
</tr>
<tr>
<td>Human</td>
<td>T lymphocytes</td>
<td>Possible T cell activation</td>
<td>(10)</td>
</tr>
<tr>
<td>Human</td>
<td>T lymphocyte</td>
<td>Volume sensitive</td>
<td>(3)</td>
</tr>
<tr>
<td>Rat</td>
<td>CNS</td>
<td>Possible cell excitability &amp; signal transduction</td>
<td>(37)</td>
</tr>
<tr>
<td>Mouse</td>
<td>Proximal tubules</td>
<td>Stabilizing HCO$_3^-$ transport</td>
<td>(151)</td>
</tr>
<tr>
<td>Human</td>
<td>MCF-7 and T47D</td>
<td>Regulating proliferation</td>
<td>(1)</td>
</tr>
<tr>
<td>Mouse</td>
<td>WEHI-231 cells</td>
<td>BCR-ligation-dependent apoptosis</td>
<td>(100)</td>
</tr>
<tr>
<td>Mouse</td>
<td>Retrotrapezoid nucleus neurons</td>
<td>CO$_2$ and O$_2$ chemo-sensing</td>
<td>(38)</td>
</tr>
<tr>
<td>Rat</td>
<td>Proximal neurons in inner retina</td>
<td>Neuronal excitability</td>
<td>(157)</td>
</tr>
<tr>
<td>Rat</td>
<td>Neurons in hippocampus</td>
<td>Contribution to properties of epilepsy</td>
<td>(68)</td>
</tr>
<tr>
<td>Human</td>
<td>Peripheral blood and T cells of RA patients</td>
<td>Correlation between expression and disease activity</td>
<td>(11)</td>
</tr>
<tr>
<td>Mouse</td>
<td>Gastrointestinal smooth muscles</td>
<td>Background current</td>
<td>(137)</td>
</tr>
<tr>
<td>Mouse</td>
<td>Gastroenstesinal tract</td>
<td>Contribution to resting potential</td>
<td>(156)</td>
</tr>
<tr>
<td>Rat</td>
<td>Carotid body</td>
<td>Regulating K$^+$ currents and acid sensing</td>
<td>(154)</td>
</tr>
<tr>
<td>Rat</td>
<td>Taste receptor cells</td>
<td>Setting resting potential and sour taste transduction</td>
<td>(89)</td>
</tr>
<tr>
<td>Rat</td>
<td>Cerebellar astrocytes</td>
<td>Not speculated on</td>
<td>(136)</td>
</tr>
<tr>
<td>Human</td>
<td>Cerebellar Purkinje cells</td>
<td>Not speculated on</td>
<td>(136)</td>
</tr>
<tr>
<td>Rat</td>
<td>Cerebellar granule neurons</td>
<td>Component of standing-outward potassium conductance</td>
<td>(23)</td>
</tr>
</tbody>
</table>

Furthermore the channel has proven to be insensitive to Ca$^{2+}$ and a wide range of known potassium channel blockers e.g. tetraethylammonium (TEA) (128) and charybdotoxin (ChTX) but can be blocked by e.g. clofilium (104) and quinine (128) (fig. 4). For a recent review on KCNK5 see ref. (20).
1.3.3 KCNK5 activation and gating
Different studies have been made to elucidate the activation mechanisms of KCNK5 and as mentioned earlier, KCNK5 has proven to be volume sensitive and thus activated by cell swelling. In addition we have demonstrated how protein tyrosine kinase activity is involved in RVD (Paper 1, fig. 1) and how cell swelling elicits a time-dependent tyrosine phosphorylation of the channel itself upon activation (Paper I, fig. 5). We have also shown how inactivation of the swelling-activated channel involves protein tyrosine phosphatases (Paper I, fig. 3).

Furthermore swelling-activation of KCNK5 has in kidney cells been proposed to be caused by an extracellular alkalinization due to Cl⁻/HCO₃⁻ exchanger mediated Cl⁻ influx (72). LTD₄ has likewise been implicated in the swelling-activation of KCNK5, since hypotonicity and cell swelling is followed by LTD₄ release (74; 76) and since addition of LTD₄ results in activation of a K⁺ current similar to the swelling-activated K⁺ current (57). In addition it was shown that desensitization of the LTD₄ receptor impairs RVD in EAT cells (61).

Of other gating mechanisms can be mentioned gating by intracellular pH and also extracellular pH both working in the same pH range though they are independent of each other. A recent study have also shown how KCNK5 is inhibited by heterotrimeric G protein subunits Gβγ an effect that could be eliminated by mutation of lysine residues of the C terminus (2) (also see (20)).

Despite all of these findings there are still many questions to be answered in regard to KCNK5 channel activation, thus we have yet to identify the tyrosine sites on KCNK5 being phosphorylated upon swelling-mediated activation (see section 7 and discussion in Paper I) and the direct connection between KCNK5 and LTD₄ has still not been shown. Furthermore though the effect of Gβγ G protein subunits has been established, the answer to how exactly it works and through which signaling pathway also remains to be answered.

1.3.4 KCNK5 in lymphocytes
KCNK5 was initially described in kidney cells and thorough studies have also been done in Ehrlich cells. The presence of KCNK5 in lymphocytes on the contrary is a relatively new observation. The first evidence of a background channel (TREK-2) present in lymphocytes was described in WEHI-231 cells, which is a murine cell line equivalent to immature B cells (101; 158). Further studies have revealed the presence of two-pore domain channels in lymphocytes e.g. TASK- 1 and TASK- 3 in human T cells (98) and in regard to lymphocytes KCNK5 has so far been described in human T cells (10) and in WEHI-231 immature B cells (100).
In B cells KCNK5 has been shown to be involved in apoptosis which is initiated by B cell receptor stimulation (100). In T cells Bittner and co-workers has proposed that KCNK5 holds a role in multiple sclerosis since an up-regulation of the channel was found in T cells purified from multiple sclerosis patients. The study from Bittner et al. led us to look into the potential role of KCNK5 in activated human T cells. We found that KCNK5 is indeed expressed in T cells and that activation resulted in a time-dependent expression pattern with an initial down-regulation followed by a strong up-regulation of the channel on protein level (Paper III, fig. 2). For more on KCNK5 in lymphocytes see (20; 35).

1.4 Cellular volume

The permeability of water across the plasma membrane is much larger than the permeability of potassium, sodium and chloride, thus the water permeability was measured to be $10^5$ times higher than sodium and potassium (51) and $10^6$ times higher than chloride (75) in EAT cells. A shift in intracellular and/or in extracellular osmolarity can be observed in response to e.g. accumulation of metabolic waste products, protein synthesis and transport over epithelia. The extracellular content has very little variation under physiological conditions, thus changes in the intracellular osmolyte composition are the main determinant for volume changes. The cell is very vulnerable to changes in cell volume which will affect cellular homeostasis e.g. is the actin cytoskeleton very susceptible to volume changes and it is seen how cell swelling results in a decrease in F-actin whereas cell shrinkage result in an increased amount of F-actin (117; 139). For more on volume regulation see e.g. refs. (56; 78).

1.4.1 Cell volume regulation

Maintaining a steady cell volume is crucial for keeping cellular homeostasis, thus a fundamental ability in mammalian cells, with only some exceptions, is the ability to volume regulate. Cells are constantly osmotically challenged by extracellular and especially intracellular changes in osmolyte content and since most mammalian plasma membranes contain aquaporins water will rapidly move across the membrane in response to the osmotic changes. These osmotic changes are in general met by activation of regulatory mechanisms bringing the cell volume (almost) back to normal see e.g. refs. (56; 78). There are though exceptions where the changed volume does not result in volume regulation (see section 1.4.2 and (56)).
1.4.1.1 Pathophysiological conditions
An altered cell volume is found in a number of pathophysiological conditions such as ischemia/hypoxia and epilepsy where an increase in intracellular osmolarity is seen. Hepatitis cirrhosis, nephrosis and hyponatremia are all examples of conditions that include a decreased plasma osmolarity, whereas increased plasma osmolarity is seen in diarrhea, uremia and diabetes mellitus/insipidus see (96; 111). Of other pathophysiological states can be mentioned cerebral edema (see (96)).

1.4.1.2 Regulatory mechanisms in response to volume changes
As stated above, osmotic challenged cells will often respond to the resulting swelling or shrinkage by initiating a regulatory response thereby returning their volume back towards the starting point. The cell uses different regulatory mechanisms in regard to swelling contra shrinkage. Regulatory Volume Decrease (RVD) is the cells response to swelling and consists of mechanisms excluding osmolytes and with concomitant water efflux as result, whereas Regulatory Volume Increase (RVI) is seen in response to shrinkage and involves the uptake of osmolytes and water (fig. 6).

As seen from the scanning electron images in fig. 5 EAT cells have clear invaginations of the membrane under isotonic conditions, letting them tolerate some swelling by unfolding of the surface thereby not disrupting the cell membrane. Shrinkage on the other hand causes more prominent invaginations (51). This is not only true in Ehrlich cells but also in lymphocytes (18) and certainly in a lot of other cell types. Since the mechanisms of RVI is not of direct interest to this work I will not go more into detail on the matter, but merely refer to fig. 6 and the extensive review by Hoffmann et al. (56).
1.4.1.3 The volume set-point

Most cells have a fine tuned volume regulation which occurs instantly upon osmotic changes making volume changes almost undetectable and the cells are said to be isovolumetric. Mammalian cells are very sensitive to volume changes and as little as a 3.5% (44) volume change can result in the activation of regulatory mechanisms – the volume set-point of the cell is reached. The volume set-point defines the border for cell volume regulation and volumes higher or below this threshold will result in volume regulation. The volume set-point is not to be viewed as a definite size, but rather a dynamic mechanism by which the cell adapts to its environment and function (52) and it is thus seen that cells that undergo RVD or RVI often will have an altered volume set point (53; 103). The volume set-point can also change accordingly to cellular function, thus proliferating cells experience a shift in the volume set-point since volume regulation in this case is not beneficial for the growing cell (see section 1.4.2.2).

1.4.1.4 RVD

When subjected to cell swelling the cell will release KCl and organic osmolytes (52) through specific channels and co-transporters which will then drive water to leave the cell and subsequently lead the cell volume back towards its starting point. Besides the volume sensitive K⁺ and Cl⁻ channels KCl can also leave the cell through transporters and so the K⁺-Cl⁻ co-transporters, the H⁺/HCO₃⁻ anion exchanger and the K⁺/H⁺ exchanger (fig. 6) are likewise important players in a RVD response (see (111)).

The exact composition and influence of the different transporters and channels involved in RVD differs from cell type to cell type, thus different K⁺ channels have been shown to be implicated in the swelling induced K⁺ efflux. Whereas the K⁺ channel picture is very diverse, it is commonly agreed that the swelling activated Cl⁻ channel holds one identity namely that of VRAC (volume regulated anion channel), though it’s

---

**Fig. 6: Ion movement during RVD and RVI**

Cell swelling results in KCl efflux through specific K⁺ (varies from cell type to cell type) and Cl⁻ channels (VRAC), the K⁺-Cl⁻ co-transporter (KCC), the H⁺/HCO₃⁻ anion exchanger (AE), the K⁺/H⁺ exchanger together with an organic osmolytes efflux (not shown here). The ion efflux drives osmotic obliged water to leave the cell and let it reach a volume close to its starting point. RVI on the other hand rely on ion up-take through transporters such as AE, the Na⁺/H⁺ exchanger NHE, the Na⁺/K⁺/2Cl⁻ co-transporter (NKCC), the Na⁺/Cl⁻ co-transporter and organic osmolytes up-take (not shown here). Own figure.
molecular identity for the time being remains unknown. The molecular identity of VRAC is very much a hot topic and it has been suggested to belong to various chloride channel families though no definite answer to the puzzle has been found. A further discussion of that subject is outside the scope of this thesis and will not be further dealt with here.

### 1.4.1.5 $K^+$ channels in volume regulation

As mentioned previously the $K^+$ channels picture is quite diverse and so far, and to my knowledge members of 9 different potassium channels subfamilies has been shown to be volume sensitive (see (56)). It should be noted that the discrepancies between the number stated here and the number of channel families listed as including volume sensitive members in ref. (56) is due to the fact that the volume sensitive channels KCNK5 was recently moved from the TASK subfamily to the TALK subfamily as described previously in this thesis (see section 1.3).

Volume sensitive potassium channels can e.g. be found amongst the family of Ca$^{2+}$-activated $K^+$ channels (47) in the BK subfamily (see (56; 111)), the IK (63; 67; 150) and SK (63; 132) subfamilies and amongst the voltage-gated $K^+$ channels has e.g. Kv1.3 (28) and Kv1.5 (4; 34) been shown to contribute to RVD in lymphocytes. Of special interest to this thesis the KCNK5 has been shown to be the volume regulating $K^+$ channel in EAT cells (104; 106) and mouse proximal tubules (7) see table 1 and (20).

### 1.4.1.6 Volume regulation in Ehrlich cells

Volume regulation in Ehrlich cells (EAT and ELA cells) have been intensely studied over the years and much knowledge has come from that. The main pathways for KCl efflux in Ehrlich cells are through specific volume sensitive $K^+$ and Cl$^-$ channels and the currents through these channels are designated $I_{K,\text{vol}}$ and $I_{Cl,\text{vol}}$ see (55).

$I_{Cl,\text{vol}}$

The outward rectified chloride current activated by cell swelling runs through the volume regulated anion channel (VRAC). It has been described as being Ca$^{2+}$ independent, tamoxifen inhibited, relative insensitive to DIDS and niflumic acid (118) and to hold a permeability sequence of SCN$^->I^->NO_3^->Br^-Cl^->F^-$ (see ref. (53)).

$I_{K,\text{vol}}$

Initially an intermediate-conductance $K^+$ (IK) channel was shown to be activated in cell attached patch studies on EAT cells (19) but later studies e.g. showing how $I_{K,\text{vol}}$ was poorly affected by clotrimazol, apamin, kalitoxin, margatoxin, TEA and ChTX which are all known inhibitors of $K^+$ channels (including Ca$^{2+}$-activated $K^+$ channels), together with the fact that $I_{K,\text{vol}}$ was activated despite buffering [Ca2+], (57) led to the conclusion that RVD in Ehrlich cells were dominated by a
Ca\(^{2+}\)-independent channel (62; 129). It was further more demonstrated how \(I_{K,vol}\) had a specific selection profile favoring entry of Rb\(^+\) and K\(^+\) which eliminated the stretch-activated K\(^+\) channel since it was proven to be non-specific (19; 108). For further information see (53). \(I_{K,vol}\) did not belong to the family of voltage-gated K\(^+\) channels either (108). One drug clofilium, did though inhibit \(I_{K,vol}\) hence evidence pointed towards the family of two-pore domain channels (106). PH sensitivity with potentiation of the current with alkalization and inhibition at acidification narrowed the field to – at that time - a TASK channel (see section 1.3) (54; 71). It was later shown that KCNK5 (TASK-2) but neither TASK-1 nor TASK-3 was present in Ehrlich cells (104).

**Activation of \(I_{K,vol}\) in EAT cells**

When swollen phospholipase cPLA\(_{2\alpha}\) is translocated to the nucleus (120) where it is phosphorylated and activated by a G-coupled process. This activation results in a release of arachidonic acid (AA) which is 3.3 times higher than seen in cells kept isotonic (145). AA which is a precursor for leukotriene C\(_4\) (LTC\(_4\)) requires the action of FLAP (5-LOX activating protein), 5-LOX and LTC\(_4\) synthase to be generated. LTC\(_4\) is subsequently transported out of the cell where it is converted to leukotriene D\(_4\) (LTD\(_4\)) by γ-Glutamyl transeptidase, here it binds to its receptor which in turn activates \(I_{K,vol}\) (57; 61; 74) (also see (53; 56)). The direct mechanisms between LTD\(_4\) and channel activation still remains to be studied.

Protein tyrosine kinase activation has been shown to play a role during RVD in various cell lines (6; 22; 25; 88) and it was speculated that tyrosine phosphorylation was also of importance in the RVD response in Ehrlich cells somewhere in the signaling pathway just described. In “Paper I” we show how tyrosine phosphorylation indeed is vital for RVD, since protein tyrosine kinase phosphorylation of the volume sensitive K\(^+\) channel (which in Ehrlich cells is KCNK5) is part of the activation mechanisms upon cell swelling. Thus we found a swelling-induced and time-dependent tyrosine phosphorylation on the channel itself in response to acute hypotonicity (Paper I, fig. 5).

