Volume Regulated Channels
- $I_{\text{Cl, swell}}$ Regulation of Cell Physiology
- Structure/Function Relation of TRPV4 and Chemical Agonists

PhD thesis
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PREFACE:

I was just thinking what song to quote – from my own generation of course. Nothing from the old Beatles generation. Something that would reflect my thoughts about finishing my PhD thesis, something of intelligence, something of importance. However, nothing came up. Not that my generation haven’t generated wonderful music! We - whom are young in the 90’s and 00’s - have been blessed with some of most exciting music ever heard. I just couldn’t find anything to quote to reflect my feelings.

Maybe, because I’m feeling a bit ambivalent about the project. It didn’t quite turn out to be, what I expected it to be, when I made the proposal. It didn’t generate the conclusions I was expecting and I didn’t perform as I expected myself to perform.

During the last years we have left the August Krogh Institute and Hans Ussing’s Biokemisk Afdeling A for history but I’m not sure my work have completely left the influence of Krogh and Ussing. For this at least I’m very happy.

I came ashore though – at least I hope so. Mostly because of my wonderful supervisors: Stine and Else. Truly the most intelligent and empathic person’s one could ever imagine. You have guided me whenever I had scientific or intellectual problems and more. You have arranged for me to visit fantastic laboratories in both Chile and Belgium at professors Andrés Stutzin and Bernd Nilius. Both of German origin, but with very different personalities. Both with a fantastic taste for science and very inspirational originality. Both in science and as persons. Definitely not everyday persons.

Stine, you sometimes seems all excuses about your perfectionism. Don’t. As annoying as it may be for the rest of us to acknowledge our own imperfections it truly is necessary. I hope one day to acquire your interest for detail and your taste for true insight but am afraid it will never work out that way.

Else, you have a very special talent of being both a very flamboyant person as well as being exceptionally practical. You can make things happen and this is what makes you a born leader. However it is the flamboyance that makes the everyday fun. You can always talk to everybody and always have interesting things to say. It is the latter that makes you inspiring.

Actually, the last three years have not been bad at all. I started alone and now have a family with wife and kid. Hence, I sit as a happy man writing. Maybe this is the reason I cannot find the music in my collection of inexcusable noise?

TAK
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>[Ca^{2+}]_i</td>
<td>Intracellular Ca^{2+} Concentration</td>
</tr>
<tr>
<td>[Cl^-]_i</td>
<td>Intracellular Cl^- Concentration</td>
</tr>
<tr>
<td>4α-PDD</td>
<td>4α-phorbol-12,13-decanoate</td>
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<tr>
<td>4α-PD H</td>
<td>4α-phorbol-12,13-dehexanoate</td>
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<tr>
<td>A9C</td>
<td>Anthracene-9-carboxylic acid</td>
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<tr>
<td>AA</td>
<td>Arachidonic Acid</td>
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<tr>
<td>ADH</td>
<td>Antidiuretic Hormone</td>
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<td>AE</td>
<td>Anion Exchanger</td>
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<td>BAA</td>
<td>Bisandrographolide A</td>
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<td>Best1</td>
<td>Bestrophin 1</td>
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<td>CF</td>
<td>Cystic Fibrosis</td>
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<td>CIC-1</td>
<td>Chloride Channel 1</td>
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<td>CLIC1</td>
<td>Chloride Intracellular Channel 1</td>
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<tr>
<td>CRAC</td>
<td>Ca^{2+} release-activated Ca^{2+} channels</td>
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<tr>
<td>DIDS</td>
<td>4,4'-Diisothiocyanatostilbene-2,2'-disulfonic acid</td>
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<td>DIOH</td>
<td>[(dihydroindenyl)oxy] alkanoic acid</td>
</tr>
<tr>
<td>EAT</td>
<td>Ehrlich Ascites Tumor</td>
</tr>
<tr>
<td>E_{Cl}</td>
<td>Nernst Potential of Cl^-</td>
</tr>
<tr>
<td>EET</td>
<td>Epoxyeicosatrienoic Acid</td>
</tr>
<tr>
<td>ELA</td>
<td>Ehrlich Lettré Ascites</td>
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<td>hKv1.2</td>
<td>Human Voltage gated K^+ channel 1.2</td>
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<tr>
<td>KO</td>
<td>Knock Out</td>
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<td>MAEC</td>
<td>Mouse Aortic Endothelial Cells</td>
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<tr>
<td>MSA</td>
<td>Methane Sulphonic Acid</td>
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<tr>
<td>NHE1</td>
<td>Na^+/H^+ Exchanger 1</td>
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<td>NKCC</td>
<td>Na^+,K^+,2Cl^- co-transporter</td>
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<tr>
<td>NPPB</td>
<td>5-Nitro-2-(3-phenylpropylamino)benzoic acid</td>
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<td>OVLT</td>
<td><em>Organum vasculosum lamina termis</em></td>
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<td>Intracellular pH</td>
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<td>Phospholipase A_2</td>
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<td>Reactive Oxygen Species</td>
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<tr>
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<td>Ruthenium Red</td>
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<tr>
<td>RVD</td>
<td>Regulatory Volume Decrease</td>
</tr>
<tr>
<td>RVI</td>
<td>Regulatory Volume Increase</td>
</tr>
<tr>
<td>SON</td>
<td><em>supraoptic nucleus</em></td>
</tr>
<tr>
<td>TRP</td>
<td>Transient Receptor Potential</td>
</tr>
<tr>
<td>TRPA</td>
<td>TRP Ankyrin</td>
</tr>
<tr>
<td>TRPC</td>
<td>TRP Canonical</td>
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<tr>
<td>TRPM</td>
<td>TRP Melastatin</td>
</tr>
<tr>
<td>TRPML</td>
<td>TRP Mucolopin</td>
</tr>
<tr>
<td>TRPP</td>
<td>TRP Polycystin</td>
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<tr>
<td>TRPV4</td>
<td>TRP Vanilloid member 1</td>
</tr>
<tr>
<td>TRPV4</td>
<td>TRP Vanilloid member 4</td>
</tr>
<tr>
<td>V_m</td>
<td>Membrane Potential</td>
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THESIS OBJECTIVES

My thesis focuses on volume regulated ion channels – their activation and role in physiology. More specifically, the thesis will focus on these different subjects:

1. Investigation of the potential roles of Cl⁻ and volume regulated Cl⁻ channels in proliferation.
2. Investigation of oncogene effect on volume activated Cl⁻ channel (I_{Cl, swell}) activation.
3. Investigation of potential differences in signaling leading to I_{Cl, swell} in adherent and non-adherent cells.
4. Investigation of the structure-function relationship of Transient Receptor Potential Vanilloid 4 (TRPV4) channel activation by agonist.
SUMMARY:
Despite the relative stability of the extracellular space of healthy higher vertebrates, mammalian cell volume homeostasis is constantly challenged by intracellular dynamics, pathological events or the migration of cells between environments of varying osmotic strength. To counter the effects of volume perturbations evolution have developed system of channels and transporters to tightly control volume homeostasis.

In the past decades evidence has been mounting, that the importance of these volume regulated channels and transporters are not restricted to the defense of cellular volume but are also essential for a number of physiological processes such as proliferation, controlled cell death, migration and endocrinology.

The thesis have been focusing on two Channels, namely the swelling activated Cl$^-$ channel (I$_{\text{Cl,swell}}$) and the transient receptor potential Vanilloid (TRPV4) channel.

I: Cl$^-$ serves a multitude of functions in the mammalian cell, regulating the membrane potential ($E_m$), cell volume, protein activity and the driving force for facilitated transporters giving Cl$^-$ and Cl$^-$ channels a major potential of regulating cellular function. These functions include control of the cell cycle, controlled cell death and cellular migration. Volume regulatory mechanisms has long been in focus for regulating cellular proliferation and my thesis work have been focusing on the role of Cl$^-$ channels in proliferation with specific emphasis on I$_{\text{Cl,swell}}$. Pharmacological blockage of the ubiquitously expressed I$_{\text{Cl,swell}}$ will decrease proliferation in several cell types, including Ehrlich cells. A differentiated expression of I$_{\text{Cl,swell}}$ in the cell cycle has been described in different cell types indicating a regulating role. In Ehrlich Lettré ascites (ELA) cells we suggest the differentiated expression of I$_{\text{Cl,swell}}$ to be protective rather than regulating, while the role of Cl$^-$ in proliferation is due to other Cl$^-$ channels regulating $E_m$.

II: The volume regulated response to hypotonic stimuli is Ca$^{2+}$ dependent in the majority of endothelial cells. TRPV4, a member of the Transient Receptor Potential (TRP) channel family is a Ca$^{2+}$ permeable non-selective cation channel, which is activated by cell swelling. Besides cell swelling, however, TRPV4 is also activated by heat and a number of synthetic compounds. Despite of intense investigation of TRPV4, the structure function relationship is still rudimentary understood. Potential agonist binding sites have been proposed in transmembrane domains 3 and 4, in congruence with agonist binding sites of TRPV1. However, the functional relationship between TRPV4 and agonist binding is not yet understood. In this thesis is further elaborate the structure/function relationship between TRPV4 and its agonists. I identifies new essential residues for agonist activation, which has the potential of explaining agonist gating in TRPV4. The thesis will further complex the understanding of the TRPV4 pharmacore as new synthetic compounds are identified to interact with TRPV4. Understanding the structure/function relationship of TRPV4 is essential for future development of specific TRPV4 agonist for treatment of diseases causes by dysfunctional TRPV4. E.g. two inherited bone dysplasias have recently been demonstrated in humans to originate from TRPV4 mutations.
RESUME
På trods a den relative stabilitet af det ekstracellulære milieu i dunde pattedyr, vil volumen homeostasen af celler konstant blive udfordret af den intracellulære dynamik, patologiske begivenheder eller cellens vandring mellem milieuer af varierende osmotisk styrke. For at modvirke disse udfordringer for volumen homeostasen har evolutionen udviklet et system af kanaler og transportere der stringent kontrolleer cellens volumen.

I de senste årtier er der opbygget evidens for, at vigtigheden af disse volumenregulerede kanaler og transportere ikke er begrænset til forsvaret af den cellulære volumen, men tillige er essential for en række fysiologiske processer, som proliferation, kontrolleret celledød, migration og nyrernes funktion.

Denne tese fokuserer hovedsageligt på to kanaler: Den svulmnings aktiverede Cl⁻ kanal (I_{Cl,swell}) og transient receptor potential vanilloid kanal (TRPV4).

I: Cl⁻ er vigtig for en række cellulære funktioner, hvor Cl⁻ regulerer membran potentialet, cellens volumen, protein aktivitet og drivenergien for en række sekundære transporter, hvilket giver Cl⁻ og Cl⁻ kanaler en stor mulighed for at regulere cellulære funktioner. Disse funktioner inkluderer kontrol af celle cyclus, kontrolleret celledød og celular migration. Volumen regulatoriske mekanismer har længe været i fokus i reguleringen af den cellulære proliferation og min tese har fokuseret på Cl⁻ kanalers rolle i proliferation med speciel fokus på I_{Cl,swell}. Farmakologisk modulering a den allestedsnærværende I_{Cl,swell} vil medføre nedsat proliferationshastighed i adskillige celle typer. Ligeledes har man i adskillige celle typer beskrevet en varieret udtryksgrad af I_{Cl,swell} i det legeblods celle cyclus, hvilket kunne antyde en reguleringe rolle. Mine data i Ehrlich celler antyder dog at I_{Cl,swell} spiller en beskyttende snarere end en reguleringe rolle, mens Cl⁻ regulerer proliferationshastigheden via membran potentialet gennem andre kanaler.

CHAPTER I: INTRODUCTION

Despite the relative stability of the extracellular space of healthy higher vertebrates, mammalian cell volume homeostasis is constantly challenged due to intracellular dynamics. The intracellular environment is dominated by impermeable colloids (proteins, nucleotides, phosphates, and carbohydrates) which are predominantly negatively charged exceeding a profound electrochemical force across the plasma membrane. Known as the Donnan effect, this would - if not countered - lead to massive uptake of cations followed by water leading to cell swelling and eventually cell bursting. This schism is avoided primarily by the activity of the Na\(^+\)/K\(^+\) ATPase (see \(^1,2\)) or in some cells by the Ca\(^2+\) ATPase and Na\(^+\)/Ca\(^2+\) exchanger \(^3\). In addition to the constant challenge to steady state volume imposed by the Donnan effect, both the intra- and extracellular osmolarity can change under certain conditions. Metabolic activity – specifically acting on these colloids – alters the cellular concentration of osmotically active particles, thus imposing a very dynamic challenge to the cellular volume homeostasis. Moreover, the intracellular osmotic environment is changed during several physiological processes like transepithelial transport of osmotically active substances, or activation of channels and transporters leading to a net loss or gain of ions (see e.g. \(^4\)). Even though the extracellular space is normally very osmotically stable a number of cell types are exposed to severe changes in extracellular osmolarity. E.g. cells of the intestinal system are exposed to very low osmolalities following water intake, and blood cells are exposed to low osmolarity when passing the same intestinal epithelium, but very high osmolarity when passing the kidney medulla during antidiuresis. Kidney cells them self’s will be exposed to very severe osmotic challenges during antidiuresis. Even cells which are normally not exposed to a changing external milieu can face external challenges during a number of pathophysiological conditions like hypo- and hypernatremia, hypoxia, hypothermia and hyperglycemia (for reviews see \(^4-6\)).

Hence it is clear that cells must have lines of defense in order to maintain a constant volume. For this purpose, cells have a number of channels and transporters that are specifically activated upon volume disturbances. Cells shrink following hypertonicity, triggering a process known as regulated volume increase (RVI). Depending on the cell type, volume increase is achieved in this process by activating the Na\(^+\), K\(^+\), 2Cl\(^-\) co-transporter (NKCC) and/or the Na\(^+\)/H\(^+\) exchanger1 (NHE1) and/or non-selective cation channels, leading to net KCl uptake followed by osmotically driven water uptake (see \(^4,6\)). Note that the Na\(^+\) taken up is exchanged for K\(^+\) by the Na/K pump. In contrast, hypotonicity will lead to cell swelling and cells responds to this by opening separate K\(^-\) and Cl\(^-\) channels, KCl cotransporters, and channels for organic anions leading to a net diffusion of KCl and organic osmolytes from the cytosol into the extracellular fluid. This loss of osmolytes is followed by osmotic water loss and volume recovery – the process known as regulated volume
decrease (RVD) (see 4, 6). In my thesis work I have predominantly been working with swelling activated ion channels, and these will be further introduced in the following chapters.

Besides maintaining cell volume homestasis, volume regulated transport mechanisms serve fundamental physiological purposes in the cell and in the organism. Thus, changes in cell volume modulate a wide variety of downstream responses, like transepithelial transport, hormone and transmitter release, cell migration (See 7), proliferation and apoptosis (see 4, 8). Finally, recent evidence support a role for volume activated channels in regulating the blood osmolarity. Thus, as discussed later in the thesis (Chapter III), volume regulated channels regulate the activity of neurons of the supraoptic nucleus as well as in neurons of the organum vasculosum lamina terminalis. These organelles of the hypothalamus constitutes the main sensory system for systemic osmolarity, and controls kidney antidiuresis via antidiuretic hormone (ADH) release from the pituitary gland (see 9-11). This way, volume regulated channels are in effect regulating the stability of the same milieu they are protecting individual cells from.
CHAPTER II: VOLUME REGULATED CL\textsuperscript{–} CHANNELS:

A Short Overview of Volume Regulated Cl\textsuperscript{–} Channels

The Volume Regulated Anion Current:
As described above (Chapter I), osmotic swelling of mammalian cells will activate separate K\textsuperscript{+} and Cl\textsuperscript{–} channels leading to KCl loss to counter the osmotic imbalance between interior and exterior milieu. While a large number of swelling-activated K\textsuperscript{+} channels of known molecular identity have been described (see 4), the Cl\textsuperscript{–} efflux pathway (I\textsubscript{Cl,swell}) is most often discussed in singular form\textsuperscript{12,13}. This is due to the fact that almost every cell type will activate a current with very specific biophysical and pharmacological characteristics (see \textsuperscript{13,14}). Activation typically starts with a delay of 30-60 s and the activated current density will be a function of the severity of cell swelling. The almost ubiquitous biophysical characteristics of swelling activated Cl\textsuperscript{–} currents are: Outward rectification, voltage dependent inactivation at potentials more depolarized than 40 mV and an Eisenmann I halide selectivity sequence (I\textsuperscript{–} > Br\textsuperscript{–} > Cl\textsuperscript{–}) (see \textsuperscript{12-15}). These characteristics are not consensus characteristics of any molecular identified Cl\textsuperscript{–} channel, and a very fierce debate concerning the molecular nature of I\textsubscript{Cl,swell} is going on – as will be elaborated below.