**1.4.1.7 Long-term effects of anisotonicity**

During long-term exposure to an anisotonic environment cells respond with late-phase volume regulatory mechanisms in contrary to the acute phase response (described in section 1.4.1.2) which takes place within minutes of cell swelling. The late-phase mechanisms are often connected with an altered gene expression and protein synthesis changes. Organic osmolytes makes good late-phase regulators since accumulation of organic osmolytes compared to e.g. electrolytes are harmless, thus an accumulation of electrolytes can upset various metabolic processes e.g. denature macromolecules and influence membrane potential (see (142)).
Long-term anisotonicity can be both hypo- or hypertonic and hypertonicity is by far the most studied of the two conditions. It has been shown how long-term hypertonicity results in an increased gene transcription of various volume regulatory genes (14) while the number of studies on long-term hypotonicity is scarce. Thus we studied the KCNK5 function and expression on various levels in Ehrlich cells upon long-term exposure to hypotonicity (Paper II). We found that the maximum current through KCNK5 in ELA cells were inhibited (Paper II, fig. 1) as were the EAT cells ability to perform RVD (Paper II, fig. 2). We further more found a decreased KCNK5 protein expression in both cell lines upon stimulation (Paper II, fig. 4) and our results thus show how a functional down-regulation of KCNK5 upon long-term hypotonicity is probably due to a decreased KCNK5 protein synthesis (see section 7 and the discussion in Paper II).

1.4.1.8 Volume regulation in lymphocytes

Lymphocytes like most other mammalian cells are subject to changing intracellular environment causing potential volume changes and subsequent regulation. T cells are of course not static in the sense that they are in constant movement around the body and thereby eligible to extracellular osmotic changes e.g. when passing around the kidney. Lymphocytes are as most other mammalian cells capable of performing RVD in response to cell swelling (18; 29; 135) and as seen in many cells the higher the osmotic challenge the lesser the recovery (18). T cells are only able to perform RVI if previously experiencing hypotonically elicited RVD – also called post-RVD RVI (29).

As seen in other volume regulating cells VRAC is responsible for the swelling activated Cl⁻ current in lymphocytes (17; 81; 85) (also see (16)), whereas the K⁺ channel picture is more diverse and different regulatory volume pathways and K⁺ channels have been suggested to play a role in T cell volume regulation including the voltage-gated K⁺ channel Kv1.3 (17; 28; 80), the Ca²⁺-activated KCa3.1 (138), and two-pore domain potassium channels (3; 12). Kv1.3 is involved in RVD in mature T cells whereas KCa3.1 is so far only implicated in RVD in thymocytes (immature T cells), thus it is seen that RVD in mature T cells is Ca²⁺-independent (16). Kv1.3 is not directly volume sensitive, but is thought to be activated by a change in membrane potential due to activation of volume sensitive anion channels causing Cl⁻ efflux, plasma membrane depolarizing and subsequent Kv1.3 activation (16; 29; 87).

KCa3.1 and Kv1.3 is by far the most studied and described K⁺ channels in T cell physiology including cell volume regulation and the role of other K⁺ channels is still object to some discussion (see e.g. (16; 112)).

Recently members of the two-pore domain potassium channel family were proposed to play a role in cell volume regulation in murine and human T cells (3; 12), thus both KCNK5 (in mouse...
and human T cells) (3; 12) and TRESK-2 (in human T cells) (3) were given prominent roles in RVD. We have likewise studied the effect of KCNK5 in human T cell volume regulation and find that the channel is expressed in these cells and that this expression is up-regulated when the T cells are activated (Paper III, fig. 2). We find that the RVD response in activated T cells is impaired (Paper III, fig. 3), contradicting findings by Andronic et al. (see section 7 and the discussion in Paper III). We furthermore found that the Cl⁻ permeability in activated and swollen T cells were inhibited compared to non-activated control cells (Paper III, fig. 4), thus we suggest that Cl⁻ is the limiting factor in the RVD response in activated T cells and that the strong up-regulation of KCNK5 has another function. We speculate whether this function could be in maintaining an electrochemical gradient favoring a sustained Ca^{2+} influx, by extruding K⁺ and thus keeping the plasma membrane hyperpolarized (see paper III and section 1.5.3.2).

1.4.2 When changes in cell volume act as signals

Not all cell volume changes will result in the activation of volume regulatory mechanisms and often the change in cellular volume is an important player in cell signaling. As described previously an altered cell volume is seen in various pathophysiological events, but changes in cell volume without any regulatory mechanisms activating is also seen under physiological conditions. It has for instance been shown that volume changes serves as signaling events in cell proliferation and migration, transport across an epithelia and in apoptosis (fig. 7) which will be further addressed below (also see ref. (56)).

1.4.2.1 Programmed cell death

Different conditions can lead to programmed cell death (or apoptosis), thus programmed cell death is part of both cell physiology (e.g. cells lacking growth factor stimulation) and pathophysiology (e.g. cells with DNA damage). If not wanted, the cells can become suicidal by undergoing programmed cells death a process that in contrast to necrosis will not damage the surrounding cells. Apoptosis is a physiological process that is essential to keep the balance in cell number (see (79)). A number of characteristics can be observed

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Figure 7: volume changes as a signaling mechanism
There are various examples of an altered cell volume acting as a signaling event and some of these examples are depicted in this figure. See the text for more details. Model based on (56).
in an apoptotic cell and one well described feature in apoptotic cell death is the initial loss of cell volume. The apoptotic loss of cell volume is called Apoptotic Volume Decrease (AVD) and will not result in RVI. The volume decrease is concomitant to a loss of K$^+$ and Cl$^-$ ions which initially does not change the K$^+$ concentration but later in the AVD it decreases significantly (123). The decrease in K$^+$ is followed by an increase in the Na$^+$ concentration. AVD was first believed to be a secondary event in apoptosis, but have shown to be an important signal in facilitating the further progress into programmed cell death (79; 93; 123). It has further more been shown that the decrease in intracellular K$^+$ concentration is vital in apoptosis and that this decrease is to some extend due to an inhibition of Na$^+$/K$^+$ pump function. The role of K$^+$ channels in apoptosis is complex and so various K$^+$ channels have been implicated depending on cell type. It is most often seen that inhibition of K$^+$ channel function and thus inhibited K$^+$ loss will protect against apoptosis, but in some cell types the opposite has been seen. For more on ion channels in programmed cell death see (79).

1.4.2.2 Proliferation
When looking at cell volume changes and cell proliferation it is well known that the proliferating cell undergo swelling to be able to produce two daughter cells of the same volume as the initial parental cell, thus the dividing cell has increased in size without activating RVD mechanisms. What is perhaps less well known and definitely less described is how this increase in volume probably serves as a signal for the cell to progress through the cell cycle (see (56; 70; 92)). It has thus been shown how proliferation in some cells can be stimulated in swollen cells and inhibited in shrinking cells (30; 77; 122; 134). It is well known that cancer cells often have dysregulated control of cell cycle progression involving changes in ion channels (119). How exactly these volume changes influence cell cycle progression is yet to be fully elucidated.

1.4.2.3 Transepithelial transport
Keeping a steady cell volume in epithelial cells constitute a challenge for the cells, since they are subject to massive changes in osmolyte composition due to absorption and secretion of ions and nutrients which are followed by water (see (119)). Though cell volume under normal conditions are without large changes due to tight regulatory mechanisms there is evidence of epithelial cell volume as a mechanism in the physiology of absorption and secretion. In that respect cell swelling has been implicated in epithelial absorption and cell shrinkage in secretion (see (56; 78)).

1.4.2.4 Migration and invasion
In cell migration there is likewise evidence of cell volume being an important signal and it has e.g. been shown that hypertonicity inhibits neutrophile migration in the lung (131) and that cell swelling plays a role in human polymorphonuclear leucocytes migration stimulated by fMLP.
In transformed renal epithelial cells it was seen that migration could be inhibited both by hypo- and hypertonicity, thus it seems that the type of impact cell volume changes has on migration, can vary between cell types. For more on the subject of volume changes as a signal (see (56)). Briefly, it is thought that a migrating cell is capable of cell swelling in the protruding part of the cell and cell shrinkage in the back. These volume changes are facilitated by specific shrinkage-activated transporters and swelling-activated channels resulting in cell protrusion in the leading end and retraction in the rear end of the cell respectively (139).
1.5 The immune system

The immune system can be divided into two parts; the innate and the acquired (or adaptive) immune system. As the name implies the acquired immune system is under constant development through the exposure to pathogens, the following immune response and the subsequent activation of memory cells. The immune system is able to remember different pathogens, an ability that is acquired throughout the numerous infections and pathogenic influence an individual undergoes. This memory function makes a second exposure to a pathogen elicit a far greater and faster immune response compared to the first exposure response, even if the second exposure occurs many years after the initial one. Immunological memory can be obtained by exposing an individual to a pathogen either by disease or by vaccination.

The innate immune system, on the contrary, is the part of the immune system that we have with us from birth and it constitutes the first barrier that meets an invading pathogen. Only when the innate immune system has proven to be insufficient, the more specialized acquired immune system is activated. Some infections can be defeated solely by an innate immune response and those infections will hardly give any symptoms or do much harm to the body.

The innate and the adaptive immune systems, though playing very different parts in the battle against intruders, should not be viewed as two distinct features, but rather as players on the same team. The two system overlaps in different ways e.g. the dendritic cells of the innate immune system plays a crucial role in the activation of the adaptive immune system since they act as antigen presenting cells (APC’s), in fact the dendritic cells are the most important of APC’s. Two other cell types can also act as APC’c namely the macrophages and the B cells.

Both the innate and the adaptive immune system relies on white blood cells or leucocytes, but whereas the major players in the innate system are the macrophages, granulocytes, mast cells and dendritic cells the highly specialized T and B lymphocytes (T and B cells) constitutes the acquired part. A small fraction of activated T and B cells end up as memory cells making a future response to the same pathogen more efficient (see above).

1.5.1 B and T lymphocytes

When encountering an antigen, it is recognized by different mechanisms being a T cell or a B cell and whereas T cells recognize antigens with the T cell receptor (TCR) B cells have immunoglobulins acting as antigen receptors. Upon antigenic binding to the TCR T cells activates, proliferate and differentiates into effector cells.
While B cells are specialized in detecting antigens of pathogens living outside the cells, T cells are specialized against pathogens living inside the host cell, thus they will always act on another cell and not the pathogen itself. So to be activated T cells have to recognize a specific antigen which can be from viruses, intracellular bacteria or from pathogens which have been internalized, degraded by the host cell and taken up by major histocompatibility complex (MHC) molecules to be displayed on the cell surface.

Whereas B cells recognize the antigen directly, T cells cannot recognize antigens unless displayed by MHC molecules. B cells when activated differentiate into plasma cells which are antibody producing (fig. 8), but since the B lymphocytes are of lesser interest in regard to this thesis they will not be further dealt with here.

### 1.5.2 T cell activation

T cell activation – from naïve to effector T cell – requires various signals and mechanisms. The activation signaling can be divided into three sub-signals; signal 1, 2 and 3. Signal 1 comprises the antigen-specific TCR activation whereas signal 2 involves the interaction between the APC and the T cell itself, resulting in the promotion of survival and expansion of the T cell. In signal 3 differentiations of the effector T cells in response to cytokine stimulation is seen. One of the co-stimulatory molecules involved in signal 2 is B7 molecules, which act on the CD28 receptor on the T cell.

**Different morphology of naïve vs. activated lymphocytes**

Inactivated or naïve lymphocytes are small, has few organelles and most of the chromatin in the nucleus is inactive. When activated the cell undergo and extreme transformation, they swell (both the nucleus and the surroundings), the chromatin becomes less dense, nucleoli becomes visible and a massive mRNA and protein synthesis occurs – the lymphocytes goes from being an inactive naïve lymphocyte to becoming an activated lymphoblast.

### 1.5.2.1 T cell subtypes

As mentioned previously activation of T cells result in proliferation and differentiation into different kinds of effector T lymphocytes such as Cytotoxic T cells, Helper T cells or Regulatory T cells (fig. 8). From the start (after their development in the thymus) T lymphocytes can be divided into two groups according to the cell surface proteins displayed. One group bears the surface protein CD4 the other carries CD8, both surface proteins being important for T cell function. There are two main classes of MHC molecules – MHC class I and MHC class II and whereas CD8+ T cells recognize MHC class I molecules, CD4+ T cells recognize antigens shown on the surface by MHC class II molecules. MHC class I molecules display peptides from the cytosol...
while MHC class II molecules display peptides from intracellular vesicles, a difference reflecting the different tasks of the two T cell subtypes.

**CD8⁺ T cells**

Cytotoxic T cells carry the CD8 surface protein (or co-receptor) and kills virus infected cells. They recognize antigen peptides generated in the cytoplasm of a cell and presented by MHC class I molecules, which is found on almost all nucleated cells. When activated CD8⁺ T cells are always bound to differentiate into cytotoxic T cells.

**CD4⁺ T cells**

Whereas CD8⁺ T cells always become cytotoxic T cells, CD4⁺ T cells can differentiate further into the subsets T\(_h\)1, T\(_h\)2, T\(_h\)17 and regulatory T cells (fig 8). T\(_h\)1 and T\(_h\)2 subsets are also known as helper T cells since they help activate B cells, T\(_h\)17 are involved in activating a neutrophile response, whereas the role of regulatory T cells is to suppress the immune system. CD4⁺ T cells recognize MHC class II molecules and their general role is to activate other cells such as the B cell. MHC class II molecules are found on B cells, dendritic cells and macrophages which are all cells of the immune system and which is in contrast to the MHC class I molecules found on numerous cell types.

![Diagram of T and B cell development and subsets](image)

**Figure 8: overview of the development of T and B cells and their subsets upon activation**

Both T and B cells originate from the common lymphoid progenitor. A B cell will stay a B cell but will after activation differentiate into antibody secreting plasma cells. T cells on the other hand can be divided into two main groups; those carrying the CD8 surface protein and those carrying CD4. CD8⁺ T cells are bound to become cytotoxic T cells upon activation while CD4⁺ cells can further differentiate into the subsets T\(_h\)1, T\(_h\)2, T\(_h\)17 and regulatory T cells. A small fraction activated T and B cells will become memory cells (not shown on this figure). Own figure.
1.5.3 When the immune system attacks the body

Sometimes the immune system turns on us in a way that is not beneficial for us. Factors in the surrounding environment can be taken for antigens and elicit an unwanted immune response as seen in e.g. allergic reactions, which as its best means a lot of discomfort for the individual, but as its worst can be life-threatening. Another type of unwanted immune reaction is not seen in response to the surroundings but to the body’s own cells and tissue – a response known as autoimmunity. One of the most important features of the immune system is its ability to regulate and to generate self-tolerance. During lymphocyte development lymphocytes with affinity to the individual will be generated since the process is strictly random, but they will be removed before entering the bloodstream. In autoimmune diseases something in the process of generating self-tolerance is not working properly with potential catastrophic consequences as a result. Of T cell mediated autoimmune diseases can be mentioned e.g. type 1 diabetes mellitus and multiple sclerosis.

1.5.3 Ion channels in T cell activation

When encountering an antigen presented by MHC class proteins on dendritic cells T cells get activated. This activation leads to clonal expansion of the antigen specific T cells and the (hopeful) destruction of the invading pathogen. Clonal expansion involves strong proliferation thereby amplifying the number of T cells bearing the TCR’s relevant for a given infection. The activation of T cells relies on numerous factors and ion channel function is essential in this process.

Three ion channels have been well described in T cell activation namely the voltage-activated Kv1.3 channel, the Ca\(^{2+}\)-activated KCa3.1 channel and the Ca\(^{2+}\) release-activated Ca\(^{2+}\) channel (CRAC). For more on the subject see e.g. (16; 112; 146). All of these channels and mechanisms will be further described in the following sections.

1.5.3.1 Ca\(^{2+}\) signaling

For a sustained activation of T cells an elevated intracellular Ca\(^{2+}\) concentration \([Ca^{2+}]_i\) is crucial (59; 152) and is generated by Ca\(^{2+}\) release from intracellular stores together with Ca\(^{2+}\) influx from the extracellular matrix. MHC class II protein presentation of an antigen and the subsequent TCR stimulation results in depletion of Ca\(^{2+}\) from intracellular stores to the cytoplasm through two different signaling pathways - an 1,4,5-inositol trisphosphate (IP\(_3\)) (58) and a cyclic ADP-ribose (cADPr) (45) mediated signaling pathway. The elevated \([Ca^{2+}]_i\) in turn activates calcineurin – a
Ca\textsuperscript{2+}-calmodulin-dependent phosphatase which dephosphorylates the transcription factor NFAT facilitating interleukin-2 (IL-2) transcription and expression, which is important in keeping the T cell activated in an antigen independent manner. A 1-2 hour rise in [Ca\textsuperscript{2+}]\textsubscript{i} is needed for sufficient NFAT stimulation and IL-2 expression, thus a sustained high intracellular Ca\textsuperscript{2+} level is therefore essential for T cell activation (24; 40; 65; 102). For a review on Ca\textsuperscript{2+} signaling in T cells see (86).