The ubiquitous nature of this current has somewhat derailed the scientific debate surrounding volume regulated channels, as furious debate about I\textsubscript{Cl,swell} initiates the moment volume regulated anion channels are proposed. However, a number of volume regulated anion channels have been identified albeit with different biophysical characteristics when compared to I\textsubscript{Cl,swell}. Also the characteristics of I\textsubscript{Cl,swell} might not be as clear as suggested above. Thus anybody working with swelling activated Cl\textsuperscript{–} currents will soon learn that the voltage dependent inactivation differs significantly between cell types (see \textsuperscript{12,13}) which could suggest additional channels and/or channel components/accessory subunits in the process. Below I shall briefly give an overview of the Cl\textsuperscript{–} channels that have been regarded of interest with respect to volume regulation.

Chloride Channel 3 (CIC-3):
CIC-3 is the most debated Cl\textsuperscript{–} channel with regards to volume regulation and both its role in volume regulation and even its function as a channel is disputed. CIC-3 was initially identified by Hume and colleagues as a volume sensitive Cl\textsuperscript{–} channel in 1997 with biophysical and pharmacological characteristics similar to I\textsubscript{Cl,swell}\textsuperscript{16}. This was rather surprising given the characteristics of other channel members of the CIC channel family (CIC-1 and -2) (see \textsuperscript{15,17,18}). Hence, these data where soon to be disputed, as other groups found it difficult to heterologously express CIC-3 channels in the plasma membrane and found different biophysical characteristics when they successfully expressed CIC-3 channels\textsuperscript{19-21}. It has later been suggested that this difference primarily is due to different splice variants\textsuperscript{22}. Moreover, independently generated mice
with disrupted CIC-3 genes failed to show difference in \( I_{\text{Cl,swell}} \) characteristics\(^{23-26}\). Although Hume and colleagues later explained these findings by compensatory mechanisms\(^{26}\) and found functional phenotypes in cardiac cells from conditional knock outs (KO)\(^{27}\), these results seem to rule out CIC-3 as the ubiquitously found \( I_{\text{Cl,swell}} \). Perhaps the most definitive argument against CIC-3 in volume regulation came from Thomas Jentsch’s group, who demonstrated that CIC-3 is more likely to be an intracellular \( \text{Cl}^-/\text{H}^+ \) exchanger, in line with the most closely related members of the CLC family (CIC 4 and 5) (see \(^{15,17,18}\)).

Whether CIC-3 is in fact a volume activated \( \text{Cl}^- \) channel is still up for rather hefty debate but it seems very clear from a number of different studies in glioma\(^{28}\), cardiac\(^{29,30}\) and smooth muscle cells\(^{31}\) that CIC-3 at the very least has a regulating role for \( I_{\text{Cl,swell}} \).

**Chloride Channel 2 (CIC-2)**

CIC-2 is a widely expressed \( \text{Cl}^- \) channel of the CIC family of channels and transporters\(^{17}\) and was among the first cloned channels to evidently be volume regulated. It was initially cloned and characterized in Xenopus oocytes\(^{32}\) and later recognized as volume sensitive in mammalian cells\(^{33,34}\). However, CIC-2 does not share biophysical properties with the mythical \( I_{\text{Cl,swell}} \), as CIC-2 shows inward rectification voltage dependent activation at hyperpolarized potentials and have an reverse halide selectivity (\( \text{Cl}^- > \text{Br}^- > \text{I}^- \)) (see \(^{15}\)).

**Bestrophin 1 (Best1)**

In the ever growing field of \( \text{Cl}^- \) channels, all the identified families seem to include volume regulated members and the Bestrophin family is no different. Bestrophin was initially identified as a monogenetic disorder, responsible for *Vitelliform macular dystrophy (VMD) also known as bests disease*, which significantly inhibits retinal vision. Electrooculography indicated \( \text{Cl}^- \) channel dysfunction and the VMD gene was later recognized as a \( \text{Ca}^{2+} \) activated \( \text{Cl}^- \) channel\(^{35}\). hBest1 and mBest2 were identified as volume sensitive channels by overexpression in HeLA, HEK293 and ARPE-19 cells, predominantly by showing inhibition to hyperosmolarity. Activation upon hyposmolarity was suggested but it was very difficult to observe due to the simultaneous activation of endogenous \( I_{\text{Cl,swell}} \). In *Drosophila melanogaster*, dual regulation of bestrophin by \( \text{Ca}^{2+} \) and volume has been described\(^{37}\).

Bestrophins have a low low-field strength selectivity of halides (\( \text{I}^- > \text{Br}^- > \text{Cl}^- \))\(^{36}\) and hBest1 is recognized by voltage dependent inactivation at negative potentials and small outward rectification\(^{35}\). Importantly, however, Bestrophin expression is relatively restricted to specific tissues limiting the physiological importance in the vast majority of tissues, although this might still be due to limited investigation efforts\(^{38}\).
Chloride Intracellular Channel 1 (CLIC1)

From a biophysical and biochemical point of view CLIC1 is one of the most interesting channels ever cloned. CLIC1 is predominantly found as a soluble protein in the cytoplasm but dimerises upon oxidation and inserts into the plasma membrane where it constitutes a functional channel\(^{39}\). CLIC1 shows outward rectification and a halide permeability sequence of F\(^-\) > Cl\(^-\) > I\(^-\)\(^{40,41}\).

CLIC1 has been investigated for involvement in volume regulation in a single study in which it was concluded to be unlikely to be involved in volume regulation\(^{42}\). The CLIC1 inhibitor Indanyloxyacetic acid 94 (IAA-94) blocked volume regulated anion currents but only at very high concentrations\(^{42}\).

TMEM16A

The most recent Cl\(^-\) channel family to be identified is the Ca\(^{2+}\) sensitive TMEM16 family of Cl\(^-\) channels. This family was remarkably recognized simultaneously and independently by three different groups\(^{43-45}\) and solves the decade long search for the Ca\(^{2+}\) activated Cl\(^-\) current of epithelia\(^{43,46}\). TMEM16A has recently been suggested to be involved in volume regulation following osmotic swelling. However, the characteristics of TMEM16A\(^{47}\) do not resemble those of I\(_{\text{Cl,swell}}^{43-45}\). Ca\(^{2+}\) dependent activation of Cl\(^-\) currents in respect to cell swelling has been described in a number of cells especially of epithelial origin, as has a dependence of RVD on extracellular Ca\(^{2+}\) (see 4). Hence, TMEM16A can most certainly be essential for volume regulation in a number of cells but it does not constitute the ubiquitous I\(_{\text{Cl,swell}}\). The impact of Ca\(^{2+}\) in RVD will be further discussed in Chapter III.

Cl\(^-\) Channels in Cell Proliferation:

Cell proliferation is one of the most tightly regulated processes and misregulation will lead to several pathophysiologies with cancer and developmental disorders being the most obvious. In spite of the fact that monovalent ion channels have been suggested as potential antiproliferative/anticancer targets in several recent reviews\(^{48-51}\), the role of monovalent ions per se in control of cell proliferation is very rudimentarily described. Ions, however, constitute very interesting molecules in this regard as their concentrations and movement across the membrane affects a very versatile number of cellular processes like membrane potential, cell volume and driving force for secondary transporters.

My thesis work has focused mainly on the role of Cl\(^-\) in cell proliferation. As a consequence of the Donnan equilibrium described in Chapter I, the cell volume is very sensitive to [Cl\(^-\)] changes (see e.g. 4) and this aspect have been dealt with in numerous studies concerning cell proliferation (see 5). This is, however, not the only role of Cl\(^-\). Three other functions need to be considered: (i) In the vast majority of cells the membrane potential (E\(_{m}\)) is hyperpolarized in respect to the equilibrium potential of Cl\(^-\) (E\(_{\text{Cl}}\)) giving Cl\(^-\) permeability a good possibility of fine tuning E\(_{m}\). (ii) Additionally important, is the fact that the Cl\(^-\) gradient
across the membrane functions as driving force for a number of coupled transporters affecting pH\textsubscript{i}\textsuperscript{52} and amino acid uptake\textsuperscript{53}. (iii) Finally, less recognized is the role of Cl\textsuperscript{-} as a second messenger (see \textsuperscript{54}).

A role for Cl\textsuperscript{-} and Cl\textsuperscript{−} channels can be speculated into all parts of the cell cycle. However, it seems especially important for the G1/S phase transition as many cell types a stopped in G1 by Cl\textsuperscript{-} channel inhibitors\textsuperscript{55-58}, although in glia and CHO-K1 cells inhibition of CIC-3 and CLIC1, respectively, inhibits cell division in the M-phase\textsuperscript{28,59}. In the following I will review the role of Cl\textsuperscript{-} channels in proliferation of mammalian cells with respect to the different roles of Cl\textsuperscript{-} ions and channels:

**Role of Cl\textsuperscript{-} in Volume Changes During Cell Cycle Progression**

It is obvious that a volume increase has to occur between cell divisions. Of course this volume increase mainly originates from an increase in cell matter and not from osmotic swelling. However, ionic movements and resulting osmotic swelling or shrinkage have been shown essential for regulating cell proliferation (see \textsuperscript{8,60}). The functional importance of volume changes during the cell cycle is best understood in glia cells, thanks to an excellent series of studies by Habela and Sontheimer\textsuperscript{28,61,62}. In DG-54 glioma cells, a significant volume decrease was observed in the M phase of the cell cycle resulting in a condensation of the cytoplasm. This in turn was shown to be a prerequisite for chromatin/DNA condensation in preparation for chromosome separation\textsuperscript{62}. This cell volume decrease is driven by a loss of Cl\textsuperscript{-}, following activation of a Cl\textsuperscript{-} conductance sensitive to CIC-3 siRNA\textsuperscript{28,61}. Notably, however, this mechanism is dependent on the very high Cl\textsuperscript{-} concentration (∼100 mM compared to 20-40 mM in most cell types) in these cells, which is decreased to 66 mM in the M phase\textsuperscript{61}, and is probably not relevant in cell types with substantially lower [Cl\textsuperscript{-}].

In Nasopharyngeal carcinoma cells (CNE-2Z), cell volume was found to increase steadily before a marked volume increase in the M-phase\textsuperscript{63} – i.e. apparently opposite of what was seen in glioma cells. Interestingly, the group was able to find a variable capability of cells to perform RVD during the cell cycle, with the fastest RVD response in G1 phase cells and the lowest in S phase\textsuperscript{63}. This change in RVD rate was paralleled by changes in the expression of I\textsubscript{Cl, swell} in these cells\textsuperscript{64} and a number of I\textsubscript{Cl, swell} antagonists inhibited proliferation of CNE-2Z cells\textsuperscript{55} as is also seen in other cell types (e.g. \textsuperscript{65-69}, CHAPTER IV and V). Unfortunately, no functional explanation has been given for the importance of the differentiated RVD response in cell cycle progression. However, with the intense increase in cell matter during the cell cycle, it could be speculated that this differentiated RVD capacity means to defend the cells from excessive volume changes during colloid synthesis. Such a mechanism would also be in line with our data in ELA cells. In these cells we see an upregulation of I\textsubscript{Cl, swell} in the S phase compared to G1\textsuperscript{65} (CHAPTER IV), but this is not reflected in the general Cl\textsuperscript{-} conductance, which is down-regulated in the S phase compared to G1\textsuperscript{69} (CHAPTER V). Hence, it seems like the volume regulatory mechanism is up-regulated during the cell cycle both in CNE-2Z and ELA
cells but not activated. It must be emphasized though, that in ELA cells we found a significant increase in the cell water to protein ratio driven by an increase in the content of Na\(^+\) and Cl\(^-\) (CHAPTER V). Hence, the increased expression of \(I_{\text{Cl,swell}}\) is either not sufficient to maintain volume homeostasis or a volume increase is necessary for S phase cells.

The Potential Role of Cl\(^-\) as a Messenger:
Probably, the role of Cl\(^-\) as a direct regulator of protein activity is the least investigated aspect of Cl\(^-\) and Cl\(^-\) channel regulation of proliferation. However, significant evidence exist for Cl\(^-\) regulating important proteins such as protein kinases and phosphatases as well as the ubiquitously expressed NKCC1 (see \(^54\)). Inhibition of the latter will inhibit proliferation of fibroblasts\(^70\), vascular smooth muscle cells\(^71\), lymphocytes\(^72\), vascular endothelial cells\(^73\) and airway smooth muscle cells\(^74\), while in Balb/c 3T3 cells NKCC1 over-expression stimulated cell proliferation\(^75\). As NKCC1 doesn’t conclusively regulate Na\(^+\) and K\(^+\), it has been suggested that NKCC1 regulates proliferation by regulation of [Cl\(^-\)]\(^54\). In ELA cells, however, we found no difference in the Cl\(^-\) concentration between G1 and S phase and was able to substitute 80 % extracellular Cl\(^-\) with methane sulphonic acid (MSA) without affecting proliferation\(^69\) (CHAPTER V). This strongly suggests, that – although several Cl\(^-\) channel inhibitors agonizes proliferation – Cl\(^-\) is not essential for ELA cell proliferation. Hence, Cl\(^-\) concentration is not a ubiquitous signal for cell proliferation.

Cl\(^-\) as a pH Regulator:
pH is essential for all chemical processes and the intracellular pH (pH\(_i\)) will affect protein folding, protein-protein interactions and the effectiveness of enzymatic processes, which make pH an essential player for physiological processes. pH\(_i\) oscillations in the cell cycle have long been known and seems to be especially important for the G1/S phase transition (reviewed in \(^76\)). Cl\(^-\) important for pH\(_i\) regulation via the HCO\(_3\^-\)/Cl\(^-\) antiporters (AE1-3), which is essential for the response following both acification and alkalization of the cytosol (see \(^76\)). Further, HCO\(_3\^-\) is essential for most cell types to grow (reviewed \(^76\)). Although an altered expression pattern of AE2 have been described in various cancers\(^77-79\) very little information have been generated for a role of AE1-3 in control of the cell cycle. Hence, AE1-3 seems to play a protective role for pH\(_i\) in cell cycle control rather than direct regulation. In line, the decreased extracellular pH reported in tumors will – as a function of the Henderson-Hasselbach equation – mediate decreased HCO\(_3\^-\) activity and eliminate the driving force for Cl\(^-\)/HCO\(_3\^-\) exchange (see \(^80\)). In ELA cells our own results show a particular high tolerance for Cl\(^-\) substation by MSA despite the very high selectivity of Cl\(^-\)/HCO\(_3\^-\) exchangers – even between halides (see \(^81\)). The regulatory role of pH\(_i\) in the cell cycle is more likely to involve NHE1 activity as reviewed elsewhere \(^76,82\).
**Cl Channels as Regulators of $E_m$:**

The membrane potential is broadly recognized to regulate the cell cycle primarily by regulating the driving force for Ca$^{2+}$ (see $^{83, 84}$). [Ca$^{2+}$], is an essential messenger for proliferation with [Ca$^{2+}$], oscillations particularly important during G1/S phase transition $^{83, 84}$ and several Ca$^{2+}$ channels, including L-type, T-type and members of the TRP channel family are involved in cancer $^{84}$. With $E_m$ in mammalian cells to a large extent dominated by the K$^+$ permeability it is obvious that a dynamic between [Ca$^{2+}$], and Ca$^{2+}$ activated K$^+$ channels exists which regulate proliferation $^{83, 84}$. However, as cells typically have a [Cl$^-$], between 20 and 40 mM in an environment with $\sim$100 mM giving $E_{Cl}$ between -32 mV and -25 mV, Cl$^-$ permeability has the potential of regulating $E_m$.