**The IP\textsubscript{3} mediated pathway**

The binding of MHC complexes (bearing antigen peptides) to the T cell receptor results in recruitment and activation of protein tyrosine kinases (PTK’s) from the families Src, Csk, Tec and Syk (see (127)). The PTK’s then phosphorylates and activates phospholipase C-γ (PLCγ) leading to the cleavage of phosphatidylinositol 4,5-bisphosphate (PIP\textsubscript{2}) into diacylglycerol (DAG) and IP\textsubscript{3}. IP\textsubscript{3} binds to the IP\textsubscript{3}-receptor in the membrane of the endoplasmatic reticulum (ER) causing release of Ca\textsuperscript{2+} from ER stores into the cytosol. When Ca\textsuperscript{2+} is depleted from ER-stores Ca\textsuperscript{2+} is released from of the EF-hand domain of stromal interacting molecule 1 (STIM1) located within the lumen of ER, thus STIM1 acts as a store depletion sensors (90; 133). The Ca\textsuperscript{2+} from the ER stores causes the STIM protein to oligomerize and translocate to activate Orai1 bound to the plasma membrane. This results in the forming of a Ca\textsuperscript{2+} selective pore – the CRAC channel which allows Ca\textsuperscript{2+} to enter the cell down its concentration gradient (124; 149; 155) (see fig. 9).

**The cADPr mediated pathway**

Another pathway resulting in Ca\textsuperscript{2+} release from intracellular thapsigargin insensitive stores is via cADPr – a pathway much lesser studied and described than the IP\textsubscript{3} mediated pathway described above. The intracellular stores releasing Ca\textsuperscript{2+} through this pathway are located close to the plasma membrane (13) and the Ca\textsuperscript{2+} release is as for the IP\textsubscript{3} pathway initiated by tyrosine kinases in response to receptor coupling see (112).

Signaling events by both pathways result in the uptake of Ca\textsuperscript{2+} through CRAC channels and results by Guse et al. (46) suggests that high [Ca\textsuperscript{2+}], via the IP\textsubscript{3} pathway is important for the initiation and cADPr signaling for the sustainment of the signal.

**1.5.3.2 K\textsuperscript{+} channels in Ca\textsuperscript{2+} signaling**

The sustained high [Ca\textsuperscript{2+}], depends on membrane hyperpolarization facilitated by K\textsuperscript{+} efflux through specific K\textsuperscript{+} channels, thus besides the release from ER stores [Ca\textsuperscript{2+}], is also influenced by voltage sensitive Ca\textsuperscript{2+} influx from the extracellular matrix through CRAC channels (fig. 9). Ca\textsuperscript{2+} influx is governed by capacitative Ca\textsuperscript{2+} entry by which the depletion of intracellular stores facilitates Ca\textsuperscript{2+} uptake (126; 144) through CRAC channels situated in the plasma membrane (114) and once activated the electrochemical gradient determines the Ca\textsuperscript{2+} influx (159). This also
means that the influx will increase in response to cell membrane hyperpolarization which can be obtained by the efflux of $K^+$ (fig. 9).

One of the most well described $K^+$ channels in T cell activation physiology is the voltage-gated Kv1.3 channel (also known as KCNA3) which was first described in 1984 (15; 27; 36; 95). The channel activates at approximately -60mV (94; 147) not far from the resting potential (148) and further depolarization causes increased conductance, thus T cell activation and the following $Ca^{2+}$ influx results in a depolarization of the membrane, Kv1.3 activation and $K^+$ efflux. This in turn results in hyperpolarization which creates the electrochemical gradient favoring sustained $Ca^{2+}$ entry (16; 112).

The Kv1.3 channel is not alone in generating the membrane potential needed for $Ca^{2+}$ entry thus the $Ca^{2+}$ activated KCa3.1 (also known as hIKCa1, IKCa1 or KCNN4) also play an important role. Activation of KCa3.1 is not dependent on membrane potential but is activated – as the name implies – with rising [$Ca^{2+}$], which is detected by calmodulin bound to the C-terminus (33; 67). KCa3.1 activation level is between 200 and 300nM [$Ca^{2+}$], which is below the concentration found in non-activated T cells (30-100nM $Ca^{2+}$ (147)). The channel is activated by the rise in [$Ca^{2+}$], mediated by store depletion and $Ca^{2+}$ influx via CRAC channels (fig. 9).

**1.5.3.3 Other ion channels described in T cells**

There are five main channel types described in T cells; Kv1.3, KCa3.1, $I_{Cl,vol}$, CRAC and TRPM7 channels but other channel types has also been proposed to play a role in T cell physiology. Amongst them are e.g. channels such as voltage-gated $Na^+$ channel (15) and the potassium channels TASK-1 and TASK-3 has likewise been reported in T cell function (98) though their roles still need further investigations and might be by some regarded as questionable (16; 146). Besides TASK-1 and TASK-3 one of those, in T cell activation relatively recent described $K^+$ channels, is KCNK5 (also known as TASK-2 or K2p5.1), thus in 2010 Bittner et al. published a paper giving KCNK5 a role in the autoimmune disease multiple sclerosis (10). Though the presence and functional importance is questioned by some (REF) preliminary data supported that KCNK5 was expressed in T cells and we thus investigated the expression pattern of KCNK5.
both on mRNA and protein levels and found a strong up-regulation of the channel upon T cell activation (see Paper III, fig. 1+2).

Bittner et al. furthermore found a significant up-regulation of KCNK5 in the T cell subtypes CD4$^+$ and CD8$^+$ in multiple sclerosis patients during acute relapses and furthermore they found KCNK5 levels to be increased in CD8$^+$ T cells. We have also looked into the KCNK5 expression pattern in various T cell subtypes, confirming an up-regulation upon activation though we find no significant difference in KCNK5 protein expression between the studied subtypes (see fig. 10, own unpublished results).

### 1.5.4 T cell proliferation

T cell proliferation is essential for the battle against pathogen intruders, since the activation and increased number of the right T cells will result in efficient fight. Clonal expansion is one of the most important dogmas in immunology and describes how T cells will proliferate upon activation. Being able to control or ultimately stop proliferation has thus gained high interest since it might be a mean to prevent autoimmune diseases.

#### 1.5.4.1 K$^+$ channels in proliferation

K$^+$ channels are shown to be involved in proliferation in various cell types and though the exact mechanisms behind the involvement of ion channels in proliferation is still somewhat uncertain the importance of ion channels are not. It is believed that ion channels help regulate...
proliferation through their potential impact on mechanisms such as membrane potential, calcium homeostasis and or cell volume regulation (see below and section 1.4.2.2).

As mentioned previously ion channels and volume regulation is thought to influence cell proliferation since a key feature of the dividing cell is the gain in volume ensuring that the daughter cells do not get smaller and smaller with every division (see section 1.4.2.2).

Different K+ channels have been implicated in cell cycle-control in various cell types where they are thought to play a role in cell cycle progression by control of the membrane potential thus it is seen that the membrane potential in early G1 phase (see fig. 11) is depolarized and followed by a hyperpolarization through G1 to the S phase (see fig. 11) – a hyperpolarization believed to be governed by K+ channels (143). In MCF-7 breast cancer cells this hyperpolarization depend on the activation of ATP-sensitive K+ channels (153) and in brown fat cells (113) melanoma cells (109) and prostate cancer cells (141) voltage-gated K+ channels was shown to be involved. The same mechanisms was also true for the two pore-domain K+ channel TASK-3 in a human lung carcinoma cell line (121). For more on the subject see (116). KCNK5 has been implicated in the regulation of proliferation in human breast cancer cell lines T47D and MCF-7 where it is believed that a increase in KCNK5 is necessary for the progression of the cells from G1 to the S phase (1). Even though we cannot draw direct conclusions from our data two things are certain, T cells proliferate upon activations and KCNK5 is at the same time heavily up-regulated (see fig. 10 A+B and Paper III).
2 Summary and conclusions

During my time as a PhD-student I have worked on the KCNK5 channel in three different set-ups. I have worked on the signaling pathway from acute cell swelling to activation of KCNK5 and RVD in Ehrlich cells, I have studied the long-term effects on channel physiology and expression pattern also in Ehrlich cells and last but not least I have studied the expression pattern and physiology in activated human T cell physiology. I have learned a great deal about the channel and in the following I will try and summarize and when possible conclude on my findings.

**Paper I**
- KCNK5 is tyrosine phosphorylated upon swelling-induced activation in a time-dependent manner
- As tyrosine phosphorylation seem to be involved in activation of the channel tyrosine phosphatases seem to be involved in the de-activation
- This tyrosine phosphorylation does not seem to be caused by neither Src nor FAK
- The JAK/STAT pathway could be up-streams of the phosphorylation and activation of KCNK5

**Paper II**
- Long-term hypotonicity (48h) reduces the maximum current through KCNK5
- Long-term hypotonicity likewise reduces RVD performance upon cell swelling
- This physiological impairment is probably due to a decreased KCNK5 protein expression

**Paper III**
- CD3/CD28 activation of human T cells result in proliferation and cell swelling
- KCNK5 is heavily up-regulated both on mRNA and protein levels in activated T cells
- Despite KCNK5 up-regulation activated T cells has impaired RVD
- RVD impairment in activated T cells is probably due to a functional down-regulation of the volume activated anion channel VRAC in these cells
3 Experimental procedures

Most of the experimental procedures are described in the three articles, but one procedure deserves a few comments (section 3.1) and some experiments are not published but are included in this thesis, thus the experimental procedures for these experiments will be explained in the following section (section 3.2).

3.1 cDNA quantification instead of reference genes
In the search of suitable reference genes for normalization of Real-Time qPCR data numerous candidates were tested but none showed satisfactory results. Instead I looked at alternative ways of normalizing my data and such an alternative was cDNA quantification using Quant-It Oligreen (Invitrogen) which was described by Lundby et al. in 2005 (91). One could argue that since the same amount of mRNA is used for each cDNA sample generated there should be no particular need for an extra normalization procedure but besides being standard method of operation when conducting Real-Time qPCR previously results verify the need for an additional normalization step. The discrepancies between mRNA starting concentration and cDNA end result could be due partly to pipetting uncertainties or slight differences in PCR reaction in each tube. Thus cDNA was quantified in each sample as described (91) and the data normalized to that value.

3.2 T cell subtypes
During my studies of KCNK5 in activated T cell physiology I also looked at the KCNK5 expression pattern in T cell subtypes besides the studies on whole T cell populations. The results are found in section 1.5.3.3 and in fig. 10.

3.2.1 Purification
T cell subtypes were purified from human peripheral blood mononuclear cells (PBMC’s) using EasySep enrichment kits from StemCell Tech. PBMC’s were previously purified from human buffy coats obtained from the blood bank at Rigshospitalet as described in paper III. The following subtypes were purified in accordance to manufactures descriptions: Human CD8⁺ T cells, Human CD4⁻ T cells, Human Naïve CD8⁺ T cells and Human Naïve CD4⁺ T. T cell subtypes were kept in the same media and under the same conditions as the heterogeneous T cell population (see paper III) and were kept either quiescent or activated using Dynabeads Human T-activator CD3/CD28 (Invitrogen, Life Sciences) in the ratio 1:1.

3.2.2 Proliferation
Proliferation was simply measured by cell count using the NucleoCounter® NC-100™ machine (ChemoMetech, Allerød, Denmark).
References


35. **Flores CA, Cid LP, Niemeyer MI and Sepúlveda FV.** B lymphocytes taken to task: a role for a background conductance K2P K+ channel in B cells. Focus on "Expression of TASK-


Ref Type: Unpublished Work


Activation of the TASK-2 channel after cell swelling is dependent on tyrosine phosphorylation

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Activation of the TASK-2 channel after cell swelling is dependent on tyrosine phosphorylation

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The swelling-activated K⁺ currents (I_{swell}) in Ehrlich ascites tumor cells (EATC) has been reported to be through the two-pore domain (K₂,2), TWIK-related acid-sensitive K⁺ channel 2 (TASK-2). The regulatory volume decrease (RVD), following hypotonic exposure in EATC, is rate limited by I_{swell} indicating that inhibition of RVD reflects inhibition of TASK-2. We find that in EATC the tyrosine kinase inhibitor genistein inhibits RVD by 90%, and that the tyrosine phosphatase inhibitor monoperoxidase(picolinate)-oxy-vanadate(V) [mpV(pic)] shifted the volume set point for inactivation of the channel to a lower cell volume. Swelling-activated K⁺ efflux was impaired by genistein and the Src kinase family inhibitor 4-amino-5-(4-chloro-phenyl)-7-(t-buty1) pyrazol[3,4-d]pyrimidine (PP2) and enhanced by the tyrosine phosphatase inhibitor mpV(pic). With the use of the TASK-2 inhibitor clotrimium, it is demonstrated that mpV(pic) increased the volume-sensitive part of the K⁺ efflux 1.3 times. To exclude K⁺ efflux via a KCl cotransporter, cellular Cl⁻ was substituted with NO₃⁻. Also under these conditions K⁺ efflux was completely blocked by genistein. Thus tyrosine kinases seem to be involved in the activation of the volume-sensitive K⁺ channel, whereas tyrosine phosphatases appears to be involved in inactivation of the channel. Overexpressing TASK-2 in human embryonic kidney (HEK)-293 cells increased the RVD rate and reduced the cell volume set point. TASK-2 has tyrosine sites, and precipitation of TASK-2 together with Western blotting and antibodies against phosphotyrosines revealed a cell swelling-induced, time-dependent tyrosine phosphorylation of the channel. Even though we found an inhibiting effect of PP2 on RVD, neither Src nor the focal adhesion kinase (FAK) seem to be involved. Inhibitors of the epidermal growth factor receptor tyrosine kinases had no effect on RVD, whereas the Janus kinase (JAK) inhibitor cucurbitacin inhibited the RVD by 40%. It is suggested that the cytokine receptor-coupled JAK/STAT pathway is upstream of the swelling-induced phosphorylation and activation of TASK-2 in EATC.

The ability of a cell to volume regulate after volume perturbations is fundamental for its homeostasis. When exposed to an increase in the internal or a decrease in the external osmolyte concentration, most mammalian cells swell and subsequently compensate for this swelling by a release of osmolytes through different membrane transporters followed by a concomitant efflux of water, a process termed regulatory volume decrease (RVD) (19). Especially swelling-activated K⁺ and Cl⁻ channels as well as volume-sensitive transporters of organic osmolytes (e.g., taurine) are important in the RVD response (16), and it has been estimated that ~70% of the loss of osmolytes in Ehrlich ascites tumor cells (EATC) during RVD is due to the loss of KCl (14). Various K⁺ channels have been shown to be implicated in RVD depending on cell type; i.e., 1) Ca²⁺-activated small conductance K⁺ (SK) channels (24, 42), intermediate conductance K⁺ (IK) channels (3, 7, 24, 25, 46), and Big conductance K⁺ (BK) channels (10, 23); 2) stretch-activated K⁺ channels; 3) voltage-dependent channels; 4) the MinK channel; and 5) the two-pore domain K⁺ channel (4, 31–35, 37). The volume-sensitive ion channels in EATC are the volume-regulated anion channel (VRAC) (38) and the TWIK-related acid-sensitive K⁺ channel-2 (TASK-2) (22, 33, 41), which belongs to the family of two-pore domain channels (K₂,2). TASK-2 is sensitive to clotrimium (35), independent of intracellular Ca²⁺ (35, 41), and highly sensitive to external pH (21). Besides being the swelling-activated K⁺ channel in EATC, TASK-2 has also been found to be involved in volume regulation in kidney cells (4) and in murine spermatozoa (1). Moreover, TASK-2 has been associated with apoptotic volume decrease in EATC cells following cisplatin exposure (40). For further details about volume-sensitive K⁺ channels see Refs. 19 and 44.

Tyrosine kinases are important under several physiological conditions such as cell proliferation, migration, differentiation, metabolism, and immune system function, but also a high number of oncogenes are tyrosine kinases (for a review see Ref. 6). There are several evidences for the role of tyrosine kinases in RVD (8). The nonreceptor tyrosine kinases Src and focal adhesion kinase (FAK) has previously been reported to be activated during hypotonic stimulation in various cell types; i.e., phosphorylation of an activation site in FAK have previously been reported in, for example, chicken retina cells (9), human umbilical vein endothelial cells (15), and in cerebellar granule neurons (30), whereas hypotonic activation of Src has been reported in cerebellar granule neurons (30), rat hepatocytes (45), and in rat hepatoma cells (2). There is however no clear evidence linking the activation of tyrosine kinases to activation of swelling-activated K⁺ currents (I_{swell}). In EATC preliminary results showed that the relatively unspecific tyrosine kinase inhibitor genistein (371 μM) strongly inhibited RVD, and the tyrosine phosphatase inhibitor monoperoxidase(picolinate)-oxy-vanadate(V) [mpV(pic)] (10 μM) significantly decreased the volume set point after RVD (17). Thus tyrosine phosphorylation appears to play a role in RVD in EATC, possibly as a part of the volume-sensing mechanism. The objective of this study was thus to examine the involvement of tyrosine kinases/phosphatases in the swelling-induced activation/deactivation of TASK-2 in EATC. We specifically test whether overexpression of TASK-2 improves the RVD re-

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sponse and whether TASK-2 is directly tyrosine phosphory-
lated following hyposmotic exposure.

**EXPERIMENTAL PROCEDURES**

**Cell Cultures: EATC, EATCC, and Human Embryonic Kidney Cells-293**

In the present investigation we have used EATC maintained under two separate conditions: 1) EATC maintained in first generation hybrids of female NMRI mice by weekly intraperitoneal transplantation of 1.5 × 10^7 cells per mouse. Six to seven days after transfection the cells were harvested and transferred to isotonic NaCl-Ringer containing 2.5 U/ml heparin and resuspended at cytotoxic 4%. 2) EATC cultures (EATCC) were obtained by transferring EATC to RPMI 1640 medium supplemented with 10% serum and 100 U/ml penicillin-streptomycin and grown at 37°C. 5% CO2. EATCC were maintained by transfer of 8.5 × 10^5 cells to 10 ml fresh RPMI 1640 medium every 3–4 days, and only passages 6–28 were used for experiments. The EATCC were introduced because we wanted to avoid the use of mice for tumor cell growth. This was supported by The Danish Society for the Protection of Laboratory Animals. Propagation of ascites cells was approved by “Dyreforsorgsstyrelsen” (2007/561–1313). J) Human embryonic kidney cells (HEK-293) were grown in Dulbecco’s modified Eagle’s medium (DMEM with GlutaMAX-I, t-glucose, and sodium pyruvate, Invitrogen) with 10% fetal bovine serum and 10 U/ml penicillin-streptomycin. Cells were subcultured every 3–4 days.

**Solutions and Materials**

For EATC the standard isotonic NaCl-Ringer solution (300 mosM) contained (in mM) 143 NaCl, 5 KCl, 1 NaHCO3, 1 CaCl2, 3.3 MOPS, 3.3 N-tris(hydroxymethyl) methyl-3-aminoethane sulfonic acid (TES), and 5 HEPES, pH 7.4. Hypotonic Ringer (150 mosM) was obtained from the isotonic solution by dilution with buffered water (water containing 3.3 mM MOPS, 3.3 mM TES, and 5 mM HEPES, pH 7.4). The NO3 buffer was prepared by adding 3.3 mM MOPS, 3.3 mM TES, and 5 mM HEPES; pH was adjusted to 7.4. Hypotonic Ringer (150 mosM) contained (in mM) 140 NaCl, 4 KCl, 2 CaCl2, 1 MgCl2, and 10 HEPES; pH was adjusted to 7.4. Hypotonic Ringer (150 mosM) was obtained as described above. Unless otherwise stated, reagents were analytical grade and obtained from Sigma (St. Louis, MO).

**Cell Volume Measurements**

Cell volume was determined using two different methods. Absolute cell volume of EATC/EATCC was estimated by electronic cell sizing by Coulter counter measurements (tube orifice 100 μm, Coulter Multisizer 3, Coulter, Luton, UK). Relative cell volume of the mono-

**Gel Electrophoresis and Western Blotting**

EATCC were lysed in 95°C lysis buffer (10 mM Tris-HCl, pH 7.4, 1% SDS, and 20 mM EDTA) with protease inhibitors (Roche Applied Science) and phosphatase inhibitors added. The lysates were subsequently homogenized, and the supernatant was collected by centrifugation. HEK-293 cells transfected with TASK-2 were lysed in 95°C lysis buffer (5 mM Tris-HCl, pH 7.4, 0.5% SDS, and 10 mM EDTA). Protein concentrations were determined using Bio-Rad DC protein assay. SDS-polyacrylamide gel electrophoresis were performed using a 10% gel (Invitrogen) and transferred to nitrocellulose membranes. The membranes were dyed with Ponceau Red (Sigma) and washed in TBST (10 mM Tris-HCl, pH 7.5, 120 mM NaCl, and 0.1% Tween 20) before being blocked in a 5% nonfat dry milk and TBST solution. Membranes were incubated with primary antibody at following concentrations: TASK-2 (Alomone) 1:500, nonphosphorylated (np) FAK Y397 (Biosource) 1:200, phosphorylated (p) FAK Y397 (Biosource) 1:200, np-Src Y416 (Cell Signaling) 1:250, p-Src Y416 (Cell Signaling) 1:250, np-Src Y527 (Cell Signaling) 1:250, p-Src Y527 (Cell Signaling) 1:250. Membranes were washed in TBST before introduction to secondary antibody (Sigma) 1:5,000. The protein bands were visualized using 5-bromo-4-chloro-3-indolyl phosphate-nitro blue tetrazolium (BCIP/NBT Kirkegaard and Perry Lab) and scanned. Protein band density was estimated using UN-SCAN-IT software.
A TASK-2 containing vector (pcDNA3.1) was kindly given to us by Dr. Sepúlveda (Chile). The TASK-2 channel was transferred to two other vectors. The first, pMT2-HA vector using PCR at the following conditions: denaturation at 98°C for 30 s followed by 25 cycles with the initial temperature at 98°C for 10 s, annealing at 65°C for 30 s extension at 72°C for 40 s, and a end extension at 72°C for 7 min. The restrictions sites XhoI and XmaI were used together with the following primers: 5′-AGGTGACCGGGGT-3′ (sense) and 5′-TCACGTGCCCCTGGG-3′ (antisense). PCR product was purified and resolved by agarose gel electrophoresis. DNA bands were cut out of the gel, purified, and subsequently cloned into the pMT2-HA vector. For all purification steps products from Qiagen was used. Sequencing was performed by Eurofins MWG Operon (Germany). Second, pcDNA3.1/myc-His vector (Invitrogen) using PCR and the restrictions sites BamH I and XhoI. The following primers were designed: 5′-AGGTGACCGGGGTCCCTTC3′ (sense) and 5′-CTGCCCCCCTGGGGTTATCTGC-3′ (antisense). PCR conditions were the following: denaturation at 98°C for 5 min followed by 25 cycles with the initial temperature at 98°C for 1 min, annealing at 62°C for 30 s and extension at 72°C for 40 s, and at the end a 7 min extension at 72°C. The rest of the procedure was identical to the one described above.

**Light scatter.** HEK-293 cells for transfection were grown on coated (poly-l-lysine) coverslips to ~50–80% confluence and transfected with a TASK-2 containing vector (pMT2-HA). For transfection we used 1 µg DNA/3 µl FuGENE 6 Transfection Reagent (Roche Applied Science) according to the manufacturer’s instructions. Experiments were performed ~24 h after transfection.

**Precipitation of TASK-2.** HEK-293 cells for transfection were grown in coated (poly-l-lysine) wells (9.6 cm²/well) to ~50–80% confluence and transfected with a TASK-2-containing vector (pcDNA3.1/myc-His). For transfection we used 1 µg DNA/3 µl FuGENE 6 Transfection Reagent (Roche Applied Science) according to the manufacturer’s instructions. Experiments were performed ~36 h after transfection. It should be noted that we tried to use the HA tag to immunoprecipitate the TASK-2 without success.

**Western blot analysis.** HEK-293 cells for transfection were grown to ~50–80% confluence in six-well trays and transfected as described above.

**RESULTS**

**Effect of Tyrosine Kinase Inhibitors on RVD and on Swelling-Activated K⁺ Efflux**

In the present investigation we used EATC grown in cell culture (EATCC) or in the abdominal cavity of mice (EATCC) to investigate the effect of the tyrosine kinase inhibitor genistein on the RVD and the swelling-induced K⁺ loss. From Fig. 1, A and B, it is seen that genistein is an effective inhibitor of RVD in EATC grown both in culture and in the abdominal cavity. Genistein was dissolved in DMSO, which can be potentially toxic to the cells, but experiments showed that the concentrations used (0.34%) did not affect the ability of the cells to perform RVD (data not shown). With the use of the recovery level after 3 min (EATC) or 4 min (EATCC), it is estimated that genistein reduced the RVD from 73% to 22% in EATC (Fig. 1C) and from 61% to 10% in EATCC (Fig. 1D). It is noted that the swelling response to hyposmotic media is much faster (in seconds) in the EATCs than that in the EATCCs, which probably reflects a difference in the number of aquaporins. This has not been tested. To investigate whether genistein directly affects the swelling-activated K⁺ efflux, we measured the K⁺ efflux in EATC in hypotonic medium in the presence and absence of genistein. 86Rb was used as a tracer for K⁺. Figure 2, A and C, shows that genistein reduces the measured 86Rb efflux and the calculated K⁺ efflux down to about 30% upon hypotonic exposure; i.e., the control cells had a mean K⁺ loss of 100 µmol·g protein⁻¹·min⁻¹, and the treated cells had a mean loss of 33.3 µmol·g protein⁻¹·min⁻¹. To exclude contribution to the K⁺ efflux by the KCl cotransporter, we substituted all internal and external Cl⁻ with NO₃⁻ by preincubating the cells for 60 min in NO₃⁻ Ringer. From Fig. 2, B and D, it is seen that the swelling-activated K⁺ efflux, now representing only efflux via K⁺ channels, is completely inhibited by genistein. Substitution of NO₃⁻ for Cl⁻ reduced the K⁺ efflux from 222 ± 13 to 171 ± 10 µmol·mg dry wt⁻¹·min⁻¹ (n = 11 for each series), indicating a K⁺ efflux via the KCl cotransporter equal to 51 ± 10 µmol·mg dry wt⁻¹·min⁻¹; i.e., 23%. This is quantitatively similar to the genistein-insensitive K⁺ flux seen in NaCl medium (Fig. 2C).

**Effect of Tyrosine Phosphatase Inhibitor on RVD and Loss Of K⁺**

As inhibition of tyrosine kinases hinders RVD (Fig. 1) and inhibits the activation of volume-sensitive K⁺ channels (Fig. 2), we investigated whether inhibition of tyrosine phosphatases could potentiate the RVD in EATC. Cell volume measurements were performed in the presence of the tyrosine phosphatase inhibitor mpV(pic) (10 µM). From Fig. 3A it is seen that in control cells a new steady-state cell volume was obtained after 3 min exposure to hypotonic medium (150 mosM), which was about 20% larger than the original cell volume. In the presence of the tyrosine phosphatase inhibitor mpV(pic), the volume recovery at time 4 min improved and amounted to 90% compared with the 70% in control cells (Fig. 3B). These findings were further substantiated through K⁺ efflux measurements. When treated with mpV(pic), the mean swelling-activated K⁺ efflux in EATC was increased to 150% compared with nontreated cells (Fig. 3C). Adding clotrim, a known
TASK-2 blocker (35), the K⁺ efflux was inhibited to 27% of the K⁺ efflux value in nontreated control cells. In the presence of clofilium plus mpV(pic), the K⁺ efflux was reduced to the efflux value seen in the presence of clofilium alone. Thus a mpV(pic)-sensitive phosphatase seems to be involved in the inactivation of the volume-sensitive K⁺ channel (TASK-2) in EATC.

To test whether inhibition of tyrosine phosphatase could prevent the effect of inhibition of tyrosine kinase, we measured the RVD response in cells treated with genistein alone and genistein in combination with the phosphatase inhibitor mpV(pic). From Fig. 3D it is seen that inhibition of the tyrosine phosphatase indeed prevented the effect of kinase inhibition.

Fig. 1. The general tyrosine kinase inhibitor genistein inhibits volume recovery in Ehrlich ascites tumor cells (EATC) and EATC cultures (EATCC). A and B: EATC and EATCC were placed in either isotonic Ringer (300 mosM) or hypotonic Ringer (150 mosM), and the absolute volume was observed as a function of time. EATC and EATCC were preincubated with the tyrosine kinase inhibitor genistein (371 μM) for 45 min before they were transferred at time 0 to hypotonic medium also containing genistein (371 μM). C and D: mean recovery after 3 min of NaCl and EATCC in hypotonic medium was estimated from 3 independent sets of experiments and shown as means ± SE. **Significantly reduced from control cells ability to regulate their volume.

Fig. 2. Genistein inhibits swelling-induced, Cl⁻-independent K⁺ efflux. EATC were incubated in isotonic NaCl Ringer (A and C) or isotonic NaNO₃ Ringer (B and D). ⁸⁶Rb, genistein (100 μM) and [³H]inulin were added 60, 45, and 5 min before initiation of efflux estimation, respectively. Cells were subsequently washed once in isotonic Ringer (300 mosM, NaCl/NaNO₃), and at time 0 resuspended in hypotonic Ringer (150 mosM, NaCl/NaNO₃) containing no genistein (control) or 100 μM genistein. Twenty samples were drawn within the first 10 min and centrifuged through a silicone oil phase as indicated in Experimental Procedures. Double samples were used to estimate the cellular K⁺ and ⁸⁶Rb content within 1 min following hypotonic exposure. ⁸⁶Rb⁺ activity (cpm/mg cell dry wt) was plotted versus time (A: NaCl Ringer; B: NaNO₃ Ringer), and the rate constant for ⁸⁶Rb⁺ efflux (min⁻¹) was determined by fitting the efflux curve to an exponential function. The K⁺ efflux (μmol·g dry wt⁻¹·min⁻¹), calculated by multiplying the ⁸⁶Rb-rate constant (min⁻¹) by the K⁺ content (μmol/g dry wt), was estimated from 5 and 8 sets of experiments in NaCl Ringer (C) and NaNO₃ Ringer (D), respectively and shown as mean values ± SE. **Significantly reduced from control cells ability to regulate their volume (P < 0.01).
Functional Effect of Overexpressing TASK-2 on RVD

To test the effect of TASK-2 overexpression on RVD, we used HEK-293 cells, which according to Niemeyer and co-workers (33) show no swelling-activated K⁺ current. From Fig. 4A it is seen that overexpression of TASK-2 cloned from EATC in HEK-293 was obtained after 24, 36, and 48 h. Using the light scattering technique, we found that TASK-2 overexpressing HEK-293 cells (Fig. 4C) had a faster volume recovery than the wild-type cells (Fig. 4B). It was estimated that TASK-2 overexpression resulted in a twofold faster volume recovery (Fig. 4D). It is noted that the TASK-2 overexpressing cells made an overshot in the RVD (Fig. 4C), which could be taken to indicate that the cellular signaling system involved in closing of the TASK-2 channel was not effective in the HEK-293 cells. This was not investigated further. It is also noted that the transfected cells showed a strong post-RVD recovery (Fig. 4B), indicating a far greater ion loss during the RVD process compared with the wild-type cells. Thus expression of TASK-2 contributed significantly to the net loss of K⁺ followed by Cl⁻ and water.

Swelling-Induced Tyrosine-Phosphorylation of TASK-2

To investigate whether TASK-2 is tyrosine-phosphorylated following hypotonic exposure, we transfected HEK-293 cells with a TASK-2 containing pcDNA3.1/myc-His vector, stimulated the cells hypotonically for 1, 5, or 10 min, and subsequently precipitated the TASK-2 channel using Ni-NTA agarose (Qiagen). Tyrosine phosphorylation was detected through SDS-PAGE and Western blotting using a phospho-specific tyrosine antibody (pY100). From Fig. 5 it is seen that TASK-2 expression was identical in isotonic- and hypotonic-treated cells (Fig. 5A), whereas the phosphorylation of TASK-2 (pY100) increased within 1 min following hypotonic exposure and appeared to be maximal after 5 min (Fig. 5B). It is noted that ANOVA test on the total experimental data set indicated no difference of the pY100 band among 1, 5, and 10 min isotonic medium. The relative specific activity for tyrosine-phosphorylated TASK-2 under hypotonic conditions (the ratio between pY100 band intensity and the TASK-2 band intensity) was increased four times within the first minute (P < 0.14), four times within 5 min (P = 0.01), and three times after 10 min (P = 0.03) hypotonic exposure. Hence, the TASK-2 channel was still functional.
tyrosine phosphorylated 10 min after hypotonic exposure, i.e., at that time the K⁺ efflux was still high (Fig. 2, A and B).

**Putative Tyrosine Kinases Involved in Swelling-Induced Activation of TASK-2**

**Src and FAK.** Various tyrosine kinase inhibitors were used in an attempt to narrow down the potential tyrosine kinase(s) involved in channel activation. As seen in Fig. 6A the volume-activated K⁺ efflux was reduced with 25% in the presence of the Src kinase inhibitor PP2 compared with controls with no inhibitor present. Hypotonic medium with and without PP2 present indicates a small but significant inhibition of the swelling-activated K⁺ efflux in EATC. The RVD, estimated as the initial reduction in cell volume (10⁻³⁵ l/min), in EATCC was significantly reduced to 35% (Fig. 6B), indicating an eventual role of Src in the RVD response. To pursue a role of Src in activation of the swelling-induced K⁺ channels and RVD we used Western blot analysis with phospho-specific antibodies against 1) Src Y416, which is in the kinase domain and is phosphorylated during activation, and 2) Src Y527, which has to be dephosphorylated to activate the kinase. From Fig. 6, C (left) and D, it is seen that 1 and 5 min of hypotonic stimulation elicited a significant decrease in the phosphorylation of Src Y416, reflecting a decreased Src activity after osmotic cell swelling. Looking at the inactivating site Y527, no change in the phosphorylation levels was observed over a 10-min time period (Fig. 6, C, right, and E). The eventual effect of cell swelling on FAK was likewise investigated using Western blot analysis and a phospho-specific FAK Y397 antibody. From Fig. 7, A (Western blot) and B (quantification), it is seen that initially (1 min after stimulation), there was no change in the phosphorylation of FAK, which would be expected if the kinase was activated during cell swelling. On the contrary there was a significant decrease in the phosphorylation levels at 5 and 10 min after hypotonic stimulation (Fig. 7B). Thus an increase in the Src and FAK kinase activities does not seem to be involved in activation of volume-sensitive channels Kᵥ, in EATCC, and hence RVD.