In ELA cells we found that the effect of substituting Cl$^-$ in the extracellular solution was dependent on the permeability of the anion substituent with a stronger effect of less permeable anions $^{69}$ (CHAPTER V) and found $E_m$ to be regulated according to Cl$^-$ permeability in the plasma membrane indicating. A number of Cl$^-$ channel antagonists inhibited proliferation in ELA cells $^{65, 69}$ (CHAPTER IV and V) and these data suggest Cl$^-$ channels regulate proliferation by regulating $E_m$. In T lymphocytes 4,4'-Diisothiocyanatostilbene-2,2'-disulfonic acid disodium (DIDS) sensitive Cl$^-$ channels are essential for Ca$^{2+}$ signals via Ca$^{2+}$ release-activated Ca$^{2+}$ channels (CRAC ) which regulate [Ca$^{2+}$], which in turn regulates proliferation $^{85}$. Hence, the role of Cl$^-$ channels setting $E_m$ is most likely regulating proliferation by setting the driving force for Ca$^{2+}$ influx and this would also be in agreement with the importance of Cl$^-$ channel activity in G1/S phase transition $^{55-58}$ in which Ca$^{2+}$ oscillations are essential (see $^{83, 84}$).

**What Channels are Important for Proliferation?**

With the limited specificity of Cl$^-$ channel inhibitors (see $^{15}$) it is very difficult to elucidate which channels have an importance for proliferation of mammalian cells. Further, the role of Cl$^-$ channels seems to be conductive rather than specific. Hence, pharmacological modulation of Cl$^-$ conductance can affect proliferation by inhibiting unregulated channels – giving a failed conclusion of regulating importance for these channels. In line, proliferation is inhibited by almost every anion inhibitor in the book, including 5-Nitro-2-(3-phenylpropyramino)benzoic acid (NPPB)$^{55, 62, 64, 68, 86}$, DIDS$^{28, 85}$, tamoxifen$^{55, 68, 69}$ (CHAPTER V), NS3728 $^{65, 69}$ (CHAPTER IV and V), IAA-94 $^{59, 86}$, Anthracene-9-carboxylic acid (A9C)$^{59}$ and niflumic acid$^{69}$ (CHAPTER V). It must be noted, though, that the effect of different Cl$^-$ channel antagonists are very cell type specific, reflecting different channel expression in different cell types.

From a more molecular perspective the literature has several examples of channels which are regulated in the cell cycle. Several studies have shown differentiation of I$_{Cl, \text{swell}}$ $^{64, 65, 68}$ (CHAPTER IV) activity following hypotonic activation$^{64, 65, 68}$ (CHAPTER IV) but as no molecular consensus candidate is known for this current
the importance of this differentiation is entirely build on pharmacology. However, a few molecular I_{Cl, swell} candidates is clearly important for cells cycle progression. In porcine arterial smooth muscle cells CIC-2 is specifically unregulated after insulin-like growth factor (IGF)-1 stimulation of proliferation and siRNA knock-down (KD) of CIC-2 inhibited IGF-1 stimulated proliferation\cite{86}. In contrast CIC-2 antisense did not affect proliferation of human leukemic cells\cite{56}. CIC-3 is significantly up-regulated in the M phase of glia cells and siRNA KD accumulated mitotic cells. CLIC1 is specifically inserted into the plasma membrane of CHO-K1 cells in the M phase and pharmacological inhibition will stop the cell cycle\cite{59}.

Based on the current knowledge it is very difficult to pinpoint one Cl– channel with importance for proliferation. It is much more likely, that different Cl– channels is important for proliferation of different cell types and in some cell types works in aggregate with other Cl– channels.

**Conclusion:**
In conclusion it is fair to say that Cl– and Cl- channels not only in theory have many possibilities of regulating the cell cycle. Depending on the cell type Cl– channels will promote proliferation by cell volume/ cytosolic condensation, direct molecular interaction or by regulating E_{m}. As well as no general regulatory mechanism for Cl– exist in proliferation, no particular Cl– channel seems to be specifically important for proliferation throughout the number of cell types investigated. Hence, specific Cl– channel inhibitors has the potential to descriminate between cancers of different origin but – unfortunately – specific Cl– channel inhibitors are yet to be described.

**I_{Cl, swell} in Cell Migration:**
The interest in Cl– channels in cancer research is mainly due to the importance that Cl– channels have for cell proliferation. There is, however, more to chloride channels and cancer, as Cl– channels are also involved in cell migration, as discussed in this section, as well as in control of cell death, as discussed in \cite{87,88} (see also \cite{89}). Migration of fibroblast-type cells involves a series of directed morphological changes in which lamellipodia protrusion and uropodial retraction is separated in time and rate\cite{7,90}. These morphological changes are supported by volume regulated ion transporters. NHE1, anion exchangers, and aquaporins are found at the leading edge and ostensibly contribute to leading edge protrusion by inducing local swelling, while retraction of the lagging edge is thought to be supported by volume decrease via activation of separate K+ and Cl– channels (Fig 1, see \cite{7,90}).
The involvement of volume regulated Cl⁻ channels in cell migration has been confirmed in nasopharyngeal carcinoma cells⁹², neutrophils⁹³ and glioma cells⁹⁴,⁹⁵. Hence, it was interesting for us to investigate whether oncogenes regulate the activity of volume regulated anion channels and whether this in turn impacted on cell migration.

We focused on the effect of proto-oncogene H-Ras on migration and I_{Cl,swell} activity. H-Ras is a member of the three member Ras gene family (H-, K- and N-Ras). Ras are small monomeric G-proteins with GTP binding and hydrolyzing capabilities. GTP bound Ras binds and regulates a large number of intracellular signaling pathways affecting proliferation, apoptosis, cell motility and cell morphology (see⁹⁶,⁹⁷). Ras genes are almost totally identical and although expression of different gene members is tissue specific, the gene family is ubiquitous. Notably, mutations in the Ras family are seen in 70 % of neoplasias affecting primarily metastasis and angiogenesis. H-Ras is especially expressed in the skin and skeletal muscles and is mutated in bladder and kidney cancers (see⁹⁶,⁹⁷).

In order to investigate whether H-Ras affects I_{Cl,swell} activity, we performed patch clamp experiments in WT and v-H-Ras expressing NIH 3T3 cells, the latter having two mutations increasing activity and lowering GTPase activity.

We found no difference in the characteristics or magnitude of the measured I_{Cl,swell} currents, indicating that H-Ras does not stimulate Cl⁻ channel expression. However, mild cell swelling (8%) induced a significantly higher Cl⁻ current in H-Ras cells compared to wild type cells, demonstrating an increased sensitivity to

**Figure 1: Cellular migration. On top:** NaCl is taken up in the leading edge via NHE1 and AE2 followed by osmotically driven water uptake mediating directed cell swelling. **Bottom:** In the retractive end, KCl is lost due to opening of separate K⁺ and Cl⁻ channels mediating cell shrinkage. Modified from⁷,⁹¹.
physiological cell swelling of the magnitude relevant in migrating cells\(^9^8\) (CHAPTER VI). In line with previous studies\(^9^9-1^0^5\), Ras activity increased cellular migration\(^9^8\) (CHAPTER VI). Importantly, migration of both WT and v-H-Ras expressing cells was dependent on Cl\(^-\) channels, as the high affinity Cl\(^-\) channel inhibitor, NS3728, inhibited migration of both cell types. Notably, the displacement was inhibited more by NS3728 in H-Ras expressing cells than in WT cells, indicating a role for Cl\(^-\) channels in directional migration which is more prominent in the presence of H-Ras.

Future studies will need to investigate how H-Ras regulates I\(_{Cl,swell}\) activity but in this context it is very interesting H-Ras suppresses integrin activation\(^1^0^6\) and H-Ras inhibits cellular adhesion\(^1^0^3,1^0^7\). Integrin as I will elaborate below is a major regulator of I\(_{Cl,swell}\).

**Signaling Mechanisms Involved in Activation of I\(_{Cl,swell}\)**

The issue of how I\(_{Cl,swell}\) is activated by cell swelling is still incompletely resolved. A number of activation mechanisms have been proposed during the years as it seems evident that I\(_{Cl,swell}\) is not mechanosensitive\(^1^0^8\) (discussed in \(^1^2\)). I\(_{Cl,swell}\) activity is heavily modulated by several intracellular signaling moieties, including arachidonic acid, G-proteins, reactive oxygen species (ROS), and the actin cytoskeleton, and is also modulated by phosphorylation/ dephosphorylation (see \(^4,1^2-1^4\)). However, the volume sensing mechanism is still elusive. Nilius and Voets showed that I\(_{Cl,swell}\) activation in CPAE cells is strictly correlated to the decreasing intracellular ionic strength rather than to cell volume\(^1^0^9\), and ionic strength has also been implicated in activating I\(_{Cl,swell}\) in CHO\(^1^1^0\) and HEK 293 cells\(^1^1^1\). Ionic strength appears to regulate the channel indirectly via other signaling mechanisms such as tyrosine phosphorylation, since tyrosine kinase inhibitors abolishes the activation\(^1^1^2\). Ionic strength as the main trigger of I\(_{Cl,swell}\) is very appealing in its simplicity and clear biophysical origin, but it will only work when cell volume is increasing because of changing environments and not during isotonic conditions, under which I\(_{Cl,swell}\) also plays a significant role (e.g. \(^4\)). Hence, there must be other means of I\(_{Cl,swell}\) activation. In three simultaneous studies, ROS were shown to activate I\(_{Cl,swell}\) in HeLa cells\(^1^1^3,1^1^4\), HTC cells\(^1^1^4\) and rabbit ventricular myocytes\(^1^1^5\). Based on studies in ventricular myocytes, Baumgartner's group has suggested a scheme in which integrins act as the volume sensor, stimulating NADPH oxidase production of ROS via Src kinases\(^1^1^5-1^1^7\). The importance of Src kinases for I\(_{Cl,swell}\) activation was confirmed pharmacologically in HTC cells by Andres Stutzins group\(^1^1^8\), pointing to a similar pathway in this cell type. Baumgarten's group very elegantly showed that integrin stretch induced via antibody-coated magnetic beads mediated I\(_{Cl,swell}\) activation, clearly demonstrating an extracellular effect of volume sensing, possibly via integrin interaction with the extracellular matrix\(^1^1^5\).
**Role of ROS in Regulation of $I_{\text{Cl,swell}}$ in Adherent Versus Suspension Cells**

A mechanism based on the interaction between integrins and the adherent surface could, however, not work in suspension cells like Ehrlich ascites tumor (EAT) cells, in which swelling activated Cl$^{-}$ efflux was initially identified$^{119}$ and $I_{\text{Cl,swell}}$ later described electrophysiologically$^{120}$. Hence, we decided to compare the volume regulatory effect of ROS in Ehrlich cells in suspension versus adherent Ehrlich cells. Suspended EAT cells shrank in isotonic solution in the presence of 500 $\mu$M H$_2$O$_2$, and H$_2$O$_2$ accelerated the RVD response following hypotonic swelling$^{121}$ (CHAPTER VII). However, the observed KCl loss was totally abolished by substituting Cl$^{-}$ with NO$_3$$^-$ (which is more permeable through $I_{\text{Cl,swell}}$ compared to Cl$^{-}$ (see 12)). In contrast, cell shrinkage was unaffected by a rise in extracellular K$^+$ concentration to a value that eliminates the electrochemical driving force for K$^+$, but was impaired when the driving force for KCl cotransport was omitted. This indicated KCl co-transport rather than channel activation. In line with this [(dihydroindenyl)oxy] alkanoic acid (DIOA) which blocks KCl co-transport$^{122}$ almost totally eliminated the H$_2$O$_2$ induced volume decrease.

In EAT cells, no Cl$^{-}$ current was activated upon exposure to 500 $\mu$M H$_2$O$_2$ in isotonic bath solution. A 33% hypotonic solution activated outwardly rectifying Cl$^{-}$ currents as previously described$^{120}$ but no additional current activation was seen by the presence of H$_2$O$_2$ $^{121}$ (CHAPTER VII). This contrasted to our findings in adherent cell types. Ehrlich Lettré ascites (ELA) cells is a cell type developed from EAT cells$^{123}$. In these cells we observed a significant Cl$^{-}$ current activation as a result of H$_2$O$_2$ stimulation in isotonic medium$^{121}$ (CHAPTER VII), comparable to the effect observed in other cell types$^{113}$-$^{115}$. During hypotonic stimulation, H$_2$O$_2$ potentiated both K$^+$ and Cl$^{-}$ channel activation.

This difference between EAT and ELA cells of course can have several explanations. A difference in intracellular signaling events is likely to constitute a major part, as $I_{\text{Cl,swell}}$ activation in EAT cells is totally insensitive to extracellular H$_2$O$_2$ in contrast to ELA cells. It is also clear, however, that the hyposmotic activation is different since H$_2$O$_2$ generation during hyposmotic stress is only seen in ELA cells$^{121}$, $^{124}$ (CHAPTER VII). Hence, it would seem that some adaptation from suspension to adherent life has occurred involving both production and sensitivity to H$_2$O$_2$. The adaptation, however, does not affect the expression of integrins. Song et al showed the same amount of $\alpha_5\beta_1$ in adherent and non-adherent Ehrlich cells but was able to show different affinity to laminin and fibronectin and explained these differences by differentiated intracellular mechanisms$^{125}$.

This adaptation is very interesting in respect to metastasis. Oncogenes, such as H-Ras, decrease integrin activity and integrin based adhesion$^{103}$, $^{106}$, $^{107}$ promoting metastasis. However, it increases $I_{\text{Cl,swell}}$ sensitivity to mild osmotic stimuli as described above. Hence, the adaptation from integrin/ROS based activation to a
different activation mechanism could be at the very core of metastasis as the continued function of $I_{Cl,swell}$ could be essential for proliferation and migration of the metastatic cells. Further studies are required to reveal the exact adaptation but it is interesting if the lack of a solid support changes the activation mechanism for $I_{Cl,swell}$ from integrin/ROS signaling to alternative signaling events.

CHAPTER III: STRUCTURE/FUNCTION RELATIONSHIP OF TRPV4 AND ITS AGONISTS

A Brief Introduction to TRPV4

The Role of Calcium in Volume Regulation:

Intracellular $\text{Ca}^{2+}$ is not a ubiquitous signal in volume regulation but increases in $[\text{Ca}^{2+}]_i$ have been described in numerous cell types particularly of epithelial origin, following cell swelling (recently reviewed\cite{4}). The rise in $[\text{Ca}^{2+}]_i$ has been described to be biphasic, with the initial phase representing $\text{Ca}^{2+}$ uptake from the extracellular medium, followed by $\text{Ca}^{2+}$ release from intracellular stores in the second phase\cite{126}. Removing extracellular calcium will inhibit RVD responses in many epithelial cell types, reflecting the importance of both $\text{Ca}^{2+}$ sensitive $\text{Cl}^-$ and $\text{K}^+$ channels for the RVD response in these cells (see \cite{4}). A number of different channels, like the L-type $\text{Ca}^{2+}$ channels\cite{127}, stretch activated $\text{Ca}^{2+}$ channels\cite{128,129} and members of the Transient Receptor Potential (TRP) channel family\cite{130,131} have been proposed to form the swelling-activated entry path of $\text{Ca}^{2+}$ from the extracellular space.

I have in my thesis work focused on the TRP channel TRPV4, and more specifically on the structure-function relationship of its activation by pharmacological agents, and thus, this channel and its pharmacology will also be the focus here.

A Short Introduction to the TRP Channel Family:

The superfamily of TRP channels are a group of cation conducting channels sharing low amino acid homology. The mammalian members of this superfamily are normally subdivided according to their primary amino acid composition into six families: TRPC (canonical), TRPV (vanilloid), TRPM (melastatin), TRPP (polycystin), TRPML (mucolipin), and the TRPA (ankyrin). All TRP channels have six putative transmembrane domains and a hydrophobic pore loop between TM 5 and 6. In general, TRP channels exhibit poor selectivity between cations but TRPM4 and -5 will only conduct monovalent cation currents while TRPV5 and 6 are the only highly $\text{Ca}^{2+}$ selective channels (see \cite{14,132,133}).

TRP channels constitute a major molecular sensing mechanism of mammals and are activated by stimuli as different as temperature, volume, pain, and a number of chemical flavors like chili, menthol, cinnamon and
mustard. TRP channels are involved in various physiological processes like pain sensing, heat regulation, taste and systemic osmoregulation (see \textsuperscript{14,132,133}). Not surprisingly, the number of channelopathies shown to involve TRP channels is increasing at a rapid rate. These involve deafness and neurodegenerative diseases, polycystic kidney disease, focal and segmental glomerulosclerosis, hypomagnesemia \textsuperscript{134} and autosomal dominant brachyolmia \textsuperscript{135}.

Several members of the TRP channel family have been experimentally activated by osmotic swelling. TRPV2 \textsuperscript{136}, TRPV4 \textsuperscript{130,131}, TRPM3 \textsuperscript{137}, TRPM7 \textsuperscript{138}, TRPC6 \textsuperscript{139}, TRPP2 \textsuperscript{140} and TRPP3 \textsuperscript{141} are all activated by cellular volume expansion and TRPV1 is involved in neuronal osmo-sensing \textsuperscript{142}. I have exclusively been working with TRPV4 and will not discuss the volume regulation of the remaining TRP channels further. For reviews of osmosensitivity of TRP channels see \textsuperscript{4,143,144}.

**The TRPV4 Channel and its Role in Volume Regulation:**

TRPV4 belongs to the TRP vanilloid channel family and was initially identified as a swelling activated nonspecific cation channel with voltage independent characteristics between -100 and +100 mV \textsuperscript{130,131}. The channel is especially highly expressed in osmotically challenged tissues including epithelial cells and neurons of the CNS, consistent with a fundamental role in osmosensing (see \textsuperscript{132,145}).

TRP channels are notorious for their promiscuous gating and TRPV4 is no different. In addition to its volume sensitivity it is also a temperature sensitive channel activating at temperatures above 20°C \textsuperscript{146}, and it is activated by a series of both natural \textsuperscript{147} and synthetic compounds \textsuperscript{148-151} (CHAPTER VIII). These activating characteristics bode for significant physiological importance. Nonetheless, TRPV4 KO mice showed no obvious phenotypes: The mice have normal growth, appearance and lifespan. Interestingly though, TRPV4 KO mice showed dysfunctional osmoregulation of blood plasma, with increased hypertonic blood and a decreased antidiuretic hormone (ADH) response after induced hyperosmolar stress \textsuperscript{11}. The systemic osmotic pressure is regulated via a complex feedback mechanism in the CNS which controls the release of ADH, which in turn controls the reuptake of water via the kidneys (see \textsuperscript{9,10}). As ADH responses were changed in TRPV4 KO mice \textsuperscript{11}, this is strongly indicative of a CNS connection. Further, TRPV4 staining in the brain revealed high TRPV4 expression in the osmosensitive hypothalamic organs *Organum vasculosum laminae terminalis* (OVLT) and the supraoptic nucleus (SON). Expression of the immediate early response protein, c-fos, which is expressed in active neuroendocrine neurons \textsuperscript{152}, diminished in TRPV4 KO mice in these organs upon hyperosmotic stress \textsuperscript{11}. This strongly suggests a role for TRPV4 in the osmosensing neurons in the hypothalamus. However, the precise mechanism involved in the neuronal role of TRPV4 is still to be described.
TRPV4 is only secondarily activated by osmotic swelling via metabolites of arachidonic acid. Phospholipase A$_2$ (PLA$_2$) hydrolysis of phospholipids into arachidonic acid (AA) and lysophospholipids has been described in several cell types following hypotonic stress, including EAT cells$^{153}$, human platelets$^{154}$, NIH-3T3 cells$^{155-157}$, CPH-100 neuroblastoma$^{158}$ and HeLa cells$^{159}$ (for review of PLA$_2$ in volume regulation see $^4$). In TRPV4 transfected HEK293 cells and mouse aortic endothelial cells (MAECs) AA activates TRPV4 but not in MAECs from TRPV4 KO mice$^{160,161}$. To activate TRPV4 AA requires metabolism by cytochrome 450 epoxygenase into epoxyeicosatrienoic acids (EET)$^{160,161}$, which is in difference to the volume activated K$^+$ channel in EAT cells, which requires lipoxigenase generated leukotrienes$^{162}$ (see $^{163}$). More specifically 5$'$,6$'$-EET activates TRPV4 directly in inside-out patches, while 11$'$,12$'$-EET, 14$'$,15-EET and 20$'$-hydroxyeicosatetraenoic acid were ineffective. 8$'$,9$'$-EET activated TRPV4 currents in the whole cell configuration but the observed currents where smaller compared to 5$'$,6$'$-EET stimulated currents.

Wolfgang Liedtkes group who initially cloned TRPV4$^{130}$ and later generated the first TRPV4 KO mouse$^{11}$ showed in an elegant study, that mammalian TRPV4 was able to rescue the retractive behavior of Caenorhabditis elegans to mechanical and osmotic stimuli in animals with mutations in TRP channel ortholog (OSM-9)$^{164}$. These results where surprising as they indicate mechanical stimulation of TRPV4, which is not seen in mammalian cells$^{130,131}$. This difference is so far not understood and more data are required. However, the presence of separate stretch activating proteins initiating signalling have been proposed$^{165}$.

**TRPV4 in Physiology and Pathophysiology:**
Despite the above-mentioned lack of obvious phenotype in TRPV4 KO mice, the role of TRPV4 in physiology is slowly being elucidated, and TRPV4 dysregulation is continuously being suggested to be involved in new pathophysilogies$^{134}$.

Of course misregulated systemic osmotic pressure is by no means a trivial issue. Patients with severe brain injuries such as traumatic brain injury, hemorrhagic stroke, ischemic stroke and subarachnoid hemorrhage frequently have abnormal ADH secretion$^{166-169}$, and disorders in water/salt balance contribute to high morbidity and mortality of these patients$^{169,170}$. Further, hypo-osmotic blood plasma are seen in numerous circumstances including after vomiting, diarrhea, acute or chronic renal dysfunction, and are a serious side-effect of numerous commonly used drugs – especially among elderly people.

TRPV4 controls bladder voiding, as has been shown in a series of recent studies in mice$^{150,171-174}$. The activation in the bladder is related to bladder pressure, hence, mechanical stretch. More studies are required to understand this activation but it does constitute an interesting parallel to the above mentioned
data in *C. elegans*. It has been shown that TRPV4 is functionally expressed in both cystic fibrosis (CF) and non-CF tracheal epithelial cell lines but is insensitive to hypotonic stimuli in CF cells\(^ {175}\). This could mean TRPV4 has an important role in tracheal fluid secretion and could pose a pharmacological target for CF patients. Finally, in mice, TRPV4 has been shown to mediate acute lung injury by disrupting the alveolar septal barrier\(^ {176}\). This could implicate a role for TRPV4 in lung injuries but more studies are needed. The only recognized human diseases related to TRPV4 are in skeletal growth. A number of gain-of-function mutations were recently identified in three different skeletal dysplasias\(^ {135, 177}\), resulting in severe pathological phenotypes.

**Structure/Function Relationship between TRPV4 and its Chemical Agonist.**

**A Few Notes about TRPV4 Antagonists:**
Unfortunately the list of TRPV4 antagonist is still short. TRPV4 is inhibited by the classic TRP channel inhibitor Ruthenium red (RR) Gd\(^ {3+}\), LA\(^ {3+}\) and the high affinity compounds RN-1734 and RN-9893 (see \(^ {132}\)). None of these antagonists are specific, something which are continuously inhibiting research into the physiological role of TRPV4.

**TRPV4 agonists and their interaction with the channel**
Activation of TRPV4 is, as already discussed, promiscuous and is activated by hyposmolarity, heat and a number of pharmacological activators. Following hyposmotic stimulation, the channel is activated by intracellular metabolites of arachidonic acid more specifically 5’,6’-EET and 8’,9’-EET\(^ {160, 178}\). Activation by hyposmolarity, AA and 5’,6’-EAT was shown to be affected by Y591A and R594A (Fig. Fig. 2A) mutation but as these mutations also affected the activation by heat and TRPV4 agonist 4α-phorbol-12,13-didecanoate (4α-PDD) this was associated with channel gating rather than 5’,6’-EAT interaction\(^ {149}\). This conclusion is based on the findings that heat and 4α-PDD activation works in separate ways to osmotic stimuli\(^ {178}\).

A number of chemical compounds have been identified to activate TRPV4 currents which include the bisandrographolide A (BAA) from the Indian medical plant *Andrographys paniculata*\(^ {147}\), a recently published chemical drug from GlaxoSmithKline (GSK1016790A) and a number of 4α-phorbol esters (Fig. 2B). The compound best described with respect to TRPV4 modulation is the phorbol ester derivative 4α-PDD\(^ {148}\), the identification of which constituted a major breakthrough for the investigation of TRPV4 currents. A binding pocket for chemical agonists has been suggested in transmembrane domains (TM) 3 and 4 (Fig. 2A). This is functionally very interesting as TRPV1 is agonized by residues in the same area (see \(^ {179}\)). Indeed 4α-PDD interacts with Y556 in TM3\(^ {178}\), corresponding to capsaicin binding tyrosine 511 in TRPV1\(^ {180}\). Although 4α-PDD doesn’t seem to bind the adjacent serine in TRPV4 as seen for capsaicin in TRPV1\(^ {178}\), we showed this residue to be very important for binding the shorter 4α-Phorbol-12,13-dihexanoate (4α-PDD)\(^ {151}\) (CHAPTER
VIII), identifying identical activation mechanisms for capsaicin and 4α-PDD in TRPV1 and TRPV4, respectively. Likewise in TM4 three residues: L584, W586 and M587 - corresponding to TRPV1 vanilloid binding residues\textsuperscript{181} - were identified as critical for 4α-PDD activation of TRPV4\textsuperscript{149}. Leu584 and Trp586 - but not Tyr556 or Ser557 - is also important for BAA activation of TRPV4\textsuperscript{149}. Hence, it is obvious from the differences between 4α-PDD, 4α-PDH and BAA that the TM3 YS motif is not essential for activation of TRPV4 by chemical agents, albeit a correlation between affinity and YS binding has been observed (EC\textsubscript{50}, 4α-PDD = 0.37 µM\textsuperscript{148}; EC\textsubscript{50}, 4α-PDH = 0.07 µM\textsuperscript{151} (CHAPTER VIII); EC\textsubscript{50}, BAA = 0.79 µM\textsuperscript{147}).

The most high affinity TRPV4 agonist described so far is the N-((1S)-1-[[4-((2S)-2-[(2,4-dichlorophenyl)sulfonyl]amino)-3-hydroxypropanoyl]-1-piperazinyl]carbonyl]-3-methylbutyl)-1-benzo thiophene-2-carboxamide.
(GSK1016790A) from GlaxoSmithKline with an EC\textsubscript{50} value between 2.1 and 18 nM\textsuperscript{150}. No structural information about the binding of GSK1016790A to TRPV4 is available but as GSK1016790A also have relatively high affinity for TRPV1 (EC\textsubscript{50}= 50 nM)\textsuperscript{182} it is feasible to speculate GSK1016790A interacts with the above discussed conserved residues in TM 3 and 4.

No recognized structural relationship exists between chemical TRPV4 agonist, but it is very clear that they share a preference for aromatic structures (Fig. 2B). In line with this it is clear from a number of studies that the 4α-phorbol head group is the determining factor for 4α-phorbol esters activity, while the side chains merely serve a positioning role for the head group\textsuperscript{149,151} (CHAPTER VIII).

**Functional perspectives for TRPV4 gating**
The structural correlation of TRPV1 and TRPV4 is indeed very interesting as capsaicin - despite common depiction as an agonist - is merely a TRPV1 sensitizer, changing the voltage dependent profile in relation to temperature activation of TRPV1\textsuperscript{183}. As TRPV4 is a voltage independent channel\textsuperscript{130, 131}, how can this similarity be explained? Well first of all the assumption of voltage independence could be wrong, given that only a voltage span between -100 mV and +100 mV has been explored and only after hypotonicity, which only activates relative small TRPV4 currents\textsuperscript{130} and is known to activate TRPV4 via separate pathways compared to heat and 4α-PDD\textsuperscript{178}. TRP channel gating charges are, similar to what is known for the voltage dependent K\textsuperscript{+} channels, found in TM3 and 4\textsuperscript{184}. Although charged residues are found in TM4 of TRPV4, none of these correspond to the gating charge residues of either TRPM8 or hKv1.2 channels. However, it must be emphasized that – as mentioned above – Y591 and R594 in TM4 (Fig. 2A) both have been suggested to support gating of TRPV4\textsuperscript{149} and these residues could potentially serve as gating charges. To finally exclude the possibility of TRPV4 as a voltage activated channel, further studies will have to investigate the voltage dependent characteristics at more extreme membrane potentials (+/- 200 mV).