**JAK2 and receptor-coupled tyrosine kinases.** To substantiate the functional role of tyrosine kinases, we investigated the effect of inhibition of JAK2 (cucurbitacin I (5)), the insulin-like growth factor I receptor [picropodophyllotoxin (13)], and the epidermal growth factor receptor [GW583340, dihydrochloride (11)] on the volume recovery after cell swelling. From Fig. 8 it is seen that cucurbitacin I reduced the recovery level after 4 min by 40%, whereas the other inhibitors had no significant effect. This indicates that the JAK/STAT3 pathway could be involved in the tyrosine phosphorylation of TASK-2 and hence in the RVD response.

**DISCUSSION**

**Tyrosine Kinases are Involved in the Initiation of RVD**

In volume measurements on EATC and EATCC using the Coulter counter, it was seen that the control cells did not reach the perfect osmometer value, which is due to the fact that the cells activate their volume-sensitive ion channels already after no more than a 5% swelling. A RVD response will thus hinder the cells in reaching the perfect osmometer value. On the contrary EATCC treated with genistein did in fact reach the perfect osmometer value, indicating that their ability to volume regulate was completely inhibited. Even after 4 min there was no sign of a RVD response. The inhibition of RVD was evident and similar in both cell lines. These results may be explained by the inhibition of either one or both of the volume-activated ion channels, which in EATC and EATCC are VRAC (Iᵥ(Cl)) and TASK-2 (Iᵥ(K)) (see INTRODUCTION) upon hypotonic swelling. The Kᵥ permeability increases much less when compared...
with the Cl\(^{-}\) permeability during cell swelling, which makes the K\(^{+}\) permeability the limiting factor for the RVD rate (27). Inhibition of RVD will thus often reflect an inhibition of the swelling-activated K\(^{+}\) channel. However, it should be noted that an almost complete inhibition of VRAC would lead to the same results. Results from efflux measurements comply with the volume measurements, and it was seen that the swelling-activation of K\(^{+}\) efflux in EATC was strongly inhibited when cells were treated with the tyrosine kinase inhibitor genistein.

This was true also when all intracellular Cl\(^{-}\) was substituted with NO\(^{-}\) to assure that all measured K\(^{+}\) efflux was via a K\(^{+}\) channel and not involving the KCl cotransporter. It is thus clear that one or more tyrosine kinases are responsible for swelling-activation of the K\(^{+}\) channels, which in EATC and EATCC are known to be TASK-2 channels. It has previously been shown that TASK-1 background current is inhibited by genistein and that an inhibitor of protein tyrosine phosphatase reduces this effect (12). In the present investigation it is demonstrated that inhibition of tyrosine phosphatases prevents the inhibitory effect of the tyrosine kinase inhibitor genistein on RVD. A similar phenomenon has been demonstrated on the volume-sensitive release of organic osmolytes (26). We assume that it is a balance between tyrosine kinase and tyrosine phosphatase activities that determines the degree of phosphorylation of TASK-2 and hence its activation following osmotic cell swelling.

Tyrosine Phosphatases are Involved in the Inactivation of RVD

When using the tyrosine phosphatase inhibitor mpV(pic), the set point for RVD in EATC (defined as the volume where the RVD response ends) was significantly lowered compared with control cells. The treated cells seemed to have lost more ions than the control cells, which could indicate that the K\(^{+}\) channel (being the inhibiting factor in RVD) was prevented from closing due to the impairment of tyrosine phosphatases. These findings were substantiated by K\(^{+}\) efflux measurements, in which it was found that inhibition of tyrosine phosphatases with mpV(pic) resulted in a significantly increased swelling-activated K\(^{+}\) efflux. Taken together these results strongly indicate that tyrosine phosphatases are involved in the closing of the volume-activated K\(^{+}\) channel TASK-2 in EATC. In a couple of experiments it was not possible to see an effect in EATCC treated with mpV(pic). We have no explanation on this apparent difference between EATC and EATCC.

Overexpression of TASK-2 Accelerates the RVD

As mentioned earlier the K\(^{+}\) conductance is rate limiting during RVD, and the insertion of more channels would in theory increase the ability of the cells to volume regulate. In accordance with this theory, the TASK-2-transfected cells showed a significantly increased RVD response, both in regard to end point and velocity. This reflects a greater ion loss during RVD in cells transfected with TASK-2 channels than in wild-type HEK-293 cells. Therefore when reintroduced to isotonic Ringer, the transfected cells responded with a strong RVI like response, a so-called post-RVD RVI response. When returning the cells to the isotonic Ringer, this now appeared hypertonic due to the excessive loss of ions during RVD, causing the cells to shrink beneath their original set point. The increased post-RVD RVI response in the cells overexpressing TASK-2 was taken to indicate a greater loss of ions during RVD in transfected cells than in the wild type. We thus have a model system in which the RVD rate can be used to evaluate effects on the TASK-2 channel expressed in this system. The clear effect of genistein on this RVD response is thus a clear evidence for tyrosine kinases being involved in activation of TASK-2 after cell swelling. Further experiments using mass spectrometry analysis will hopefully give us a more detailed picture of the activation pattern of the channel.

Swelling-Induced Tyrosine Phosphorylation of TASK-2

Several tyrosine kinases have been demonstrated to be activated following cell swelling, and tyrosine kinase inhibitors have been shown to block VRAC as well as the volume-sensitive efflux pathway for organic osmolytes (19, 28, 29, 36). With respect to tyrosine kinases and swelling-activated K\(^{+}\) channels, it has been shown that Src modulates the activity of, for example, the swelling-activated Kv channels (1.3 and 1.5) and the BK channels (19). In the present investigation we find that swelling of HEK-293 cells overexpressing TASK-2 channels resulted in a time-dependent phosphorylation of the channel, which was evident within the period 1 to 10 min after hypotonic treatment. Patch-clamp experiments performed on cell-attached patches with EATC have indicated that the volume-sensitive K\(^{+}\) channel is activated within 65 s after hypo-
tonic exposure (7). Whole cell patch-clamp experiments in EATC cells revealed that the volume-sensitive K⁺/H⁺ channels were detectable within 200 s after shift in solution (35). Whole cell patch-clamp experiments cannot be used to evaluate how long the channels remain open after hypotonic challenge because the cells do not volume regulate. The rate constant, calculated from our efflux data shown in Fig. 2B, was high and unaltered in the time period 1 to 10 min after hypotonic exposure, indicating that the K⁺ channel was activated within the whole time frame. This is in accordance with the tyrosine phosphorylation pattern for TASK-2. Although it might not be possible to compare time scales for TASK-2 activation in...
EATC cells with time scales for tyrosine phosphorylation of TASK-2 in HEK-293 cells, we suggest that direct tyrosine phosphorylation is involved in swelling-induced activation of TASK-2. We cannot exclude that tyrosine phosphorylation is also involved upstream in the signaling sequence from cell swelling to activation of the K⁺ channel.

Tyrosine Kinases Involved in RVD

As genistein is a known broad-spectrum tyrosine kinase inhibitor, the results give no information to which tyrosine kinase(s) could be involved. Experiments using the more specific Src kinase inhibitor PP2 showed a significant inhibition of the volume-sensitive K⁺ efflux in EATC (Fig. 6A) as well as of the concomitant RVD response (Fig. 6B) when introducing the cells to a hypotonic solution together with the application of PP2. As these experiments are performed in the presence of intracellular Cl⁻, it might however be possible that this small inhibition of the swelling-activated K⁺ efflux could represent an inhibition of the swelling-activated KCl cotransporter. We had bemtanide present in these experiments, but bemtanide, which is an excellent inhibitor of NKCC1, is not very efficient toward KCl cotransport. Even though PP2 (a Src kinase family inhibitor) showed a small, but significant, inhibition of the RVD and the K⁺ efflux, neither the Src kinase nor FAK seemed to be activated during 10 min of hypotonic stimulation in EATCC when analyzed by Western blotting. As described in the INTRODUCTION, swelling-induced activation of these kinases has been reported in different cell types, but they do not seem to play an important role in RVD in EATCC. With respect to tyrosine kinase activity coupled to membrane-bound receptors, we found a significant inhibition of the RVD following addition of cucurbitacin I, an inhibitor of the JAK/STAT pathway that is associated with cytokine signaling. Direct roles of cytokine receptors in volume sensing has been reported (43), although the knowledge is yet rather limited but interesting to pursue. Several reports points to a role for growth factor receptors in cell volume sensing (19). However, we see no effect of the growth factor receptor tyrosine kinase inhibitors picropodophyllotoxin and dihydrochloride (GW583340) on the cell volume recovery in the present investigation. From the present data a likely working hypothesis is that cell swelling activates the cytokine receptor coupled JAK/STAT pathway, which is upstream of the swelling-induced phosphorylation and activation of the TASK-2 in EATC.

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KCNK5 is functionally down-regulated upon long-term hypotonicity in Ehrlich ascites tumor cells

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KCNK5 is functionally down-regulated upon long-term hypotonicity in Ehrlich ascites tumor cells

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Abstract
Background/aims: Regulatory volume decrease (RVD) in response to acute cell swelling is well
described and KCNK5 (also known as TASK-2 or K_2P5.1) has been shown to be the volume
sensitive K⁺ channel in Ehrlich cells. Very little is on the other hand known about the effects
of long-term hypotonicity on expression and function of KCNK5, thus we have investigated the
effect of long-term hypotonicity (24h – 48h) on KCNK5 in Ehrlich cells on the mRNA, protein and
physiological levels.

Methods: Physiological effects of long-term hypotonicity were measured using patch-clamp and
Coulter counter techniques. Expression patterns of KCNK5 on mRNA and protein levels were
established using Real-Time qPCR and western blotting respectively.

Results: The maximum swelling-activated current through KCNK5 was significantly decreased
upon 48h of hypotonicity and likewise the RVD response was significantly impaired after both 24
and 48h of hypotonic stimulation. No significant differences in the KCNK5 mRNA expression
patterns between control and stimulated cells were observed, but a significant decrease in the
KCNK5 protein level 48h after stimulation was found.

Conclusion: The data suggest that the strong physiological impairment of KCNK5 in Ehrlich cells
after long-term hypotonic stimulation is predominantly due to down-regulation of the KCNK5
protein synthesis.
Introduction

Mammalian cells, with some exceptions, are susceptible to changes in intra- or extracellular amount of osmolytes due to the combination of aquaporins present in the membrane and an osmotic pressure across the membrane. An acute decrease in extracellular or increase in intracellular osmolarity respectively will in most cells result in water uptake and thus in cell swelling. The cell homeostasis is very vulnerable to volume changes and to counteract swelling the cell possess different regulatory mechanisms involving efflux of osmolytes through ion channels and membrane transporters – a response known as regulatory volume decrease (RVD). It is estimated that 70% of the osmolyte efflux in Ehrlich Ascites Tumor (EAT) cells during RVD is KCl efflux [3] and the remaining 30% is due to the efflux of organic osmolytes [4]. For a detailed description of the mechanisms of volume regulation see [1, 2]. In EAT cells and Ehrlich Lettré Ascites (ELA) cells the volume sensitive channels are the two-pore domain K⁺ channel KCNK5 (also known as TASK-2 - TWIK-related Acid-Sensitive K⁺ channel 2 or K₂p.5.1) [5-7], and the Volume Regulated Anion Channel (VRAC, I_Clav) [8]. KCNK5 has besides in Ehrlich cells [5-7] also been shown to be involved in RVD in other cell types including mouse proximal tubules [9], T lymphocytes [10, 11], murine spermatozoa [12] and retinal glial cells [13]. It has previously been shown that the rate limiting factor in RVD in EAT cells is the volume sensitive K⁺ efflux and thus the efflux through the KCNK5 channel [14], making an altered RVD response very likely to be due to changes related to this channel. For a recent review on KCNK5 see [15].

Response to hypotonicity can be divided into an acute and a long-term phase. The acute phase which includes RVD happens within minutes of cell swelling. During long-time exposure to osmolarity changes, the cell uses other mechanisms to ensure a steady intracellular environment, such as an altered gene expression and an altered protein synthesis [16]. The role of KCNK5 in acute volume regulation is well described in different cell types (see above). It has previously been shown how the KCNK5 channel is tyrosine phosphorylated after acute cell swelling in an time dependent manner [17] and recently it has been shown how KCNK5 is inhibited by Gβγ subunits of heteromeric G protein and thereby how a G protein coupled mechanism also can regulate the KCNK5 channel [18].

Effects of long-term hypertonicity is well studied and is known to elicit an increase in gene transcription of a number of osmoregulatory genes, which will affect the uptake and synthesis of organic osmolytes (see [2, 19]). On the other hand only very limited work has been done on the effects of long-term hypotonicity on cells, thus a potential role for KCNK5 in long-term regulation has never been studied. Preliminary studies (referred in [2]) using micro array screening suggested that the expression of KCNK5 is 2.7 times down-regulated after 48 hours in a hypotonic medium correlating with preliminary measurements of the maximum swelling-correlation.
activated KCNK5 current. The purpose of this study was thus to examine the effect of long-term hypotonicity on mRNA-, protein- and functional levels of the KCNK5 channel.

Experimental procedures

Solutions and materials
Hypotonic media (180mOsm) for long-term hypotonic stimulation was obtained by diluting growth media with buffered water, containing 5 mM HEPES, 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (p/s), pH 7.4. The solution was sterile filtered before use. Patch-clamp experiments: hypotonic NaCl Ringer’s solution (180mOsm) contained in mM: 71 Na⁺, 5 K⁺, 1 Mg²⁺, 1 Ca²⁺, 38 Cl⁻, 33 gluconate, 10 HEPES, pH 7.4. Isotonic Ringer’s solution (300mOsm) was generated by addition of 110 mM mannitol and contained only 5 mM HEPES. The pipette solution contained in mM: 2 Na⁺, 116 K⁺, 1.2 Mg²⁺, 42.4 Cl⁻, 62 gluconate, 10 HEPES, 30 mannitol, 10 EGTA, 2.5 ATP and 0.1 GTP. Coulter counter experiments: hypotonic Ringer’s solution (180mOsm) contained in mM: 72 Na⁺, 74.5 Cl⁻, 2.5 K⁺, 0.5 Mg²⁺, 0.5 SO₄²⁻, 0.5 HPO₄²⁻, 0.5 Ca²⁺, 3.3 MOPS, 3.3 TES and 5 HEPES, pH 7.4. Isotonic Ringer’s solution (300mOsm) was obtained by the addition of sucrose.

Cell cultures
ELA cells and EAT cells were cultured in RPMI₁₆₄₀ media supplemented with 10% FBS and 1% p/s at 37 °C and 5% CO₂. EAT and ELA cells were maintained by transferring 1 ml cell suspension to 10 ml fresh RPMI₁₆₄₀ medium every 3-4 days. ELA cells were loosened from the surface by trypsination, 1.5 ml 0.25% trypsin/EDTA solution per T-75 culture flask. Only passages 6-30 were used for experiments.

Patch-clamp measurements
ELA cells were kept in isotonic (300mOsm) or hypotonic medium (180mOsm) for 24 or 48 hours and transferred to 25 mm cover-slips after which they were placed in a chamber mounted on an inverted microscope (Zeiss Axiovert 10, Carl Zeiss, Germany). Solution shifts were generated using gravity-fed and pump-suction mechanisms. The current was measured using standard whole-cell patch-clamp using a suitable amplifier (Axopatch 200B, Axon instruments, California, USA). A Digidata 1200 Interface board and pClamp7 software (Axon Instruments) were used to generate voltage-clamp command voltages, and to digitize data. In the cell attached configuration, prior to establishing the whole-cell configuration the fast capacity transients were eliminated. Following whole-cell formation, the series resistance and the whole-cell capacitance were compensated and annulled with mean values that did not change significantly during experiments [20]. Pipettes were made from Vitrex glass capillary tubing with an outside
diameter of 1.7 mm (Modulohm, Herlev, Denmark) using a Narishige PP-830 puller (Tokyo, Japan) and had resistances of ≈4 MΩ. All experiments were carried out at 37 °C.