Another possibility of course is that identified chemical agonists activate TRPV4 directly, possibly via additional binding sites. In this respect, it is notable that we found an additional 4α-PDD binding site in TM5 (Y621)\textsuperscript{185} (CHAPTER IX) of TRPV4 (Fig. 2A). This extra binding site could explain non-voltage dependent gating of TRPV4, as the pore region is situated between TMS and 6\textsuperscript{186}, and the gating therefore must change the 3D architecture of this part of the channel for channel gating. Binding TMS indeed could induce such a structural change. As aromatic residues stack in the TMS α-helix with Y621 in the very middle\textsuperscript{185} (CHAPTER IX), binding this residue could interfere with the stabilizing effect of π-π interactions between aromatic residues\textsuperscript{187,188} making a more flexible TMS structure, hence increasing the opening probability. As no [Ca\textsuperscript{2+}]\textsuperscript{2} response was observed even at very high 4α-PDD concentrations in TRPV4-Y621L transfected cells, Y621 seems essential for 4α-PDD mediated TRPV4 activation. This strongly advocates for a direct compound gating mechanism via Y621.
Conclusion
In conclusion, it is clear that TM3 and 4 are very interesting in respect to develop new synthetic drugs against TRPV4 dysfunction related diseases. However, it must also be recognized that the functional understanding of these domains is still in not satisfactory. Further work will have to address the questions whether chemical agonists activate TRPV4 by modulating the voltage dependent characteristics of TRPV4 as seen for agonist mediated TRPV1 activation or whether TRPV4 is directly gated by chemical compounds potentially via interfering with TM5 α-helix stability.
REFERENCES:


CHAPTER IV: CELL CYCLE-DEPENDENT ACTIVITY OF THE VOLUME- AND CA\textsuperscript{2+}-ACTIVATED ANION CURRENTS IN EHRlich LETTRE ASCITES CELLS

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Cell Cycle-Dependent Activity of the Volume- and Ca^{2+}-Activated Anion Currents in Ehrlich Lettre Ascites Cells

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Recent evidence implicates the volume-regulated anion current (VRAC) and other anion currents in control or modulation of cell cycle progression; however, the precise involvement of anion channels in this process is unclear. Here, Cl^{-} currents in Ehrlich Lettre Ascites (ELA) cells were monitored during cell cycle progression, under three conditions: (i) after osmotic swelling (i.e., VRAC), (ii) after an increase in the free intracellular Ca^{2+} concentration (i.e., the Ca^{2+}-activated Cl^{-} current, CaCC), and (iii) under steady-state isotonic conditions. The maximal swelling-activated VRAC current decreased in G1 and increased in early S phase, compared to that in G0. The isotonic steady-state current, which seems to be predominantly VRAC, also decreased in G1, and increased again in early S phase, to a level similar to that in G0. In contrast, the maximal CaCC current (500 nM free Ca^{2+} in the pipette), was unaltered from G0 to G1, but decreased in early S phase. A novel high-affinity anion channel inhibitor, the acidic di-aryl-urea NS3728, which inhibited both VRAC and CaCC, attenuated ELA cell growth, suggesting a possible mechanistic link between cell cycle progression and cell cycle-dependent changes in the capacity for conductive Cl^{-} transport. It is suggested that in ELA cells, entrance into the S phase requires an increase in VRAC activity and/or an increased potential for regulatory volume decrease (RVD), and at the same time a decrease in CaCC magnitude.


A volume-regulated anion current (VRAC) with a number of characteristic biophysical and pharmacological properties (for reviews, see Nilius et al., 1997a; Okada, 1997) is well described as a major player in the regulatory volume decrease (RVD) process after cell swelling (Hoffmann and Dunham, 1995; Stutzin and Hoffmann, 2006). In addition, VRAC has been assigned important roles in the control of cell cycle progression (Nilius, 2001). In agreement with this, broad-spectrum Cl^{-} channel inhibitors have been found to reduce the rate of proliferation in various cell types (Voets et al., 1995; Shen et al., 2000; Wondergem et al., 2001; Xiao et al., 2002; Jiang et al., 2004; Rouzaire-Dubois et al., 2004), and to arrest SiHa human cervical cancer cells in G0/G1 phase (Shen et al., 2000).

The possible relation between maximal VRAC current and cell cycle progression is controversial. In nasopharyngeal carcinoma CNE-2Z cells, VRAC activity was high in G1 phase, down-regulated in S phase, and again increased in M phase (Chen et al., 2002). Correspondingly, a rapid RVD response and a very small cell volume were found in the G1 phase in CNE-2Z cells (Wang et al., 2002). A similar pattern is seen in glioma cells (Ullrich and Sontheimer, 1997) although the volume sensitive anion current described in these cells is probably different from VRAC, since it exhibits time-dependent activation, in contrast to the typical time-dependent inactivation of VRAC (Ullrich and Sontheimer, 1996; Ullrich, 1999). In contrast, in SiHa human cervical cancer cells, VRAC was reported to be down-regulated in G0/G1 and increased in S (Shen et al., 2000). Finally, in mouse fibroblasts, no differences were seen between VRAC magnitude in S phase compared to G0/G1 (Doroshenko et al., 2001; for an overview of studies of Cl^{-} channels in cell cycle progression, see Nilius, 2001). Notably, such studies have addressed the magnitude of the Cl^{-} currents activated by osmotic cell swelling as induced by a reduction in extracellular osmolarity, while a physiologically more relevant parameter may be the basal isotonic Cl^{-} conductance. Moreover, evaluation of the relevance of changes in Cl^{-} conductance for cell cycle progression has been complicated by a lack of high-affinity, specific Cl^{-} channel inhibitors (see e.g., Nilius et al., 1997a; Jentsch et al., 2002; de Tassigny et al., 2003).

In addition to VRAC, other Cl^{-} channels have been reported to exhibit cell cycle dependence. Thus, a voltage-gated Cl^{-} current was linked to cell cycle progression in ascidian embryonic cells (Villaz et al., 1995) and in human glioma cells (Ullrich and Sontheimer, 1996, 1997; Ullrich, 1999). Moreover, the activity of the C. elegans CLH-3 channel, a CIC-2 ortholog, has been proposed to be correlated with meiotic maturation of oocytes (Rutledge et al., 2002). Since changes in the free intracellular Ca^{2+} concentration ([Ca^{2+}]) are known to play a major role in cell cycle progression (Munaron et al., 2004; Schreiber, 2005), it seems likely that changes in Ca^{2+}-activated Cl^{-} currents (CaCC) could also play a role, however, this has to our knowledge not yet been directly investigated in any cell type.
The aim of this study was to contribute to the understanding of the relationship between Cl− currents and cell cycle progression by investigating: (i) the pattern of swelling-activated, Ca2+-activated, and basal isotonic Cl− currents, respectively, during the cell cycle, and (ii) the effect of a novel high-affinity Cl− channel inhibitor on swelling- and Ca2+-activated Cl− currents, on RVD, and on cell growth.

The model systems used were Ehrlich Lettre Ascites (ELA) cells, in which we have recently characterized VRAC (Klausen et al., 2006). These cells are an adherent subtype of Ehrlich Ascites Tumor (EAT) cells, in which we have previously characterized VRAC and CaCC in detail (Hoffmann, 1978; Hoffmann et al., 1986; Christensen and Hoffmann, 1992; Pedersen et al., 1998). As a Cl− channel inhibitor, we employed a new compound of the family acidic di-aryl-urea NS3728 which has been shown to inhibit VRAC in HEK293 cells as well as the Cl− conductance in human erythrocytes with affinities in the nanomolar range (Helix et al., 2003).

We demonstrate that in ELA cells, the maximal swelling-activated Cl− current varies during the cell cycle, decreasing from G0 to G1, and increasing from G1 to S, such that the current in S is also significantly higher than that in G0. In contrast, the maximal CaCC is unaltered from G0 to G1, but decreases significantly from G1 to S phase. The isotonic steady-state Cl− current, which seems to be predominantly VRAC, also varies with cell cycle progression, decreased in G1 and increased again in early S phase to a level similar to that in G0. The anion channel inhibitor NS3728, which inhibits VRAC, CaCC, and RVD, attenuates ELA cell growth, suggesting a possible mechanistic link between cell cycle progression and cell cycle-dependent changes in the capacity for conductive Cl− transport and/or volume regulation.

MATERIALS AND METHODS

Reagents and stock solutions

All chemicals were from Sigma (St. Louis, MO), Merck (Darmstadt, Germany) or J.T. Baker (Deventer, Holland) unless otherwise stated. 4, 4′-Diisothiocyanato-2, 2′-stilbenedisulfonic acid (DIDS) was dissolved at 50 mM in DMSO, and niflumic acid was dissolved at 5 mM in DMSO. NS3728 was a kind gift from Neurosearch A/S, and was dissolved in DMSO (resulting in a final concentration of DMSO of 0.1% in experiments, a concentration shown to have no influence on VRAC or CaCC). 3H-Thymidin (37 MBq, 1 mCi/ml) was obtained from New England Nuclear (Wellesly, MA) and diluted in the normal growth media. Ultima Gold Scintillation fluid was from Packard, Meriden, CT.

Cell suspensions and media

ELA cells were grown at 37°C and 5% CO2 in RPMI-1640 medium (Sigma-Aldrich, St. Louis) containing 10% fetal calf serum and antibiotics (100 U/ml penicillin, 100 μg/ml streptomycin) and incubated at 37°C, 5% CO2 in a humidified 5% CO2 incubator. Cells were passaged every 3–4 days by gentle trypsinization with 0.05% trypsin-EDTA supplemented with 0.5% fetal calf serum and 1% penicillin/streptomycin (Invitrogen, Carlsbad, CA).

Synchronization of cell cultures

ELA cells (verified at non-confluence stage) were temporarily arrested in the defined G0 stage of the cell cycle by 24 h of serum starvation. [3H]thymidine incorporation was subsequently measured following addition of 10% fetal calf serum. As seen, S phase was reached at approximately 15 h after release from starvation, while after 5 h, the majority of the cells were estimated to be in G1 (Meníli et al., 2004). Data shown are representative of two independent experiments.

Electrophysiological experiments

Solutions

CaCC. In order to suppress inward cation currents, permeable cations were substituted with the weakly permeable cations NMDG and Cs+. Composition of the bath solution in these studies was (in mM): 1.5 CaCl2; 1 MgCl2; 10 NaCl; 140 NMDG-Cl; 10 HEPES; 10 glucose. The pH of the solution was adjusted to 7.4 using HCl. The Ca2+-buffered pipette solution contained (in mM): 100 Cs-aspartate; 40 CsCl; 1 MgCl2; 5 EGTA; 10 HEPES; 4 Na2-ATP, and the amount of CaCl2 required to adjust the free Ca2+ concentration to 50 nM (1.191), 100 nM (1.925), 250 nM (3.055), and 500 nM (3.801), respectively (calculated using the program Cabuf, G. Droogmans). For nominally Ca2+-free conditions, CaCl2 was omitted from the pipette solution, which otherwise had the same composition as above. Pipette solution pH was adjusted to 7.2 using CsOH. The osmolarity of the bath solution was 315 mOsm and the osmolarity of the pipette solution was 290 mOsm, leaving the cells mildly shrunk to avoid contamination of the measured anion current by VRAC.

VRAC. Before hypotonic exposure, cells were perfused with an isotonic solution (300 mOsm) containing (in mM): 90 NaCl; 1 MgCl2; 1 CaCl2; 10 HEPES; 110 mannitol. The pH was adjusted to 7.4 using TRIS. The hypotonic solution (190 mOsm) was obtained by omitting mannitol from the isotonic solution.
The intracellular pipette solution (295 mOsm) contained (in mM): 90 CsCl; 2 MgCl₂; 10 EGTA; 10 HEPES; 1.5 Na₂-ATP; 0.1 Na₂-GTP; 80 mannitol, pH adjusted to pH 7.4 using TRIS. It was previously established that VRAC in EAT cells does not require a permissive (Ca²⁺) (Pedersen et al., 1998).

**Experimental procedures.** Membrane currents were recorded from single ELA cells by whole-cell patch-clamp technique. All electrophysiological experiments were performed at room temperature. The cells were mounted in a perfusion chamber on an inverted microscope, and solutions were changed using a gravity-feed and pump suction mechanism. Cells were continuously perfused at a rate of 2 ml/min, with a complete exchange of the bath solution every 0.5 min. Patch pipettes were pulled from borosilicate glass capillaries of 1.7 mm OD. Pipettes had resistances in the range of 4.5–6.5 MΩ when filled with intracellular solution (see above) and using an Ag/AgCl wire as the reference electrode. Pipette offsets, capacitive transients, and series resistances were compensated on the patch-clamp amplifier (EPC7, List Electronic, Darmstadt, Germany or Axopatch 200B, Axon Instruments, Foster City, CA). Cells with $R_{	ext{p}}$ < 11 MΩ in the whole-cell configuration were discarded. Currents were digitized using an AD-converter at 500 Hz and filtered with a built-in four-pole Bessel filter at 2 or 3 kHz. Data acquisition and analysis were done using CED and pClamp7 software.

**Electronic cell sizing**

Cell volume was measured by electronic cell sizing using a Coulter Multiziser II (Coulter, Luton, UK) with a tube orifice of 100 μm. The instrument was calibrated using 15 μm Coulter CC size standards L1.5 (Coulter Beckman, Luton, UK) latex beads. ELA cells were synchronized by serum starvation as above, and at the indicated time points after re-addition of serum, were washed in PBS, trypsinized and suspended in normal growth medium to inhibit trypsin activity. Cells were diluted at 25,000 cells/ml in the iso- and hypotonic solutions also used in the AD-convertor at 500 Hz and filtered. Cell size was followed for 3 min. Mean cell volume was calculated as the median of the cell volume distribution curves at the relevant time points.

**Estimation of growth rate (increase in cell number)**

ELA cells were seeded in triplicate in 25 cm² T-flasks at 6.0 × 10⁴ cells per flask in 4 ml RPMI-1640 medium with 10% FBS and 1% penicillin/streptomycin. NS3728 was included in the media at different concentrations and 1/1000 DMSO was included in control experiments. To maintain a constant concentration of active inhibitor throughout the experiment, media were changed every 24 h. On the third day after seeding (confluence <80%) cells were harvested by standard trypsination and counted on a haemocytometer. To exclude dead cells from the count, 0.04% trypan blue was added before counting and trypan blue-stained cells were not included in the results. Very few trypan blue-stained cells were seen.

**Data treatment and statistical analysis**

Data are presented as mean ± SEM with n denoting the number of cells tested. For comparison of data sets, Student’s t-tests for paired and unpaired data were employed as appropriate.

In whole-cell patch-clamp experiments, pipette potentials were corrected for the liquid junction potentials using the pClamp7 (Axon) junction potential calculator function. Junction potentials were calculated at 4.9 and 19.1 mV, respectively, in solutions for measuring VRAC and CaCC.

In all experiments, % inhibition was calculated as:

\[ \% \text{ inhibition} = \frac{P_{\text{control}} - P_x}{P_{\text{control}}} \times 100 \]  

(1)

where $P_{\text{control}}$ and $P_x$ represent values of the parameter under investigation with or without the given inhibitor in the extracellular medium. From equilibrium concentration-response experiments, the potency of inhibition was calculated by fitting to a Hill equation. Fitting procedures were performed in SigmaPlot 2001 v. 7.0 (SPSS, San Jose, CA).

Since CaCC exhibits current rundown (see Fig. 3) steady-state equilibrium was difficult to obtain. Two strategies were employed to circumvent this problem. First, the rate of rundown (a) was estimated for every individual cell by linear fit in the initial minute after $I_{\text{max}}$ and currents measured in the presence of NS3728 were compensated for rundown using the equation $I_{\text{corr}} = I_t + a - t$, where $t$ is the time from $I_{\text{max}}$. Linear descriptions of the rundown should apply in intervals shorter than 5 min (see Fig. 3). $I_{\text{corr}}$ was then used to determine the steady-state inhibition. Second, the inhibitory potency of NS3728 on CaCC was further evaluated via non-equilibrium studies at a single drug concentration using Michaelis–Menten kinetics. This was deemed necessary since, in principle, inhibitor potency might be underestimated at low concentrations of NS3728 and in the late phase of the exponential inhibition curve at higher concentrations, due to small fluctuations in rundown rate. The use of Michaelis–Menten kinetics is justified since the Hill constant for NS3728-mediated inhibition of this current was not different from 1. To minimize confounding effects of rundown, these analyses were performed only at 10 μM NS3728, where inhibition occurs very rapidly, and current rundown, therefore, is not expected to change within the evaluated interval $V$. The non-equilibrium version of the Michaelis–Menten equation (see Jenkinson, 1996):

\[ I_t = I_0 \left( 1 - \frac{C}{C + IC_{50}} \right) \left( 1 - \exp \left( \frac{(IC_{50} + k_{\text{on}})}{C} \right) \right) \]

(2)

where I$_t$ is the measured current at the time (t), $IC_{50}$ and $k_{\text{off}}$ are the on and off rates, was rewritten as:

\[ -\left( \frac{I_t}{I_0} - 1 \right) = \frac{C}{C + IC_{50}} \left( 1 - \exp \left( \frac{(IC_{50} + k_{\text{on}})}{C} \right) \right) \]

(3)

which is a simple exponential function, from which the values of $(IC_{50} - 1), (C/(C+IC_{50})), \text{ and } (C \cdot k_{\text{on}} \cdot k_{\text{off}})$ were calculated by fitting current traces to an exponential decay function. From these values, the $IC_{50}$ value could be calculated.

**RESULTS**

**Cell cycle-dependent changes in maximal VRAC activity in ELA cells**

ELA cells were synchronized by 24 h of serum starvation, temporarily arresting them in the G0 stage of the cell cycle. Cell cycle stages were confirmed by measuring [³H]thymidine incorporation after release from serum starvation (Fig. 1), and by evaluation by Western blotting of cyclin D protein levels, which reached their maximal level at time 5 h after release from serum starvation ($n = 4$, data not shown), consistent with observations in other cell types (Memili et al., 2004). Based on these experiments, the 5 and 15 h time points after release were designated G1 and S phase, respectively.

We have recently described the biophysical and pharmacological properties of VRAC in ELA cells (Klausing et al., 2006), hence, the current was not further characterized here. The maximal VRAC current density measured during a 5–6 min exposure to hypotonic solution (190 mOsm) was taken as an indication of the level of VRAC expression (see Discussion). As seen in Figure 2, the current density was modest in G0-arrested (serum-starved for 24 h) cells (52.53 ± 7.31 pA/pF at +150 mV), decreased significantly ($P < 0.05$) in G1 (27.32 ± 7.39 pA/pF at +150 mV) and nearly doubled ($P < 0.01$) relative to the G0 phase in S (96.15 ± 12.43 pA/pF at +150 mV). Importantly, cell size, estimated as the cellular capacitance (Fig. 2, insert), or by electronic cell sizing did not differ detectably between the three cell cycle phases studied (isotonic cell size as measured by
Cell cycle-dependent changes in maximal CaCC activity in ELA cells

ELA cells were synchronized and arrested in G0 as described above. The maximal CaCC current density measured at 500 nM free Ca\(^{2+}\) in the pipette solution was used as an estimate of the level of CaCC expression. As seen (Fig. 4) the CaCC current density was significantly decreased in the S phase (50.96 ± 5.15 pA/pF at +150 mV) when compared to that in G0 (74.30 ± 7.92 pA/pF at +150 mV, \(P < 0.05\)) and G1 (70.03 ± 9.06 pA/pF at +150 mV, \(P < 0.05\)). The \(V_{\text{rest}}\) for the currents measured in G0 and S phase were not significantly different from the calculated Cl\(^{-}\) equilibrium potential of ~29 mV, while \(V_{\text{rest}}\) for the current measured in G1 was slightly but significantly more positive.

Cell cycle-dependent changes in the isotonic Cl\(^{-}\) current

Since cell volume was not detectably different between G0, G1, and S phase, we next wished to evaluate whether changes in anion currents also occur in non-swollen cells. We recently found that the isotonic current in ELA cells appears to be predominantly VRAC (Klausen et al., 2006), however, in order to further establish this important point, a series of additional measurements were performed. As previously reported (Klausen et al., 2006), the isotonic current measured in ELA cells under strongly \([\text{Ca}^{2+}]_i\) buffered conditions (zero added \(\text{Ca}^{2+}\) and 5 mM EGTA in the pipette solution, i.e., no contribution from CaCC) was outwardly rectifying, the \(V_{\text{rest}}\) was 5.46 ± 0.68, close to the Cl\(^{-}\) equilibrium potential of 0 mV under these conditions, and the current exhibited time-dependent inactivation.

An increase in \([\text{Ca}^{2+}]_i\) activates a strongly outwardly rectifying Cl\(^{-}\) current, CaCC in ELA cells

Since changes in \([\text{Ca}^{2+}]_i\) have been shown to play a major role in cell cycle progression (see Munaron et al., 2004; Schreiber, 2005), we wished to determine whether the magnitude of CaCCs might also exhibit cell cycle dependence. CaCCs have not previously been investi-
at potentials more depolarized than 40 mV (Fig. 5A). Furthermore, the isotonic current was inhibited by DIDS in a voltage-dependent manner (Fig. 5E). Finally, the current was inhibited by cell shrinkage, indicating that it is a volume sensitive current, the set point of which is somewhat below the steady-state isotonic volume. Taken together, these characteristics support the notion that the isotonic current measured under these conditions is predominantly VRAC. Based on our previous findings, however, a minor contribution from a Na⁺-dependent current to the isotonic current observed under these conditions cannot be excluded (Klausen et al., 2006).

We next evaluated the magnitude of the isotonic current in G0, G1, and S phase. Similar to the maximal swelling-activated VRAC current, the isotonic Cl⁻ current exhibited a significant decrease in G1 compared to G0, and increased again in the S phase (Fig. 6).
variance with the maximal swelling-activated VRAC, the isotonic current was, however, not increased in S phase compared to G0 phase.

**Effect of the anion channel inhibitor NS3728 on VRAC and the isotonic current**

In order to establish a mechanistic link between the observed changes in Cl\(^{-}\)/C\(_{0}\) conductance during cell cycle progression, high-affinity, specific inhibitors of the currents involved, VRAC and CaCC, were required. The acidic di-aryl-urea NS3728 has been found to potently block swelling-activated anion currents in HEK293 cells with an IC\(_{50}\) value around 400 nM (Helix et al., 2003). In agreement with these findings, NS3728, when present in the bath solution, inhibited VRAC in ELA cells in a concentration-dependent manner, with estimated IC\(_{50}\) values of ~400 and ~300 nM at \(-55\) and +40 mV, respectively, and with a Hill coefficient of around 2 at both potentials (Fig. 7). Inhibition of VRAC by NS3728 was reversible upon removal of the drug from the bath solution (not shown). Inclusion of NS3728 (10 \(\mu\)M) in the pipette solution had no effect on either the activation rate or the maximal VRAC current in EAT cells (not shown), supporting the view that this compound acts as a channel blocker rather than by affecting intracellular signaling cascades.

Measurements of RVD rates in ELA cells are confounded by the fact that these cells must be trypsinized for electronic cell sizing. The effect of NS3728 on RVD was therefore evaluated in the parental suspension cell line, EAT cells. As expected given the requirement for VRAC activation in RVD in the great majority of cells studied, including EAT cells (see Introduction), NS3728 inhibited RVD in EAT cells in a dose-dependent manner, with an estimated IC\(_{50}\) value of 588 nM, very close to the estimated IC\(_{50}\) value of about 540 nM for NS3728-mediated inhibition of VRAC in...
these cells (not shown). Consistent with this reflecting
inhibition of VRAC, osmotic cell swelling was unaffected
by NS3728, which also did not inhibit the swelling-
activated K⁺ current (I_{Kvol}) in ELA cells (C. Hougaard
and E.K. Hoffmann, unpublished data).

NS3728 also inhibited the isotonic current in a dose-
dependent manner. Thus, inhibition by 0.5 μM NS3728
was 30 ± 2.1% at −55 mV and 47 ± 6% at +40 mV, and
inhibition by 1 μM NS3728 was 37 ± 6% at −55 mV and
61 ± 3% at +40 mV (n = 6 and 8 for 0.5 and 1 μM,
respectively). This is slightly less than the inhibitory
effect calculated for VRAC, possibly reflecting the
contribution of a Na⁺-dependent component to the
isotonic, but not to the hypotonic current (Klausen
et al., 2006).

**Effect of NS3728 on CaCC**

As seen in Figure 8, perfusion of ELA cells with a bath
solution containing NS3728 significantly inhibited
CaCC (here activated by 500 nM [Ca²⁺]). The steady-
state inhibition of CaCC by NS3728 was calculated after
correction for the rundown exhibited by this current (see
Fig. 3), as described in Materials and Methods. NS3728
inhibited CaCC in an apparently voltage-dependent
manner, with IC₅₀ values of 2.1 and 0.7 μM at −95 mV
and +50 mV, respectively (Fig. 8C). However, small
fluctuations in rundown rate might result in under-
estimation of inhibitor potency (see Materials and
Methods). We therefore also analyzed the inhibitory
effect of 10 μM NS3728 by non-equilibrium Michaelis–
Menten kinetics, as detailed in Materials and Methods.
Non-equilibrium analysis resulted in an IC₅₀ value of
1.05 μM at +50 mV, in good agreement with the
value obtained assuming equilibrium kinetics, while at
−95 mV, the IC₅₀ value obtained using non-equilibrium
kinetics was 1.49 μM, substantially smaller than that of
2.1 μM obtained assuming steady state. Underestima-
tion of inhibitor potency by non-equilibrium kinetics at
negative potentials could in part reflect the slight
contamination from a Ca²⁺-insensitive, potentially
NS3728-insensitive current (Fig. 3D).

**Effect of NS3728 on proliferation of ELA cells**

Since we found here, for the first time, that cell cycle
progression is associated with differential modulation
not only of VRAC, but also of CaCC, it was of interest
to evaluate the effect of NS3728, an inhibitor of both
currents, on cell growth in ELA cells. The doubling time
for ELA cells is approximately 22 h, and cell growth was
estimated from the increase in cell number after 3 days
of non-confluent growth (Fig. 9). It should be noted,
however, that this method does not distinguish between
inhibitory effects on cell proliferation and stimulatory
effects on cell death (see Discussion). As seen, in the
presence of NS3728 in the growth medium, the increase
in cell number over time was attenuated with an IC₅₀
value of 550 nM, that is, highly similar to that found for
inhibition of VRAC. It should be noted that the growth
experiments are carried out at 37°C whereas all other
data are obtained at room temperature. Since VRAC
sensitivity to NS3728 does not appear to be temperature
dependent (n = 2, data not shown), we do not expect this
to affect the sensitivity to the inhibitor. The inhibition
curve saturated at about 40%, indicating that the
capacity for conductive Cl⁻ efflux was rate limiting,
yet not essential, for cell growth.

**DISCUSSION**

In this study, we investigated the cell cycle-dependent
changes in maximal swelling- and Ca²⁺-activated Cl⁻
currents as well as in the basal isotonic Cl⁻ conductance
in ELA cells. Moreover, to assess the possible import-
tance of these currents for cell cycle progression, we
evaluated the effects of a high-affinity chloride channel
inhibitor on cell growth.

**Maximal VRAC currents are modulated
during cell cycle progression in ELA cells**

Previous studies investigating VRAC currents during
the cell cycle have not attempted to differentiate
between G0 and G1 (Shen et al., 2000; Doroshenko
et al., 2001; Chen et al., 2002). Such a differentiation
was made in a study of the cell cycle-dependent expression
of a glioma-specific chloride current (Ullrich and Sonthei-
mer, 1997), but as mentioned in the Introduction, this
current seems to be different from VRAC. Here, we
found that the maximal VRAC current in ELA cells was
high in G0, decreased in G1, and increased again in S
phase, to a level higher than that in G0. This implies that
the expression of VRAC was down-regulated in G1 and
up-regulated in S, compared to G0. In accordance with
this, an increase in VRAC current in S phase compared to
that in G0/G1 was previously reported in SiHa human
cervical cancer cells (Shen et al., 2000). It may be noted
that cyclin D was detectable in serum-starved ELA cells

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and only increased gradually after release from starvation, suggesting that this procedure does not fully synchronize the cells. It is therefore to be expected that Cl⁻ current changes during cell cycle progression are in fact even more substantial than those observed here. A limitation which this study shares with all previous investigations is that the molecular identity of VRAC is still not known, and hence, it is not possible to distinguish between an increased amount of VRAC protein and post-translational regulation of a latent pool of channels by cell cycle-dependent signaling events.

With respect to post-translational regulation, it is noteworthy that the cytoskeleton undergoes dramatic rearrangements during cell cycle progression, and that cell cycle-dependent regulation of a glioma-specific Cl⁻ channel was tentatively linked to the cytoskeletal

**Fig. 7.** Effect of the anion channel inhibitor NS3728 on VRAC in ELA cells. A: Current density over time after exposure to increasing concentrations of NS3728. Currents were measured under hypotonic (190 mOsm) conditions using the fast ramp protocol described in Figure 3 at increasing concentrations of NS3728 in the bath solution as indicated; representative of four independent experiments. B: Representative I/V relationships obtained from the fast voltage ramps at the time points indicated by the numbers 1–5 in part A. C and D: Dose-response curves for the inhibitory effect of NS3728 at −55 and +40 mV, respectively. Currents were measured under hypotonic (190 mOsm) conditions using the fast ramp protocol, and inhibition was calculated by the Hill equation as indicated in Materials and Methods (n = 3–4 independent experiments at each concentration).
changes during cell cycle progression (Ullrich, 1999). A similar mechanism may be envisaged for VRAC, which in ELA cells (Klausen et al., 2006), and in many other cell types (see Pedersen et al., 2001) is modulated by F-actin reorganization. Other possible mechanisms include the Rho-Rho kinase pathway, which has been implicated in cell proliferation/cell cycle progression (Anderson et al., 1995; Aznar and Lacal, 2001), and which has been shown to be important for VRAC regulation in a variety of cell types, including ELA cells (Klausen et al., 2006).

The functional significance of the observed changes in maximal VRAC current during cell cycle progression is yet to be determined, however, it seems reasonable to suggest that it relates to differential requirements for cell volume regulation. Thus, VRAC down-regulation in G1 may be involved in the volume increase seen in some cells (yet apparently not in ELA cells) in association with the G1/S transition (Bussolati et al., 1996), while VRAC up-regulation and thus increased capacity for RVD might be functionally important in S phase (for a related viewpoint, see Shen et al., 2000). Notably, there is also evidence that K+ channels are regulated in a cell cycle-dependent manner, and K+ channel activity has been proposed to be particularly important during early G1 phase (see Wonderlin and Strobl, 1996; Kunzelmann, 2005). The effect of Cl-/Ca2+ channel activity must, therefore, be seen in the context of the concurrent K+ channel activity: if K+ channels are active, the predominant effect of Cl- channel activation is expected to be KCl loss and cell shrinkage, whereas if K+ channel activity is limiting, the predominant effect may be membrane depolarization. It is noteworthy in this regard that cell shrinkage has been shown to be required for [Ca2+]i oscillations, which in turn are required for cell proliferation (Ritter et al., 1993; Lang et al., 2005).

Cell cycle-dependent changes in maximal CaCC activity in ELA cells

The CaCC in ELA cells was found to exhibit biophysical and pharmacological properties similar to those which we have described for this current in EAT cells (Christensen and Hoffmann, 1992; Pedersen et al., 1998) and which have also been found in other cell types (Arreola et al., 1996; Sullivan et al., 1996; Nilius et al., 1997b; Qu et al., 2003; Boese et al., 2004). The molecular identity of the CaCC in these cells is not known. It has
been reported that mClCA, mCLCA2, and MCLCA3 are not expressed at the mRNA level in EAT cells and that CaCC in these cells is distinct from those carried by mCLCA (Papasotiriou et al., 2001). Other possible candidates are the bestrophins, which exhibit anion selectivity and DIDS sensitivity similar to that of CaCC but are only slightly outwardly rectifying (Fischmeister and Hartzell, 2005).

Consistent with the findings in EAT cells, CaCC was substantial even at near-physiological Ca\(^{2+}\) concentrations (~78 pA/pF at a holding potential of ~40 mV and a [Ca\(^{2+}\)]\(_i\) of 100 nM). In other words, only a slight increase in [Ca\(^{2+}\)]\(_i\) is required for activation, hence, CaCC is poised to play a major physiological role in these cells. Changes in [Ca\(^{2+}\)]\(_i\) are known to play a major role in cell cycle progression (Munaron et al., 2004c), and it seems likely that this could in part be secondary to cell cycle-dependent changes in CaCC. However, to our knowledge, this is the first study addressing the cell cycle dependence of CaCC. From the present studies we conclude that the maximal CaCC current in ELA cells is unaltered between G0 and G1, and significantly decreased from G1 to early S phase. The possible dependence of CaCC. From the present studies we conclude that the maximal CaCC current in ELA cells is unaltered between G0 and G1, and significantly decreased from G1 to early S phase. The possible dependence of CaCC.