IK was measured as previously described in [5]. Briefly, the membrane potential was clamped in pulses of 500 ms at the equilibrium potential for Cl⁻ which was 5 mv during isotonic conditions and 0 mv under hypotonic conditions and these reversal potentials have previously proven to be good estimates [5, 6, 21]. The voltage correction was due to a dilution of the intracellular ion compositions as a result of hypotonicity and the following cell swelling. The current increase was measured after 300 sec of hypotonic stimulation and taken relative to the cell membrane surface (pF).

**mRNA measurements**

ELA and EAT cells were stimulated with hypotonic media (180 mOsm) or isotonic media (300 mOsm) for 24 or 48 hours and lysed in RNA lysis buffer (Macherey-Nagel, Düren, Germany). RNA was purified from lysates using NucleoSpin® RNA II (Macherey-Nagel, Düren, Germany) according to manufacturer’s instructions. Reverse transcriptase PCR with SuperScript II and oligo(dT)₁₂₋₁₈ primer (Invitrogen, Life Technologies, Naerum, Denmark) was used to generate cDNA from the purified mRNA and performed on an Eppendorf Mastercycler. In a total of 10 µl ddH₂O, dNTP mix (500 µM) and oligo(dT)₁₂₋₁₈ primer (500 ng) was mixed on ice, 1 µg mRNA was added and incubated for 5 min at 65 °C. The solution was put on ice and briefly centrifuged. 4 µl 5 x first strand buffer, DTT (10 µM) and 1 µl ddH₂O was subsequently added and the solution incubated for 2 min at 42 °C. 1 µl SuperScript II was added and incubated at 42 °C for 50 min followed by incubation at 70 °C for 15 min and ending at 4 °C. Real-time qPCR was performed in triplicates using the Stratagene MX4000 PCR system, Brilliant® II SYBR® Green QPCR Master Mix (Stratagene, Agilent Technologies) and the following primers: mKCNK5 forward: 5’-GTC AAG GCC ACT TGG TGA GG 3’ and mKCNK5 reverse: 5’-TGC TGG TGA AGG TGG ACT CA-3’ (KCNK5 GenBank accession number NM_021542) and ARP: mARP forward: 5’-CGA CCT GGA AGT CCA ACT AC-3’ mARP reverse: 5’-ACT TGC TGC ATC TGC TTG-3’ (GenBank accession number NM_007475), which was used as reference gene. 10 µl Master Mix, 0.4 µl forward and reverse primer (200 nM final concentration), 0.4 µl 500 x diluted ROX II reference dye (40 nM final concentration), 7.8 µl ddH₂O and 1 µl cDNA was mixed and quantification was done using the following cycles: 95 °C 10 min and 95 °C 30 sec, 58 °C 1 min, 72 °C 30 sec x 40. Standard curves were done in order to measure primer efficiency which was adjusted for in calculations. Primers were selected using Primer3 software and purchased from MWG Eurofins (Germany). Quantification was carried out using the Pfaffl method [22].
**SDS-PAGE and western blotting**

ELA and EAT cells were stimulated with isotonic or hypotonic media for 24 or 48 h and subsequently lysed in 95°C lysis buffer (10 mM Tris-HCl pH 7.4, 1% SDS and 20 mM EDTA) with protease inhibitors (Roche Applied Science) and phosphatase inhibitors added. SDS-PAGE and western blotting were performed as previously described [17]. Briefly, we used a SDS-PAGE-western blotting system form Invitrogen (Lfe technologies) and with the following antibodies; KCNK5 (Alomone Lab., Israel) 1:250 and β-actin (Sigma-Aldrich) 1:1000. Since we use two different antibodies on every blot, membranes were cut around 50kDa letting us visualize both KCNK5 and β-actin (65 and 42 kDa respectively) using BCIP/NBT. Protein bands were quantified using UN-SCAN-IT software.

**Cell membrane protein labeling and purification**

EAT and ELA cells were grown in 40% hypotonic medium (180mOsm, standard medium diluted with buffered water, see above) or isotonic medium (300mOsm, standard medium) for 48h before cell membrane purification. Cell membrane proteins were biotinylated and purified using Pierce Cell Surface Protein Isolation Kit (Thermo Fisher Scientific) according to manufactures instructions, with two exceptions; after lysis and before purification a sample was drawn from the lysate and protein concentration measured. These samples were used as a control of total KCNK5 protein content under the two circumstances. SDS-PAGE and western blotting was used to analyze the amount of KCNK5 in whole cell lysate as well as purified samples (see above).

**Cell volume measurements**

Absolute cell volume was measured by electronic cell sizing using the Coulter Multisizer II (Coulter, Luton, UK) with a tube orifice of 100 µm. For each experiment 2.5x10^6 cells were used and cell volume was determined as the median of the cell volume curves after calibration with latex beads (15 µm). Cells were either kept in isotonic (300mOsm) medium or in a 40% hypotonic medium for 24 or 48h. Prior to cell volume measurements cells were centrifuged and resuspended in standard medium (isotonic) were they were kept for 30 min in order to acclimatize before experiencing a hypotonic chock. The duration in standard medium allowed the cells to adapt to the changed environment without influencing protein synthesis and gene transcription. It should be mentioned that the cells shrink when transferred from the hypotonic into the isotonic medium and thus perform a RVI process. As KCNK5 channels are inhibited in shrunken cells [7, 18] we have included the 30 min isotonic acclimatization period and have controlled that there is no significant difference between the cell volumes in the three experimental groups. The mean isotonic, 24h hypotonic pre-treatment and 48h hypotonic pre-treatment cell volumes where 803.7±61.5 µm^3, 829.7±58 µm^3 and 783.7±46.9 µm^3 respectively, with no significant difference between the control and pre-treated cells.
All Ringer’s solutions were micro-filtered (Millipore, 0.45 µM) before use. Volume recovery was estimated as \( \frac{V_{\text{max}} - V_{\text{4 min}}}{V_{\text{max}} - V_{\text{iso}}} \), where \( V_{\text{max}} \), \( V_{\text{4 min}} \) and \( V_{\text{iso}} \) are the maximal cell volume, cell volume at time 4 min and cell volume under isotonic conditions, respectively.

**Statistics**
Student’s T-test and one-way ANOVA was used to test for statistical significance. 95% and 99% levels of significance were shown by one star or two stars respectively.

**Results**
*Patch-clamp studies revealed a decreased maximum swelling-activated current in ELA cells upon 48h of hypotonicity*
Fig. 1 shows the maximum swelling-activated \( K^+ \) current through the volume sensitive \( K^+ \) channel and it is seen that after 24h there is a decreased (not significant) \( K^+ \) current. This is supported by experiments measuring \( K^+ \) efflux after 24h hypotonic incubation using \(^{86}\text{Rb}^+\) as a tracer. We found that the rate constant for the swelling-activated \( K^+ \) efflux in stimulated cells was lower than in control cells. At 12 minutes after cell swelling the rate constant was 0.0134 in stimulated cells compared to 0.0154 in the control cells (data not shown), thus supporting the results obtained by patch-clamp. After 48h of hypotonicity the maximum swelling-activated current through KCNK5 was significantly decreased from 28.7 ± 7.2 pA/pF to 7.0 ± 2.1 pA/pF corresponding to a decrease of 75.6%.

*Long-term exposure to hypotonicity (24 and 48 h) decreased RVD in EAT cells*
In addition we studied the effect of 24 and 48 hours hypotonicity (180mOsm) on RVD in EAT cells (Fig. 2) and found that after both 24h and 48h RVD was significantly decreased both when looking at % recovery after 4 min (fig. 2A+B) and at the initial rate of RVD (fig. 2A+C). 24h of hypotonic stimulation resulted in a decreased RVD when re-exposing the cells to a hypotonic shock, with a recovery after 4 minutes of 19.3 ± 1.33% compared with 41.5 ± 2.51% seen in the control cells (fig. 2B), corresponding to a 53% decrease. When looking at the initial rate of RVD, 24h of hypotonicity resulted in a relative decrease in the rate of RVD to 46.5±3.79% compared to untreated control cells (fig. 2D). After 48h the initial rate of RVD was further decreased to 30.8±3.69% compared to untreated control cells equal to a 69% RVD inhibition (fig. 2D). The % recovery after 4 min was decreased to 12±1.51% compared to 41.5±2.51% of the control cells corresponding to a 71% decrease after 48h of hypotonicity.

*There was no significantly change in mRNA levels in EAT and ELA cells upon long-term hypotonicity*
To further elucidate the nature of the decreased physiological function of the KCNK5 channel we investigated the expression of KCNK5 on mRNA and protein levels.
Real-Time qPCR was performed on treated (48h of hypotonicity) and untreated (control) EAT and ELA cells and fig. 3 shows the mRNA expression data for ELA (Fig. 3A) and EAT (Fig. 3B) cells upon long-term hypotonicity. It is seen that even though there is a 10.6% decrease in mRNA levels in 48h hypotonically treated ELA cells compared to the untreated control cells the decrease is not significant. In the EAT cells an apparent increase in KCNK5 mRNA was detected, though the difference was also not significant.

**KCNK5 protein levels were significantly decreased in EAT and ELA cells upon 48h of hypotonic exposure**

Western blot analysis on EAT and ELA cells revealed a significant decrease in KCNK5 protein expression levels after 48h, but not after 24h of hypotonically stimulation. Fig. 4A shows how the mean KCNK5 protein level in EAT cells is approximately the same in both control cells and after 24h of hypotonicity, but is significantly decreased to 70±4.4% after 48h of treatment. The same analysis in ELA cells likewise revealed a significant difference between protein amount in control and treated cells (fig. 4B). After 48h of hypotonicity a ≈30% decrease to 70.9±12.2% was seen, while an apparent increase (not significant) after 24h was observed. The results were confirmed in a single experiment by measuring cell surface KCNK5 protein amount using membrane protein biotinylation and showing how 48h of hypotonic treatment decreases the amount of KCNK5 protein inserted into the membrane by approximately 50%, thus confirming and not very different from the decrease seen in total KCNK5 protein (see fig 4C).

**Discussion**

Since the KCNK5 channel is a very important player in the RVD response in Ehrlich cells, we investigated a possible effect of long-term hypotonicity on RVD and on expression of KCNK5. The RVD measurements were performed on the EAT cell line using a Coulter Counter. EAT cells were chosen for these volume measurements since the most accurate volume measurements are obtained on cells in suspension. A significant impairment of RVD was found both after 24 and 48 hours of long-term exposure to the hypotonic medium. The inhibited RVD performance in EAT cells could reflect a decreased function of either KCNK5 (K+) or of VRAC (Cl-). However since the KCNK5 channel is the rate limiting factor for RVD in these cells [14] and since patch-clamp measurements showed a decreased maximum swelling-activated current through KCNK5 upon long-term hypotonic stimulation, the results were taken to indicate a down-regulation of the KCNK5 channels or reduced ability of the KCNK5 channel during RVD.

The physiological impairment of RVD and of the maximum swelling-activated K+ current through KCNK5 channels could be due to regulation on a number of levels such as I) a down-regulation of KCNK5 protein synthesis II) KCNK5 mRNA transcription III) post-translational modifications
resulting in an inhibited channel function IV) an unknown regulatory mechanism “turning off” the channel V) an internalization of membrane embedded channels or a combination of the above. We found that there was no significant difference between the mRNA amount of KCNK5 in control cells compared to hypotonic stimulated cells which were true in both EAT and ELA cells. Since there was no significant difference in the mRNA expression pattern between control and stimulated cells we conclude that the physiological changes most likely are not due to regulation on the transcriptional level. It might be argued that cells with down-regulated KCNK5 expression could have died before 48h and this is why no decrease in mRNA level is seen. However, the fact that we do see a clear down-regulation at the protein level argues against the possibility.

Although there was no effect of long-term stimulation on gene transcription levels, we found significant lower levels of KCNK5 protein after 48h of hypotonicity in both cell lines, thus indicating a higher degree of KCNK5 protein degradation or decreased synthesis of the KCNK5 protein. Our results were further substantiated in one experiment, where it was shown how 48h of long-term hypotonicity resulted in a decrease in KCNK5 protein present in the plasma membrane, thus supporting the theory that long-term hypotonicity decreases KCNK5 protein expression and may in addition cause an altered KCNK5 sorting to the membrane. As the decrease in membrane bound KCNK5 was not very different from the total decrease in KCNK5 protein, we have not looked further into this.

At least three factors could influence this change in KCNK5 protein expression level: the lowered ion strength, the decrease in K⁺ concentration and the decreased Cl⁻ concentration all generated by the response to the hypotonic medium. We speculate if e.g. the decreased K⁺ concentration could prompt the cells to down-regulate the K⁺ leak-channel KCNK5 in order to govern the smaller amount of K⁺ more strictly. The lowered Cl⁻ concentration could result in the same mechanisms, but since we are dealing with a significant decrease in KCNK5 protein expression, we suggest that the physiological changes are predominantly due to alterations in potassium rather than chloride level.

To our knowledge not much work has been published on long-term effects of hypotonicity on protein synthesis but it should be noted that it has been shown that protein synthesis is up-regulated in hepatocytes of air-breathing walking catfish upon hypotonicity (≈ 2 hours) [23]. Since we are dealing with a down-regulation of the KCNK5 protein this is thus not likely to be caused by a general effect on the protein synthesis but rather by a specific effect on the KCNK5 protein synthesis or breakdown.
There is a time discrepancy between the physiological data and the protein expression data obtained in EAT cells, since there is a significant impairment of RVD in EAT cells already after 24h of hypotonicity but the down-regulation of KCNK5 protein amount is first detectable after 48h of hypotonicity. It is likely that this is caused by a regulatory inhibition of the channel followed by a later decrease in protein synthesis. This has not been further investigated.

Taken together our results show that the clear physiological changes of the KCNK5 channel in Erhlich cells upon long-term hypotonic stimulation is predominantly due to a decreased protein synthesis. We cannot rule out other regulatory mechanisms as contributors to the down-regulation of the maximum swelling-activated KCNK5 current seen after long-term hypotonicity. We have previously shown how protein tyrosine kinases are vital in KCNK5 channel opening and that tyrosine phosphatases are important for the closing of the channel upon a swelling-mediated activation of the channel [17], and it has furthermore been suggested that KCNK5 activation can be modulated by G protein Gβγ subunits [18]. Thus an altered tyrosine kinase/phosphatase profile or changes in the G protein coupled mechanism could potentially be involved in the functional inhibition of KCNK5.

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Conflict of interest
The authors declare no conflict of interest.
References


Fig. 1 Activity of KCNK5 current in ELA cells upon long-term hypotonic stimulation
Cells were kept in isotonic (300mOsm) or hypotonic medium (180mOsm) for 24 or 48 hours. The maximum swelling-activated K⁺ current was measured using standard whole-cell patch-clamp. The current increase was measured after 300 sec of hypotonic stimulation (n=3) and taken relative to the cell membrane surface (pF). Student’s T-test was used to test for statistical significance and one star (*) indicate a 95% significance level.

Fig. 2 RVD in EAT cells
A Coulter counter was used to measure the volume of isotonic control (300m) and long-term (24 or 48h) hypotonically (180 mOsm) treated EAT cells. A: representative figure showing relative RVD over time (sec) for untreated control cells and cells kept in hypotonic medium for 24 or 48 h. B: Mean volume recovery of n=5 experiments after 4 min was calculated as (V_{max}-V_{4min})/(V_{max}-V_{ iso}), where V_{max}, V_{4min} and V_{iso} are the maximal cell volume, cell volume at time 4 min and cell volume under isotonic conditions, respectively. C: initial rate of RVD or the slope was calculated (n=5) using linear regression on the linear part of the RVD curve, from maximum volume to the end of linearity. One-way ANOVA was used to test for statistical significance and two stars (**) indicate a 99% significance level.

Fig. 3 mRNA levels of KCNK5 in ELA and EAT cells
ELA (A) (n=4) and EAT (B) (n=5) cells were stimulated with hypotonic (180 mOsm) or isotonic media (300 mOsM) for 24 or 48 hours and Real-Time qPCR was performed. Student’s T test or one-way ANOVA was used to test for statistical significance.