It should be noted that the above experiments were done at zero [Ca\(^{2+}\)]\(_i\), to prevent CaCC activation. Since Ca\(^{2+}\) influx is critical for the G0/G1 transition (Wonderlin and Strobl, 1996), CaCC might well be activated at this point, however, the physiological relevance of this cannot be addressed by the whole-cell patch-clamp analyses applied in the present study, since conditions of unperturbed [Ca\(^{2+}\)]\(_i\) are required.

**The acidic di-aryl-urea NS3728 inhibits VRAC and CaCC in ELA cells with high affinity**

Tertiary amines, like tamoxifen, fluoxetine, and verapamil (for a review, see Nilius et al., 1997a; Droogmans et al., 1999; Maertens et al., 1999) are typically VRAC blockers but with quite a promiscuous pharmacological profile. The classical Cl\(^{-}\)-channel blockers like NPPB and DIDS, di-aromatic, 2-substituted acids linked by various chains, tend to be of rather low, and similar, affinity for VRAC and CACC while niflumic acid frequently has higher affinity for CaCC than for VRAC (de Tassigny et al., 2003). Specific high-affinity inhibitors of VRAC and CaCC are, however, lacking (de Tassigny et al., 2003), and since the molecular identity of VRAC is also unknown, this precludes the establishment of unequivocal mechanistic links between this current and cell cycle progression. We therefore tested a new high-affinity VRAC inhibitor, the acidic di-aryl-urea compound NS3728 (Helix et al., 2003).

Here, we found NS3728 to be a voltage-independent, reversible blocker of VRAC with the highest affinity (IC\(_{50}\) = 410 nM at negative membrane potentials) of any VRAC inhibitor yet tested in EAT and ELA cells. NS3728 was also found to inhibit CaCC in ELA cells, in a voltage-dependent manner and with an estimated IC\(_{50}\) of around 1.5 \(\mu\)M at negative potentials when estimated from non-equilibrium kinetics. With respect to the specificity of NS3728 for VRAC and CaCC, it may be noted that NS3728: (i) does not affect \(I_{\text{Kvra}}\) (C. Hougaard and E.K. Hoffmann, unpublished data); (ii) acts via a site accessible from the extracellular space, in accordance with the view that the compound acts as a channel blocker; (iii) is charged at neutral pH, and hence likely to exhibit limited membrane permeability. Neither intracellular effects nor, for example, plasma membrane receptor-mediated effects of NS3728 on cellular signaling pathways can of course not be strictly excluded. However, it may be noted that 10 \(\mu\)M NS3623, which is structurally related to NS3728, exhibited no activity in 60 different receptor-binding assays (Helix et al., 2003).

**Effect of NS3728 on proliferation**

Broad-spectrum Cl\(^{-}\) channel inhibitors have been shown to attenuate cell proliferation and arrest a variety of cell types in the G0/G1 phases (Voets et al., 1995; Shen et al., 2000; Wondergem et al., 2001; Chen et al., 2002; Xiao et al., 2002; Jiang et al., 2004; Rouzaire-Dubois et al., 2004). Although VRAC was proposed to be the...
channel of interest in most of these studies, the poor selectivity and low affinity of the Cl-channel antagonists used has precluded the exclusion of contributions from other Cl-channel types (roles of which in cell cycle progression have also been suggested; Villaz et al., 1995; Schlachter et al., 1996; Ulrich and Sontheimer, 1997; Ulrich, 1999), and/or other non-specific effects.

NS3728 dose-dependently inhibited the growth of ELA cells populations with an IC50 value of 500 nM, that is, very similar to that for inhibition of VRAC and slightly lower than that for inhibition of CaCC (1.5 μM) in these cells. IC50 values obtained in patch-clamp and growth experiments are not directly comparable, however, the close accordance of these values supports the view that VRAC and/or CaCC may play an important role in cell cycle progression in ELA cells. Future studies should address this question employing methods allowing the distinction between effects on cell proliferation and cell death. Some previous studies have reported 100% inhibition of cell growth by traditional Cl-channel inhibitors (Voets et al., 1995; Shen et al., 2000; Wondergem et al., 2001). The maximal inhibition of growth by NS3728 was only about 40%, indicating that the activity of VRAC and/or CaCC is rate-limiting, yet non-essential.

In conclusion, in ELA cells, the maximal swelling-activated Cl-current varied during the cell cycle, decreasing from G0 to G1, and increasing from G1 to S, such that the current in S was also significantly higher than that in G0. In contrast, the maximal CaCC was unaltered from G0 to G1, but decreased significantly from G1 to S phase. The isometric steady-state current, which appeared to be predominantly VRAC, also varied with cell cycle progression, decreasing from G0 to G1 similar to the swelling-activated current, and increasing again in S phase to a level similar to that in G0. A high-affinity inhibitor of VRAC and CaCC in ELA cells attenuated cell growth, suggesting a possible mechanistic link between cell cycle progression and cell cycle-dependent changes in the capacity for conductive Cl-transport.

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CHAPTER V: MONOVALENT IONS CONTROL PROLIFERATION OF 
EHRLICH LETTRE ASCITES CELLS

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Monovalent Ions Control Proliferation of Ehrlich Lettre Ascites cells
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Running title: ionic movements in the cell cycle

Keywords:
Cell cycle, chloride channel, VRAC, TASK-2, NHE1
Abstract

Despite the importance of K⁺ channels for proliferation in several cell types neither K⁺ concentration nor intracellular K⁺ content were significantly changed during cell cycle progression ELA cells. In contrast, both Na⁺ and Cl⁻ concentrations as well as intracellular content were reduced in the transition between G0 and G1 phase followed by a significant increase in both Na⁺ and Cl⁻ content between G1 and S phase resulting in cell swelling. Substituting extracellular K⁺ with the impermeable cation NMDG⁺ did not affect the rate of proliferation in ELA cells, whereas substituting Na⁺ with NMDG⁺ had a strong inhibitory effect on ELA cell proliferation showing the importance of Na⁺ for cellular proliferation. During Na⁺ substitution NHE1 activity seems to be upregulated which affects the pH regulation of the cells. The effect of Cl⁻ substitution was pronounced using the two impermeable anions gluconate and glucuronate whereas Cl⁻ substitution with the much more permeable anion MSA only resulted in minor inhibition indicating a role for anion permeability rather than a direct role of Cl⁻ ions for cell proliferation. Cl⁻ permeability (estimated from the shift in Eₘ when substituting extracellular Cl⁻ with the impermeable gluconate) was significantly reduced in S phase compared to the G1 phase and accordingly Eₘ was hyperpolarized in S phase cells. The broad specter Cl⁻ channel inhibitor DIDS did not affect proliferation and niflumic acid only had minor effects at very high concentrations whereas NS3728, which blocks VRAC and CaCC channels and Tamoxifen, which blocks VRAC potently strongly inhibited proliferation. A clear regulation of Cl⁻ channels during the cell cycle was observed, thus both CLIC1 and CIC-2 where significantly downregulated in the biotinylated fraction (the cell membrane fraction) in the S phase compared to G1, indicating a role of these channels for the intrinsic Cl⁻ permeability changes during the cell cycle. TMEM16A expression did not change. Our data strongly suggest that Na⁺ is predominantly involved in cell cycle progression through regulating pH, while Cl⁻ is at least in part regulating proliferation by fine-tuning of Eₘ.

Introduction

Several studies have shown that the cell cycle progression depends on an increase in cell volume and is delayed in osmotically shrunken cells (see (Lang et al. 142-60; Hoffmann, Lambert, and Pedersen 193-277; O'Neill C995-C1011). In fibroblasts, it was specifically shown that the cell volume was increased in the G1 – S phase transition(13). While it is obvious that cell volume must increase at some point during the cell cycle in order for the cell to end up as two cells of similar size, the precise mechanisms involved are poorly understood. Cell volume can change during the cell cycle by two distinct processes. One is accumulation of osmolytes through increased production or through metabolism of larger molecules, while the other
process works through the movement of osmotically active substances across the plasma membrane. Specifically the movement of the monovalent ions Na\(^+\), K\(^+\), H\(^+\) and Cl\(^-\) is important. In addition to their role in regulating the cellular water content and thus volume, changes in the cellular content of free, monovalent ions regulate cellular pH, transmembrane potentials (\(E_m\)), and the activity of a wide range of proteins with important functions in the cell cycle including Cyclin B1, Cyclin dependent kinase 2 (cdc2) (Putney and Barber 44645-49), mitogen activated protein kinases (MAPK) (Pedersen et al. 735-50; Pedersen et al. 195-201) and Na\(^+\), K\(^+\), Cl\(^-\), cotransporter1 (see(10)).

In agreement with these central functions of monovalent ions, channels and transporters controlling their cellular concentrations have repeatedly been shown to be important for cellular proliferation, and Na\(^+\), K\(^+\) and Cl\(^-\) channels have all been shown to be dysregulated in various cancers (Lang et al. 142-60; Kunzelmann 159-73; Schonherr 175-84). However, the specific roles of monovalent ions in cell cycle progression are poorly understood and changes in ionic activity during the cell cycle have obtained little focus. Especially the role of Chloride is elusive. In glia cells, Cl\(^-\) loss during mitosis is essential for cytoplasmic condensation assisting chromatin condensation (Habela et al. 750-57; Habela, Olsen, and Sontheimer 9205-17; Habela and Sontheimer 1613-20). However this mechanism is based on the atypically high [Cl\(^-\)], in glia cells and in other cells Cl\(^-\) channel blockers will accumulate cells in the G1 rather than in the M phase (Tang et al. 775-85; Chen et al. 253-67; Jiang et al. 27-34; Li et al. 267-71). Also blockers of K\(^+\) channels inhibits the progression to the S phase leaving cells in G1 (see (Kunzelmann 159-73; Wonderlin and Strobl 91-107)). This is thought mainly to reflect K\(^+\) ions and channels setting the driving force for [Ca\(^{2+}\)] oscillations but K\(^+\) channel inhibitors will also affect pH, (see (Berridge, Bootman, and Roderick 517-29; Kunzelmann 159-73; Lang et al. 142-60). Na\(^+\) is central in setting the intracellular pH via the ubiquitous NA\(^+\)/H\(^+\) exchanger1 (NHE) (see (12)) but is also very important for regulating [Cl\(^-\)], via Na\(^+\), K\(^+\), 2 Cl\(^-\) co-transporter (NKCC) during cell proliferation (see (10)).

In the present paper we investigated the role of the main monovalent ions in the proliferation of Ehrlich Léttre Ascites (ELA) cells during the G1/ S phase transition. We found significant differences in [Na\(^+\)], and [Cl\(^-\)] but not in [K\(^+\)], between the different phases of the cell cycle, and in congruence, cell proliferation was inhibited by substituting Na\(^+\) with non-permeable ions. Inhibition of Cl\(^-\) channels inhibited cell cycle progression and the Cl\(^-\) permeability was high in G1 and decreased at the G1/ S transition. Our data strongly suggest that Na\(^+\) is predominantly involved in cell cycle progression through regulating pH, while Cl\(^-\) is at least in part regulating proliferation by fine-tuning of the membrane potential. In addition, VRAC may, as previously discussed, also contribute to cellcycle progression by counteracting challenges to the cell volume in the S phase (7).

Materials:
If not otherwise stated all materials were purchased from Sigma-Aldrich (St. Louise, MO) or Baker (Deventer, The Netherlands). DRAQ5 was from Biostatus Limited. CLIC1 Antibody was a kind gift from Samuel N Breit, St. Vincent Hospital and University, Sidney, Australia, CIC-1 and 2 antibodies were obtained from Alpha Diagnostics (San Antonio, Tx) and Alamone Labs (Jerusalem, Israel) respectively. CIC3 antibodies where kind gifts from William J. hatton, University of Nevada, USA. The mitosis specific alexa fluor 488 conjugated antibody Phospho-Histone H3 (ser10) was obtained from Cell Signalling Technology ( Danvers, MA).
anti Cyclin D antibody came from Upstate (Lake Placid, NY). NHE1 antibody was produced by Chemicon (now MilliPore). DIDS, Niflumic Acid, Tamoxifen, EIPA (Sigma Aldrich, St. Louise, MO) and NS3728 (Neurosearch, Ballerup, DK) stock solutions were prepared in DMSO. Antagonists were further diluted in DMSO to maintain constant DMSO concentration (1:1000) in working solution.

**Methods**

**Cell culture:**

Ehrlich Lettré Ascites (ELA) were maintained in RPMI-1640 (Sigma-Aldrich) medium supplemented with 10% FBS and 1% Penicillin/streptomycin at 37 °C in 5% CO₂ atmosphere. Passages 11-28 was used for experiments.

**Flow cytometry:**

Cells where synchronized in the quiescent phase by serum starvation for 72 h and sampled with 1 h intervals following release in serum containing medium. Cells were trypsinized and harvested by centrifugation (600 g; 5 min.; 4 °C). After 1 x wash in PBS cells where slowly resuspended in absolute ethanol during continuous mixing and fixed for 30 min on ice. Cells were collected by centrifugation (600 g; 5 min.; 4 °C), resuspended in ice cold PBS and were transferred to Partec 30 μm filters and filtrated during centrifugation. Supernantant was removed and cells incubated in Triton buffer (1% BSA, 0.25% Triton-X 100 in PBS) for 15 min. Cells were collected by centrifugation (600 g; 2 min.; 4 °C) and resuspended in blocking buffer (1% BSA in PBS). Alexa fluor 488 conjugated Phospho-histone H3-ser10 Ab (1:20) was added (for specific staining of metotic cells) and incubated 1h. Cells were washed 3x in blocking buffer and resuspended in PBS containing 30 μM Draq5 for DNA staining. Flow cytometry was performed on a DAKO CyAn and analyzed using DAKO Summit 4.3 software.

**Cell surface protein purification:**

Cell surface proteins were purified by biotinylation and avidin pull down via Cell Surface Purification kit from Pierce (Rockford, IL) according to manufacturer’s instructions. In short: Cells where grown in 10 cm Petri dishes and synchronized as above. Cells were quickly washed with 2 x 8ml Ice Cold-PBS and incubated with 10 ml Sulfo-NHS-SS-Biotin (2.5 mg) in PBS for 30 min at 4°C. The reaction was quenched and the monolayer was collected using a rubber-policeman pooling 4 Petri dishes for each sample. Cells where washed and collected by centrifugation (500 x g, 4°C, 3 min.). Pellet was re-dissolved in 500 μl lysis-buffer containing Halt Protease inhibitor cocktail (Pierce, Rockford, IL) and homogenized by 5 x 2s sonication every 10 min for half an hour and clarified by centrifugation (10,000 x g, 4°C, 2 min). 50 μl total lysate was collected for protein measurement and western blotting while 425 μl lysate was incubated 60 min in rotating immobilized NeutrAvidin gel column at RT. The column was washed 3x by centrifugation (1000 x g, 25°C, 1 min.) and was incubated with 400 μl NuPage LDS sample buffer (Invitrogen) containing 53 mM DTT for 60 min at RT. Finally, biotinylated surface proteins were eluted by centrifugation (1000 x g, 25°C, 2 min)

**SDS-PAGE and Western blotting**

SDS-PAGE was carried out under denaturating and reducing conditions using 10% Bis-Tris gels with NuPage MOPS SDS running buffer and a Novex XCell (E19001) system (Novex, San Diego, CA). The protein
concentration of cleared total cell lysates was estimated using a BCA protein kit (Bio-Rad, Hercules, CA) with BSA as standard (Pierce, Rockford, IL). The amount of protein loaded per well was 30 µg for total lysates and an amount corresponding to 155 µg total lysate protein for purified surface proteins. Separated proteins were electrotransferred to Protran nitrocellulose membranes (Whatman, Dassel, Germany), membranes were stained with 1% Ponceau S Red solution (Sigma-Aldrich, St. Louis, MO), blocked (5% non-fat dry milk in 1xTBST (0.01M Tris-HCl pH 7.4, 0.15M NaCl and 0.1% Tween 20) for 75 min, incubated for 2 h with primary antibody in blocking buffer, washed extensively in TBST, and incubated for 1 h in with alkaline phosphatase-conjugated secondary antibodies in blocking buffer, all at room temperature. Membranes were washed extensively in TBST and developed using BCIP/NBT solution (Kirkegaard and Perry Laboratories, Gaithersburg, MD). Band intensity was evaluated by densitometric scanning using the program Un-Scan-IT (Silk Scientific Inc., Orem, Utah).