Fig. 4 KCNK5 protein levels in Ehrlich ascites tumor and Ehrlich Lettré ascites cells.
Cells were isotonic (300mOsm) or hypotonically (180mOSm) treated for 24 or 48 hours and SDS-PAGE and western blotting was performed on the lysates using antibodies against KCNK5 and β-actin. A: representative western blot and mean protein levels (n=3) in EAT cells. B: representative western blot and mean protein levels (n=9) in ELA cells. C: membrane purification (n=1) showing KCNK5 protein amount inserted into the membrane under control and 48h hypotonic conditions. Student’s T-test was used to determine statistical significance and one star (*) represents a statistical significance level of 95%.
Fig. 1

Maximum swelling-activated current through KCNK5 in ELA cells

I_{KCNK5} (pA/pF)

Control  24 h  48 h

*
Fig. 2

A  Regulatory volume decrease in EAT cells

B  RVD response after long-term hypotonicity

C  Initial rate of RVD after long-term hypotonicity
Fig. 3

A  KCNK5 mRNA levels in ELA cells

B  KCNK5 mRNA levels in EAT cells

Control  Hypo 48h  Control  Hypo 48h
Fig. 4

A  KCNK5 protein expression in EAT cells

B  KCNK5 protein expression in ELA cells

C  Membrane fraction upon 48 h hypotonicity
6 Paper III

The potential role of KCNK5 in activated T cell physiology with specific focus on cell volume control

The potential role of KCNK5 in activated T cell physiology with specific focus on cell volume control

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Summary

The potential role of the two-pore domain potassium channel KCNK5 (also known as TASK-2 and K_2P5.1) in activated T cell physiology has only recently been described and for years the main focus of potassium channels in T cell physiology have been on the voltage-gated K^+ channel Kv1.3 and the Ca^{2+}-activated KCa3.1. So far KCNK5 has been described to be up-regulated in multiple sclerosis patients and to be implicated in the volume regulatory mechanism regulatory volume decrease (RVD) in T cells. KCNK5 has furthermore been shown to be involved in RVD in Ehrlich cells, proximal tubules and spermatozoa.

Here we further elucidate on the potential role of KCNK5 in activated T cell physiology, thus we investigated the time-dependent expression pattern of KCNK5 in activated T cells together with its role in RVD.

We find that KCNK5 is indeed up-regulated in CD3/CD28 activated T cells both on mRNA and protein levels, but despite this strong up-regulation we find the RVD response to be inhibited in activated T cells compared to non-activated control cells. In addition we find that T cells in response to activation increase their volume and swell more than non-activated control cells when subjected to hypotonicity. We furthermore find the swelling-activated Cl^- permeability in activated T cells to be strongly decreased and thus find the RVD inhibition predominantly to be due to the decreased Cl^- permeability. Our results suggest that the up-regulation of KCNK5 in activated T cells does not play a volume regulatory role and we speculate that it might play a role in hyperpolarizing the cell membrane and thus increasing the Ca^{2+} influx.
Introduction

The importance of ion channels in T cell activation and activated T cell function is established. Especially the role of the voltage-gated Kv1.3, the Ca\(^{2+}\)-activated Kca3.1 and Ca\(^{2+}\) release-activated Ca\(^{2+}\) channel (CRAC) is well known. For reviews on ion channels and T cells see (8; 30; 34). Activation of the T cell when encountering an antigen requires a sustained rise in intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) which is generated by IP\(_3\) mediated Ca\(^{2+}\) release from stores in ER. This in turn mediates the activation of CRAC channels allowing Ca\(^{2+}\) entry from the cytosol. This Ca\(^{2+}\) influx and subsequent rise in [Ca\(^{2+}\)]\(_i\) activates KCa1.3 and also causes a depolarization activating the voltage sensitive Kv1.3 K\(^+\) channel. Activation of Kv1.3 and KCa3.1 causes K\(^+\) efflux and hyperpolarization of the plasma membrane allowing further Ca\(^{2+}\) entry via the CRAC channels in the membrane. The sustained increase in [Ca\(^{2+}\)]\(_i\) is essential for the transcription and expression of interleukin 2 (IL-2) which in turn is essential for keeping the T cells activated without the requirement of further antigen stimulation (see (23)). Other ion channels, however, have been implicated in T cell activation. The leak conductance channel (TREK-2) was described in WEHI-231 cells which is a murine immature B cell line (27; 37). Further studies have also revealed the presence of two-pore domain K\(^+\) channels in lymphocytes e.g. TASK-1 and TASK-3 (25) and of special interest to this study Bittner and co-workers suggested that the two-pore domain potassium channel KCNK5 (also known as TASK-2 or K\(_{2p5.1}\)) has a role in T cell activation and in multiple sclerosis (5).

KCNK5 has previously been shown to be volume sensitive and to be implicated in the volume regulatory process of regulatory volume decrease (RVD) in response to cell swelling in various cell types and tissues including Ehrlich Ascites Tumor (EAT) cells (19; 29), mouse proximal tubules (4), human and murine spermatozoa (2; 3) and murine T lymphocytes (6). Recently it was shown that two-pore domain potassium channels are also involved in volume regulation in human T cells (1). For a recent review on KCNK5 see (12).

T cells like most other mammalian cells are subject to changing intracellular environment causing potential volume changes and subsequent regulation. T cells are in constant movement around the body and thereby subjected to extracellular osmotic changes e.g. in the kidney and as such, T cells are as most other mammalian cells capable of performing RVD in response to cell swelling (7; 10; 11; 14).

As seen in all volume regulating cells the volume regulated anion channel (VRAC) is responsible for the swelling-activated Cl\(^-\) current (8) whereas different K\(^+\) channels have been suggested to play a part in T cell volume regulation including the voltage-gated potassium channel Kv1.3 (9; 22), the Ca\(^{2+}\)-activated KCa3.1 (33), and two-pore domain potassium channels (1; 6).
KCNK5 is up-regulated during T cell activation and is also involved in volume regulation (1; 5) but there is no description on the time dependency of the expression pattern of KCNK5 during T cell activation and its physiological consequences. This is the subject of the present paper and in addition we demonstrate a strong decrease in the swelling-activated Cl\(^-\) permeability during the activation which inhibits the RVD process in the large activated T cells.

**Experimental procedures**

**Solution and Materials**

*Volume measurements*: Hypotonic Ringer’s solution (180mOsm) contained (in mM): 71.5 NaCl, 2.5 KCl, 0.5 MgSO\(_4\), 0.5 Na\(_2\)HPO\(_4\), 0.5 CaCl\(_2\), 3.3 MOPS, 3.3 TES and 5 HEPES, pH 7.4. Isotonic Ringer’s solution (300mOsm) was obtained by addition of sucrose. *Cl\(^-\) permeability*: the hypotonic (150mOsm) low Na\(^+\) Ringer’s solution contained (in mM): 70 NMDGCl\(^-\), 0.8 NaCl, 5 KCl, 1 K\(_2\)HPO\(_4\), 1 MgSO\(_4\), 1 CaCl\(_2\), 3.3 MOPS, 3.3 TES and 5 HEPES, pH 7.4.

Gramicidin and Clofilium was purchased from Sigma and used in the concentrations 1\(\mu\)M and 100\(\mu\)M respectively.

*Purification and maintenance of cells*

Human T cells were purified from buffy coats from healthy donors. Human buffy coats were obtained from the blood bank at Rigshospitalet, Copenhagen, Denmark. All procedures were performed at room temperature. The T cell population was purified from buffy coats using RosetteSep™ (Human T Cell Enrichment Cocktail, StemCell Tech.) accordingly to manufactures description. After purification any residual red blood cells was lysed using RBC lysis buffer (eBioscience). Purified T cells were kept in RPMI\(_{1640}\) GlutaMax medium supplemented with 30 U/ml IL-2, 10% FBS and 1% P/S at 37 °C and 5% CO\(_2\).

*RNA purification and Real-Time qPCR*

T cells were stimulated for 2, 4, 8, 12, 24, 48, 72 and 144 hours CD3/CD28 beads (Invitrogen) and RNA was purified from lysates using NucleoSpin® RNA II (Macherey-Nagel) according to manufacturer’s instructions. Reverse transcriptase PCR with SuperScript II (Invitrogen) and oligo(dT)\(_{12-18}\) primer (Invitrogen) was used to generate cDNA from the purified mRNA and performed on a Eppendorf Mastercycler as previously described (20). Real-Time qPCR was performed using a Stratagene MX4000 real-time PCR system, Brilliant® II SYBR® Green QPCR Master Mix (Stratagene, Agilent Technologies) and the following primers: hKCNK5 forward: 5’-ACCACCCACTCATCTCCAC-3’, hKCNK5 reverse: 5’-AGTGCTGGTAAGGTTGGACT-3’, hKv1.3 forward: 5’-CAGTTCAGGTGCTACGTAC-3’, hKv1.3 reverse: 5’-TGTCTCCCGGTGTTAGAGT-3’, hKCa3.1 forward: 5’-ACTGGGCACCTTTCAGAC-3’ and KCa3.1 reverse: 5’-
ACGTGCTTCTCTGCTTGTT-3’. A total volume of 20 μl containing 1 μl of the cDNA, 200 nM of primers, and 10 μl 2× MasterMix was used.

Despite testing numerous potential reference genes none of them lived up to standards and instead determination of total cDNA concentration was used to correct the data obtained. To determine cDNA content upon PCR reaction and to normalize samples Quant-iT™ OliGreen® ssDNA Assay Kit was used as described in (24). Standard curves were also done to measure primer efficiency which was corrected for in calculations. Primers were selected using Primer3 software and purchased from MWG Eurofins (Germany). Quantification was carried out using the Pfaffl method: (26)

**Western blotting**

T cells were stimulated for 2h, 4h, 8h, 12h, 24h, 48h, 72h or 144h days with CD3/CD28 beads and lysed in 95°C lysis buffer (10 mM Tris-HCl pH 7.4, 1% SDS, 20 mM EDTA) with protease inhibitors (Roche Applied Science) and phosphatase inhibitors added. SDS-PAGE and western blotting was performed as previously described (19). We used the following antibodies and concentrations: KCNK5 (TASK-2) (Alomone Lab., Israel) 1:100, β-actin (Sigma-Aldrich) 1:1000.

**Volume measurements by Coulter Counter**

**Volume measurements**: T cells were stimulated with CD3/CD28 beads for 2h, 4h, 8h, 12h, 24h, 48h, 72h or 144 before the absolute cell volume was measured by electronic cell sizing using the Coulter Multisizer II (Coulter, Luton, UK) with a tube orifice of 80 μm. Before measurements calibration was performed using 15 μm latex beads. Approximately 2.5x10^6 cells were used per experiment and cell volume was determined in either isotonic or hypotonic Ringer’s solutions (all micro-filtered before use). Volume recovery was estimated in two different ways, by % recovery after 3 min as previously described (19) and by calculating the initial rate of RVD measured as the slope of the recovery part of the RVD curve. Cl⁻ permeability: to determine if the Cl⁻ permeability was decreased in activated T cells we used a method previously described in (17) with some minor alterations, thus we used clofilium as a blocker of potassium channel KCNK5 and a NMDG Na⁺ substitution Ringer’s solution (see above). Briefly, the principle of the experiments is as follows: the addition of clofilium blocks the KCNK5 channel allowing the cells to swell when subjected to hypotonicity but inhibiting K⁺ efflux and thus RVD. When adding gramicidin a high cation permeability is introduced making Cl⁻ the limiting factor. This way makes it possible to indirect measure the Cl⁻ permeability since a cell shrinkage will reflect the cells Cl⁻ permeability which then can be measured in relative values.
Statistical methods
Statistical significance was determined by Student’s T-test or one-way analysis of variance (ANOVA) where one star represent statistical significance at a 95% level, two stars at a 99% level and three stars at a >99% significance level.

Results

KCNK5 and KCa3.1 is highly up-regulated on mRNA level in CD3/CD28 activated T cells
When activating the T cells for 2, 4, 8, 12, 24, 48, 72 and 144 hours with CD3/CD28 beads both KCa3.1 and KCNK5 were up-regulated while no significant change was seen in Kv1.3 (fig. 1A) mRNA expression level compared to un-stimulated controls using Real-Time qPCR. An initial down-regulation of KCa3.1 (fig. 1B) mRNA level was observed upon 2 and 4 h of stimulation followed by an increasing up-regulation after 8, 12 and 24 h reaching a stable level of a 13 fold up-regulation after 48 h and with statistic significant values after 72 and 144 h (p<0.05). KCNK5 (fig. 1C) showed the largest up-regulation of the three with a 115 fold peak-increase in mRNA level following 24 h of T cell activation when comparing with the non-activated T cells. No initial down-regulation was observed regarding KCNK5 thus a 4 fold increase was already seen after 2 h. Statistical significance was only seen at 24h (p<0.05) when using ANOVA test though the up-regulation seem clear. This could be due to the rather large standard deviations seen in the data. When using Student’s T test on the data-set comparing each test value to its respective control statistical significance is found at times 2, 4, 8 and 144h (p<0.05).

KCNK5 is also highly up-regulated on protein level
Figure 2 shows a biphasic KCNK5 protein expression pattern with an initial significant decrease (p<0.001) during the initial stimulation (2 and 4h) of the cells with CD3/CD28 beads followed by a still increasing protein expression after 8, 12, 24 and 48 h. The expression peaks upon 72 h of stimulation with a 277% increase compared to the quiescent control cells with a subsequent slight decrease to 209% at 144 h of activation both time-points showing significant more KCNK5 protein in activated T cells than in the controls (p<0.001 and p<0.05 respectively). The activation of the T cells resulted in fast proliferation of more than 300% measured 72 h after CD3/CD28 stimulation compared to non-stimulated control cells (seen in two experiments, data not shown).

Activation causes isotonic cell swelling, decreased RVD ability and increased hypotonic swelling
Since the KCNK5 channel is a known volume regulator we tested the activated T cells ability to perform regulatory volume decrease (RVD) upon hypotonicity. From the volume measurements
depicted in fig. 3 it is seen that the activated T cells when measured in isotonic Ringer’s solution are larger (fig. 3A+B) – as described many times before, but it is also seen that in hypotonic Ringer’s solution the activated cells swell more than the non-activated control cells (fig. 3C). After 8 h of CD3/CD28 stimulation T cells in isotonic Ringer’s solution begin increasing their volume and after 24 h the mean volume is significantly (p<0.001) larger than the control cells with a mean volume of 171.6 µm^3 compared to 118.8 µm^3 of the control cells. A plateau is seen at times 72 and 144 h where the activated cells have significantly (p<0.001) increased their volume by 213% (fig. 3B).

Figure 3A+C shows how both activated and non-activated cells swell in hypotonic Ringer’s solution but to a different degree. CD3/CD28 stimulated T cells have increased maximum swelling starting at 8 h of stimulation, with highest measured values after 72 h and a small decrease seen after 144 h. At 72 h their maximum swelling reaches a value of 309.3 µm^3 compared to a gain of volume of 71.9 µm^3 seen in control cells equivalent to a 4 times increase. Maximum swelling values 24, 72 and 144 h after stimulation are significantly larger than control values (p<0.001).

Besides swelling more when hypotonically challenged, activated T cells have poorer RVD performance compared to control cells. As seen from fig. 3A both stimulated and non-stimulated T cells swell and perform RVD but when measuring % recovery after 3 min (19) and the initial rate of RVD (see experimental procedures) stimulated T cells show much less RVD capability than unstimulated control cells. As a control the RVD response in non-stimulated control cells was measured as a function of time, and it is seen that the recovery percentages of non-activated T cells are not significantly different from each other at the measured time-points (average recovery of 36% after 3 min). From fig. 3D it is seen how the relative recovery of activated T cells decreases beginning after 8 h of stimulation and with significant decrease at 12, 24, 72 and 144 h. After 72 h of CD3/CD28 stimulation the cells have the lowest ability to perform RVD with a recovery of 13.6% compared to 35.7% (data not shown) of the un-stimulated control cells and equivalent to a 60% decrease (fig. 3D). After 144 h the cells seem to regain a small fraction of RVD performance now with an absolute mean recovery of 15.5% or 53% relative to the control. When measuring RVD by the initial rate of shrinkage the results are similar – activated T cells have a lesser RVD performance than control cells (fig. 3E). It is seen that the initial rate of RVD is relatively faster in all controls when comparing them to the activated T cells though the values are only significantly lower for 8, 12, 24, 72 and 144 h. 8 h of CD3/CD28 stimulation results in a 26% decreased RVD rate of (p<0.05) followed by a further decrease after 12 and 24 h to 56% and 53% respectively (p<0.001). Stimulation for 72 and 144 h results in a slightly faster RVD with rates of 67% (p<0.01) and 63% (p<0.001) compared to that of the control cells.
The Cl$^-$ permeability of activated T cells are inhibited

Even though KCNK5 is a well-known player in RVD, the considerable up-regulation of the channel in activated T cells does not seem to increase the cells ability to perform RVD on the contrary RVD is inhibited in CD3/CD28 activated cells. Since RVD is driven by the extrusion of KCl we speculated if the Cl$^-$ permeability of the activated cells could be decreased. To determine if a decreased Cl$^-$ permeability could be responsible for the inhibited RVD we used the method described in materials and methods and in (17). As described in (17) we use the rate of RVD after addition of gramicidin in a low Na$^+$ media as a measure of the swelling-activated Cl$^-$ permeability. As seen in fig. 4 the addition of gramicidin to hypotonically swollen non-stimulated control cells result in a significant faster RVD (higher Cl$^-$ permeability) with a 34.3±1.02% recovery compared to -0.102±1.24% (statistically not different from 0) seen in the CD3/CD28 activated T cells. Thus there is a significant decrease (p<0.05) in the Cl$^-$ permeability in non-stimulated T cells when comparing them to their non-activated counterparts.

Discussion

In the current study we find that the activated T cells enlarge upon activation which is a well described feature in the activated and proliferating T cells (31; 36). We find that this enlargement occurs in a time-dependent manner which coincides with the massive up-regulation of KCNK5 protein. The strong up-regulation is in agreement with earlier findings (1; 5). Thus we here confirm these findings while adding a time profile. The time profile described here shows an initial (after 2 and 4 hours of CD3/CD28 stimulation) and significant decrease in KCNK5 protein expression in activated T cells which correlates with a minimal though not significant early shrinkage and decreased maximum swelling when comparing the activated T cells to non-activated control cells.

CD3/CD28 T cell activation causes an initial loss of cell number which in two experiments was found to be of 56% and 41% after 2 and 4 hours of stimulation respectively. This could reflect that only the activated cells survive whereas the stimulated but non-activated cells undergo programmed cell death. The initial decrease in cell number is only seen in activated cells and is therefore not due to purification procedures or culturing conditions. The loss of cell number after 2 and 4 hours correlates with the decrease in KCNK5 protein and we speculate if this decrease could occur in order to protect the T cells from apoptosis. It should be noted that the initial cell loss does not influence the results obtained by western blot, since protein expression measurements are performed on the same amount of total protein at each time-point. It has been shown that the same channels involved in RVD are also active in apoptosis thus apoptotic volume decrease (AVD) is a vital signal in the cells commitment to apoptosis (21). We therefore speculate if the down-regulation of KCNK5 protein expression seen in the heterogenic T cell
population including both apoptotic and surviving/proliferating cells reflect an initial protection against apoptosis whereas the later up-regulation of KCNK5 protein is involved in the increased proliferation in the activated T cell.

CD3/CD28 activated T cells not only get larger when measured in isotonic Ringer’s solution they also swell significantly more when subjected to a hypotonic Ringer’ solution as if they have an inhibited KCl efflux. Despite the up-regulation of the potent volume regulator KCNK5 we also find the activated T cells to have an inhibited RVD performance upon cell swelling compared to the control cells. Since an up-regulation of KCNK5 protein expression with a decreased RVD might seem contradictory we speculate if the up-regulation occurs in order to facilitate proliferation. Proliferating cells are enlarged to keep daughter cells the same size as the parental cell a feature vital for successful proliferation, thus a strong RVD mechanism would be problematic for the activated T cell whose main objective is to increase in size and number to sufficient fight off a pathogenic intrusion. The KCNK5 up-regulation will help insure a hyperpolarization of the cell membrane and thus a sustained Ca\(^{2+}\) influx vital for the long-term activation of the T cells keeping them activated in an antigen-independent manner (13; 16; 18; 28; 35) this would facilitate optimal conditions for the T cell expansion. Andronic et al. find the RVD performance in stimulated and non-stimulated T cells to be the same, an observation contradicting our findings (1), but Andronic et al only measure the 20 min volume recovery and from their figure 2 we calculate the initial rate of volume recovery to be slower in stimulated cells compared to non-stimulated cell as found in the present study. With respect to maximum swelling we and Deutch and Lee (15) find the stimulated cells to swell more than non-stimulated control cells an observation contradicting what was found by Andronic et al (1). We do not have an explanation for these apparent differences.

Since the RVD in activated T cells was found to be inhibited despite the strong up-regulation of KCNK5 protein we speculated whether the swelling-mediated Cl\(^{-}\) permeability in activated T cells could be inhibited thereby setting a limit for the cells RVD capacity. We tested this and found that the Cl\(^{-}\) permeability through the volume sensitive anion channel VRAC indeed was inhibited in the activated T cells compared to control cells. This means that regardless of the increase in KCNK5 protein quantity the cells will not perform RVD as long as there is a functional down-regulation of VRAC since the KCl efflux during RVD is an electroneutral process. It should be noted that our results are in conflict with those published by Deutsch and Lee in 1988 (15) where it is shown how phytohemagglutinin activated human peripheral blood lymphocytes (PBL) respond to gramicidin in a set-up similar to ours by decreasing cell volume, and it is thus speculated that K\(^{+}\) is the rate limiting factor of RVD in activated T cells. A major difference seems to be that Deutsch and Lee are not adding gramicidin until 30+ minutes after cell swelling whereas we add it immediately after cell swelling when the channels are maximally stimulated.
We have previously shown in Ehrlich cells that the volume activated channels close before 15 min after swelling activation (17) and Sarkadi and co-workers show that it is also true in human lymphocytes (32). Another difference between our experiments and those presented by Deutsch and Lee is the fact that they used PBL’s which contained T cells, B cells and monocytes whereas we use purified T cells.

In summary we suggest that the KCNK5 up-regulation detected in CD3/CD28 activated human T cells helps ensure a hyperpolarization of the membrane thus favoring a sustained Ca\(^{2+}\) influx and T cell activation. We further suggest that to keep the cells from loosing volume thereby supporting a strong proliferation the Cl– channel VRAC is functionally down-regulated.

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**Conflicts of interest**
The authors declare that they have no conflict of interest.
Reference List


Fig. 1: mRNA levels of KCNK5, KCa3.1 and Kv1.3 determined by Real-Time qPCR
mRNA levels were determined using Real-Time qPCR and primers against KCa3.1 (A), Kv1.3 (B) and KCNK5 (C). ANOVA test was used to test for statistical significance on 5-8 independent experiments (n=2 for 48h). One star (*) indicates a 95% significance level.

Fig. 2: KCNK5 protein expression
T cells were purified from human buffy coats and stimulated with CD3/CD28 activation beads for 2, 4h, 8h, 12h, 24h, 48h, 72h or 144h. SDS-PAGE and western blotting was performed using antibodies against KCNK5 and β-actin. Protein bands were visualized and pixel density calculated. ANOVA was used to determine statistical significance on 3-10 independent experiments and one star (*) represents a statistical significance level of 95% whereas (*** ) indicate a significance level >99%. No significance was seen after 2 and 4 h using the statistical test ANOVA but when using Student’s T-test both times showed significance here represented by (###) indicating a level of significance of >99%.

Fig. 3: RVD in CD3/CD28 stimulated T cells and non-activated controls
T cells were purified from human buffy coats and stimulated with CD3/CD28 activation beads for 2, 4, 8, 12, 24, 72 or 144 hours before absolute volume was measured using a coulter counter. Stimulated T cells and non-stimulated controls were measured in either isotonic (300mOsm) or hypotonic (160mOsm) Ringer’s solution. A: representative figure showing RVD over time (min) for untreated control cells and CD3/CD28 stimulated T cells. B: Initial mean volume measured in isotonic Ringer’s solution C: Mean maximal swelling seen in hypotonic Ringer’s solution. D: Mean volume recovery after 3 min was calculated as (V_{max}-V_{3min})/(V_{max}-V_{iso}), where V_{max}, V_{3min} and V_{iso} are the maximal cell volume, cell volume at time 3 min and cell volume under isotonic conditions, respectively. E: initial rate of RVD (the slope) was calculated using linear regression on the linear part of the RVD curve, from maximum volume to the end of linearity. ANOVA test was used to test for statistical significance on 5-7 independent experiments and one star (*) represent a 95% significance level whereas two (**) and three stars (*** ) indicate significance levels of 99% and >99% respectively.

Fig. 4: Cl⁻ permeability in RVD in CD3/CD28 activated and non-activated T cells
T cells were purified from human buffy coats and stimulated with CD3/CD28 beads for 72 hours. Activated T cells and non-activated control cells were treated with 100μM clofilium for 30 min to block the potassium channels. The cells were swollen in hypotonic low Na⁺ NMDG Ringer’s solution and their volume was measured using a coulter counter. After one min. 1 µM gramicidin was added to facilitate cation flux thereby letting the Cl⁻ flux being the limiting factor. Student’s T-test was used to test for statistical significance on 3 independent experiments and two stars (**) indicate a significance level of 99%.
7 Final thesis discussion, future work and perspectives

Throughout my time as a PhD student I have worked with the two-pore domain potassium channel KCNK5 in various cell systems and with different focus points in mind. My contribution to the field has mainly been on the channel's role in cell volume control, and I have thus worked with its short time activation, effect of long-term anisotonicity and the potential role of KCNK5 in activated T cell physiology. In this section I will focus on the perspectives of my work presented in the three papers included in the thesis. Each of the papers holds their own discussion section and I will thus refer to those, but at the same time try to look a bit further into the perspectives and future work to be done.

Protein tyrosine phosphorylation of KCNK5 upon acute cell swelling

In Paper I we show how over-expression of KCNK5 channel protein enhances RVD in human embryonic kidney (HEK)-293 cells which confirms earlier findings (104), indicating that the K⁺ efflux upon swelling is rate-limiting for the RVD response in this set up (Paper I, fig. 4). We furthermore show how protein tyrosine kinases (PTK’s) play an important role in the opening of the channel, since inhibiting PTK’s resulted in an inhibited RVD response in response to hypotonic cell swelling (Paper I, fig. 1) and how protein tyrosine phosphatases are important in the closing of the channel (Paper I, fig. 3). We test whether the Src kinase (Paper I, fig. 6) or the focal adhesion kinase (FAK) (Paper I, fig. 7) could be the PTK’s involved, but our results suggest that neither of them are likely candidates. On the contrary we find the Janus kinase (JAK) could be a potential candidate (Paper I, fig. 8). PTK phosphorylation could be involved at different steps in pathway, from cell swelling to KCNK5 channel (called TASK-2 in the paper, see section 1.3) activation and K⁺ efflux (53), and we found that cell swelling resulted in a direct tyrosine phosphorylation of KCNK5 itself with a time course following the time course of the RVD response (Paper I, fig. 5). We have not tested the potential role of PTK’s in other steps of the pathway and thus cannot rule out the possibility that tyrosine kinases and tyrosine phosphatases could also be involved somewhere else in the pathway from cell swelling to KCNK5 activation – a question that could be interesting to look further into.

Our findings opens up for a lot of new questions, as to which tyrosine phosphorylation site (or sites) are phosphorylated upon swelling-activation of the channel and which kinase (or kinases) are involved?

When submitting the KCNK5 sequence (GenBank accession number NM_021542) to the NetPhos 2.0 server predicting tyrosine phosphorylation sites, 5 possible sites are identified (fig. 12).
Future experiments using e.g. mass spectrometry could help answer the questions of which site/sites are phosphorylated upon swelling-induced activation of the channel and which kinases are involved. Answering those questions would be really beneficial for the further and full story of PTK's in the swelling-activation of KCNK5.
As described in section 1.3.3 there are various other gating and activation mechanisms involved in the swelling-induced activation of KCNK5 and we cannot exclude the possibilities of other gating mechanisms being involved.

**Long-term hypotonic stimuli and its consequences for KCNK5**

Paper II deals with the long-term effects of hypotonicity on KCNK5 channel expression and physiology. In patch-clamp (Paper II, fig. 1) and Coulter counter experiments (Paper II, fig. 2) we find that there is a decreased maximum current through the channel and at the same time, we see an inhibited RVD response after cell swelling, when subjecting the cells to long-term hypotonicity. The RVD results could also reflect an inhibition of VRAC or perhaps a combination of decreased KCNK5 and VRAC activities. Since the molecular identity of VRAC remains unknown, it would not be possible to measure mRNA or protein expression, but the volume activated Cl⁻ current through VRAC is measurable and it would be interesting to see what potential effect long-term hypotonicity would have on VRAC. It should though be noted that K⁺ efflux have been shown to be rate-limiting of RVD in Ehrlich cells, thus it is very likely that the physiological changes seen is due to K⁺ channel regulation.

Since we speculated that the physiological impairment was likely to be caused by changes related to K⁺ efflux, we studied the KCNK5 expression pattern both on mRNA and protein levels. We found a down-regulation of the KCNK5 channel protein (Paper II, fig. 4) suggesting that the functional inhibition of the K⁺ current and the RVD response is indeed likely to be due to lowered amount of KCNK5 protein.

Not much work has to my knowledge been done on the long-term effects of hypotonicity, thus we haven’t got much to relate our findings to, nor to help explain the exact mechanisms behind, which again leaves some questions un-anwered. Future experiments could, and should, aim at explaining the mechanisms behind the functionally down-regulation of the KCNK5 channel. We suggest that the physiological impairment is the effect of a decreased KCNK5 synthesis or increased KCNK5 protein degradation, but we cannot exclude the possibility of a contribution from one or more still un-known regulatory mechanisms. Such mechanisms could e.g. involve direct or indirect regulation of the channel by one or more of the mechanisms described in section 1.3.3., a decreased protein insertion into the membrane by an altered protein sorting on its way to the membrane, internalization of KCNK5 proteins already embedded in the membrane or post-translational modifications. Since our results show a lowered KCNK5 protein expression and since we (in one experiment) show an approximately equal decrease in KCNK5 inserted into the membrane we do favor the idea that an altered KCNK5 protein expression is the main reason for the changes observed rather than a decreased recruitment to the membrane.
We find that there is some discrepancy between the physiological data and the protein expression in which a physiological effect is already seen after 24 hours of stimulation whereas the inhibited protein expression is first detectable upon 48 h of hypotonicity, thus suggesting that something else causes initial impairment of the channel. We therefore speculate that the cell uses other regulatory mechanisms (see section 1.3.3 and above) initially in the response to the acute environmental change until an altered KCNK5 protein expression can be effective.

As to the question of why the cell down-regulates the KCNK5 protein and thus introduce an impaired physiological function in response to long-term hypotonicity different factors could be involved. As the long-term hypotonic stimulated cell experiences a loss of cellular ionic strength and a decrease in cellular KCl concentration it is very likely that the cell down-regulates potassium channels to protect the cell potassium homeostasis. As described in section 1.2 K\textsuperscript{+} channels and thus potassium ions are involved in numerous physiological mechanisms e.g. setting the membrane potential, proliferation secretion of hormone and transmitters, thus the cellular potassium concentration is important for many cellular functions. Furthermore a decrease in intracellular K\textsuperscript{+} is seen in apoptosis (see section 1.4.2.1) thus a functional down-regulation of the channel in a low K\textsuperscript{+} media could help protect the cell.

**KCNK5 in activated T cell physiology**

We got the opportunity to work together with Novo Nordic on the project of KCNK5 in T cell activation and thus the previously cell systems studied were changed from a murine cell line to primary human T cells and the focus point changed to T cell activation. I have not regretted taking a directional shift since the work on T cell physiology proved to extremely exciting and the chance to work together with an industrial firm to be very educational. I can only regret that limited time means that there is still work to be done before this story is complete.

While changing cell type and host species we still worked on KCNK5 and its activation, involvement in volume regulation and expression pattern. A potential role for KCNK5 and other two-pore domain K\textsuperscript{+} channels in lymphocyte biology is by some regarded as questionable (112; 146), but recent publications from different groups have made the presence and physiological function of two-pore domain K\textsuperscript{+} channels including KCNK5 in lymphocytes more than likely (3; 10; 11; 35; 98; 100). Paper III ads to the increasing evidence for a role for KCNK5 in activated T cell physiology, thus we find the channel to be massive up-regulated both on mRNA and protein levels upon T cells activation (Paper III, fig 1C and fig. 2). The roles of the two potassium channels Kv1.3 and KCa3.1 in T cell activation has long been known and is undoubtedly of great importance in T cell cell activation and physiology, thus we also looked at the mRNA expression of Kv1.3 and KCa3.1. We found Kv1.3 mRNA to be expressed in T cells but with no change in expression level upon T cell activation. KCa3.1 mRNA is on the other hand, like KCNK5 mRNA,
was also up-regulated in activated T cells. Kv1.3 is believed to be of most importance in regulating membrane potential in quiescent T cells, whereas KCa3.1 is up-regulated upon activation, though some variation in expression profile is seen in different T cell sub-populations (see (16)).

Since we find that KCNK5 is so heavily up-regulated with no resulting increase in RVD (Paper III, fig. 3) we suggest that KCNK5 holds another function in activated T cells namely as a important player in hyperpolarizing the plasma membrane upon activation and thus facilitating a sustained Ca\(^{2+}\) influx (see section 1.5.3.) in co-operation with Kv1.3 and KCa3.1 and possible other K\(^+\) channels. Since the RVD response in activated T cells is inhibited even though KCNK5 is up-regulated we also investigated the volume sensitive Cl\(^-\) permeability after T cell activation in order to see if a down-regulation of the volume sensitive Cl\(^-\) current could account for the decreased RVD performance seen in activated T cells, and we did find this to be the case (Paper III, fig. 4).

In future experiments it would be interesting to perform patch-clamp measurements of the Cl\(^-\) current upon T cell activation, to confirm our indirect findings of a decreased permeability. Furthermore membrane potential and proliferation measurements on KCNK5 knock-down T cells would help elucidate the potential importance of KCNK5 in activated T cell function.

All in all new knowledge has come from the work presented here though it has also raised several new questions as research often does and only time can tell if and how these questions will be answered.
8 Appendix

Co-authorship statement for papers I, II and III can be found on the following pages.