**Ion and water content:**

Cells were grown and synchronized as described above in Petri dishes. To estimate the intracellular water space, cells where incubated 1 h before experiments with 0.72 nM of the non-metabolizable hexose, 3-O-[^14]C-methyl)-D-Glucose (GMO) (27.6 nCi/ml) which equilibrates across the membrane through facilitated transport(8). Experiments were initiated by rapid wash in 3 x 4 ml ice cold 0.1 M Mg(NO\(_3\))\(_2\) with 55mM D-mannitol (295 mOsmol/L) containing 1 mM phloretin to inhibit GMO transport during washing. Cells were lysed by adding 2.5 ml EtOH which was allowed to evaporate. The water soluble pool was extracted by rehydration for 1 h in 1 ml H\(_2\)O. Finally, the protein fraction was recaptured by NaOH containing 0.5% sodium deoxycholate.

The Content of K\(^+\) and Na\(^+\) in the water soluble pool was measured by flame spectrophotometry using a FLM3 flame photometer (radiometer, Copenhagen, DK). Cl\(^-\) content in the water soluble pool was measured using a CMT10 chloride titrator (radiometer, Copenhagen, DK).

Ninhydrin positive reactants were measured by spectrophotometry. 15 µl Ninhydrin reactant (sigma-Aldrich) was added 30 µl and the mixture was boiled at 98°C for 10 min. exactly and cooled to room temperature. 75 µl EtOH was added the solution and the reaction was evaluated by measuring absorbance at 570 nm using Fluostar-Optima microplate reader (BMGLabtech, Offenburg, Germany).

**Cell proliferation assay:**

Cell proliferation was estimated by measuring BrdU incorporation using a chemiluminiscent Cell Proliferation assay (Roche Diagnostics, Mannheim, Germany) according to manufacturer’s instructions. In short: Cells where seeded in black 96 well dishes with 6.000 cells pr. well. 24 h after seeding the medium was changed to the relevant substitution or inhibitor containing medium and the cells where incubated for another 24 h. Two h before measurement, 10 µl 10 µM BrdU in culture medium was added. After incubation the cells were fixed for 30 min and incubated with monoclonal peroxidase conjugated BrdU antibody for 90 min. After thorough washing, 100 µl peroxidase substrate solution was added and chemiluminiscence was measured on a Fluostar-Optima microplate reader (BMGLabtech, Offenburg, Germany) within the first 10 min.

**Transmembrane potential measurement:**

$E_m$ were estimated using the potential sensitive dye DiBaC$_{3}(4)$(4). Cells where seeded on #1 coverslips and synchronized as described. Coverslips where mounted in an open perfusion chamber on an inverted microscope (Nikon Eclipse Ti) and continuously perfused using RPMI-1640 salt solution (in mM: 0.423 Ca(NO$_3$)$_2$, 0.406 MgSO$_4$, 5.4 KCl, 102.7 NaCl, 5.6 Na$_2$HPO$_4$, 11 glucose, 25 HEPES, pH 7.4). Flourescent signals exited at 470 nm where continuously measured at 520-560 nm in regions of interest (ROI) surrounding individual cells using EasyRatioPro imaging station (PTI, Seefeld, Germany). After a steady background signal was achieved, cells where 0.5 µM DiBaC$_{3}(4)$ was added. Signals were allowed to stabilize (40-50 min) before solution change. Data for all cells were corrected for ROI area (pixel$^2$) and corrected for background fluorescence, continuously measured in cell free area of individual experiments. Calibration of fluorescence signals to membrane were calculated from Nernst potentials at different extracellular Na$^+$ concentrations in the continuous presence of 10 µM gramicidin as previously described(3).

**Intracellular pH ($pH_i$) measurements:**

Intracellular pH was estimated using the cell permeant fluorescent dye BCECF, essentially as described (Pedersen et al. 19716-27). Cells were seeded on HCl and ethanol cleaned coverslips and loaded with BCECF-AM for 30 min at 37°C in RPMI 1640 salt solution (see above). Cells were placed in an angle of 50° respective to the lighting source in a termostated and perfused cuvette of a PTI Ratiomaster spectrophotometer. Fluorescence was measured at 525 nm from excitation wavelength 445 nm and 495 nm. All data were subtracted background signals obtained from unloaded cells. 445/494 nm ratios was converted to intracellular pH via a 7-point nigiricin/ high K+ calibration as described(1).

**Results:**

**Changes in cellular NaCl and water content during the cell cycle:**

While the ionic composition of the extracellular environment generally remains constant the cytoplasmic compartment can potentially change its ionic content over time. As the ionic gradient across the plasma membrane set the electrochemical driving force for these ions, we initially investigated ionic concentrations throughout the cell cycle. To discriminate between cells of the different cell cycle phases cell where synchronized by serum starvation. Cyclin D is only expressed in the presence of mitogens, hence, Quiescent cells do not express cyclin D (Malumbres and Barbacid 153-66;Ortega, Malumbres, and Barbacid 73-87) as opposed to cycling cells. We saw a marked reduction in cyclin D1 expression following 72 h serum starvation (Fig 1A) indicating cells going in to quiescence. 72 h serum starvation was previously described to lead to deep G0 phase in human diploid fibroblasts (TIG-1)(Yabuta et al. 4878-92). 72 h serum starvation was also seen increasing the amount of cells with G0/G1 phase DNA content to almost 90% (Table1). Following re-addition of serum to the growth medium Cyclin D1 expression normalized within the first 6-8 h and we will refer to cells as G1 cells after 8 h release. S phase was, in congruence with what we previously observed in ELA cells(7), predominant after 15-16 h after release (Table1) and these cells will be referred to as S phase.

Whereas the sample protein content did not differ between different samples (Fig. 2A insert), the amount of water per protein content increased in S-phase compared to G0 phase (Fig 2A), implying an increase in the amount of intracellular osmotic substances. As will be discussed below, the water uptake could be explained by an uptake of NaCl.
Despite the importance of K⁺ channels for proliferation in several cell types neither K⁺ concentration nor intracellular K⁺ content were significantly changed during cell cycle progression (Fig. 2B). In contrast, both Na⁺ and Cl⁻ concentrations were reduced by 15.0 mM and 11.3 mM, respectively, in the transition between quiescence and G1 phase (Fig. 2C and D), corresponding to a 5-8 µmol/mg protein NaCl loss. This will decrease the intracellular osmolarity 10-15 mOsmol/kg. As the water content did not change between G0 and G1 (Fig. 2A) the intracellular osmotic pressure must be kept constant via other means. However we did not find any significant changes in the content of either protein (Fig. 2A insert), K⁺ (Fig 2B insert) or ninhydrin positive reactants (n=4, data not shown).

The loss of NaCl from G0 to G1 was more than compensated in the S-phase. When results from paired experiments were compared, a significant increase in both Na⁺ (276 ± 27 to 330 ± 26 nmol/ mg protein, n=8, p< 0.05) and Cl⁻ (284 ± 25 to 345 ± 28 nmol/ mg protein, n=8, p< 0.05) was observed between G1 and S phase. This was accompanied by water uptake, i.e. the cells swelled.

**Na⁺ and Cl⁻ substitution inhibits cellular proliferation:**

Substituting extracellular K⁺ with the impermeable cation NMDG⁺ did not affect the rate of proliferation in ELA cells (Fig. 3A). In contrast, substituting Na⁺ with NMDG⁺ had a strong dose-dependent inhibitory effect on ELA cell proliferation which was significant after 40% substitution (Fig 3B). 100% Na⁺ substitution reduced the rate of proliferation to about one-third of the control rate showing the importance of Na⁺ for cellular proliferation.

Although significant, the effect of Cl⁻ substitution was less pronounced than the effect of Na⁺ depletion (Fig. 3C). We substituted Cl⁻ using three different anions: MSA which exhibits intermediate permeability via Cl⁻ channels (2; 19) and do not affect cell volume, ionic strength and pH (11) and the two more impermeable anions gluconate and glucoronate. The maximum inhibitory effect (45.5 ± 5 %) was observed by using the two impermeable anions whereas Cl⁻ substitution with MSA only resulted in minor inhibition. This indicates a role for anion permeability rather than a direct role of Cl⁻ ions for cell proliferation.

The clear difference between Na⁺ and Cl⁻ substitution points to the existence of independent functions of the two ions in the cell cycle (see discussion).

**Sodium substitution alkalizes pH, and upregulates NHE1 activity:**

As the effect of substituting Na⁺ and Cl⁻ on cellular proliferation was different, we speculated whether the two ions function via different signaling events. Further, [Cl⁻]ᵢ was not affected by Na⁺ substitution (n=5, not shown). Given the importance of Na⁺/H⁺ exchanger, NHE1 for proliferation in numerous cell types (see (12)) we next investigated the effect of the NHE1 specific antagonist EIPA on ELA cell proliferation. As seen in Fig. 4, EIPA potently inhibited BrdU incorporation with an IC₅₀ value of 14.5 µM.

To investigate whether NHE1 is differentially expressed during the cell cycle we synchronized cells by serum starvation and followed NHE1 expression as a function of time after release. NHE1 shows two different bands in western blots due to the presence of immature unglycosylated and mature glycosylated forms. Mature proteins was especially obvious in the purified biotinylated protein fraction representing cell surface proteins, which shows that NHE1 in the plasma membrane – as expected - is predominantly glycosylated. In congruence with our previous observations in three different cell lines (Schneider et al.
163-76) we found a tendency for NHE1 upregulation in the biotinylated cell surface fraction of G0 cells (P= 0.052, n=3). No difference was observed between G1 and S phase (data not shown). To determine how NHE1 activity and thus pH regulation was affected during Na+ substitution, intracellular pH was measured by the fluorescent pH sensitive dye, BCECF, and showed a significant alkalization of cells kept for 24 h in Na+ free growth medium (Fig. 5A). During these experiments we also examined the cells responsiveness to a NH4Cl induced acid load. Interestingly the recovery rate from acid load was 4x increased (P< 0.01) in sodium depleted cells when compared to cells grown in normal RPMI1640 medium (see Fig. 5B). Hence we investigated whether EIPA, was able to inhibit pH recovery after acid load and found an almost complete inhibition of recovery (Data not shown). This clearly show, NHE1 as the main player in pH regulation in ELA cells and NHE1 activity seems to be upregulated during Na+ substitution.

Cl- permeability decreases between cell cycle G1 and S phases:

BrdU assays suggested a role for anion permeability in regulating cell cycle progression (see above). As measuring the small isotonic Cl- currents constitutes a major difficulty in nystatin perforated patch clamp studies due to difficulties in bringing non-permeable cations to the cytoplasm, we assessed the Cl- permeability indirectly via Em measurements using the membrane permeable anionic fluorescent dye DiBaC4(3), which equilibrates in a Nernstian manner across the plasma membrane. Cl- permeability was estimated from the shift in Em when substituting extracellular Cl- with the impermeable gluconate (Fig 6A). These experiments showed a significant reduction in the Cl- sensitive potential of ELA cells in S phase compared to the G1 phase, indicating a significant reduction in the Cl- permeability (Fig. 6B). Accordingly, substituting with the more permeable anion MSA had a significantly smaller effect (Fig. 6A).

Em is depolarises in G1 to both G0 and S-phase cells:

Em was hyperpolarized in S phase cells (-69.6 ± 0.7 mV) when compared to cells in G1 phase (-62.9 ± 1.2 mV) (Fig. 6C). A hyperpolarization is to be expected due to the observed reduction of Cl- permeability in S phase (Ec was between -20- -30 mV). However, G1 phase cells were also depolarized when compared to quiescent cells. This difference cannot be explained by altered Cl conductance since no significant difference in ∆Em upon Cl- substitution was observed between G0 and G1 (Fig 6B). It is, however, possible that the small difference in ∆Em between G0 and G1 could turn out to be significant if more experiments were conducted.

Cl- channel antagonists inhibits cellular proliferation:

Another way of investigating the role of Cl- permeability in ELA cell proliferation is by pharmacologically inhibiting Cl- channel conductance. We used four different Cl channel inhibitors (Fig. 7) The broad specter Cl- channel inhibitor DIDS did not affect proliferation (Fig. 7A) and niflumic acid only had minor effects at very high concentrations (Fig. 7B). NS3728, which blocks VRAC and CaCC channels in ELA cells (7) inhibited BrdU incorporation with an IC50 value of 41 ± 2 µM (n_Hill = 4, Fig. 7C). Tamoxifen, which blocks VRAC potently inhibited proliferation with an IC50 value of 5.8 ± 0.8 µM (n_Hill= 1.3, Fig. 7D). Notably, both compounds inhibited progression of the cell cycle almost 100%, in contrast to Cl- substitution (see Discussion). It must however be emphasized that in the substitution studies only NaCl was substituted hence leaving 5.4 mM KCl in the medium.
**Cl- channels are differentially translocated to the plasma membrane during cell cycle:**

To identify molecular candidates for the role of Cl\(^{-}\) channels in cell cycle regulation, we investigated the expression of known Cl\(^{-}\) channels: In the CIC family of channels and transporters CIC-2 yet neither CIC-1 nor CIC-23 appeared to be present in ELA cells (Fig. 8A). The recently cloned Cl\(^{-}\) channels CLIC1 and TMEM16A where both present in ELA cells. To investigate whether these channels where differentially expressed during the cell cycle, plasma membrane proteins where isolated by biotinylation. From these experiments a clear regulation of Cl\(^{-}\) channels during the cell cycle was observed. Both CLIC1 and CIC-2 where significantly downregulated in the biotinylated fraction in S phase compared to G1, indicating a general downregulation of Cl\(^{-}\) channel translocation to the cell surface during the S-phase. A similar pattern was not observed in the total lysates, where neither CLIC1 nor CIC-2 expression was significantly changed. TMEM16A expression did not change in neither the biotin-avidin purified lysates nor in total lysates. The downregulation of CIC-2 and CLIC1 chloride channels in the S phase agrees well with the decreased Cl\(^{-}\) permeability in the S phase (fig. C) indicating that a fundamental role of these channels for the intrinsic Cl\(^{-}\) permeability changes seen during the cell cycle.

**Discussion:**

Channels and transporters are increasingly being investigated as molecular targets for anti cancer drugs (Arcangeli et al. 66-93;Le Guennec et al. 189-202;Villalonga et al. 212-23;Conti 135-44) despite a small knowledge of the proliferatory effects of ions. In this study, we report changes in monovalent ion content and concentration between the different phases of the cell cycle in ELA cells and describe their possible effects on the cell cycle.

ELA cells shoved a marked decrease in NaCl concentration between Go and G1 phase. Neither the ionic content of NaCl nor the water content changed significantly. Hence, the significant concentration loss was apparently due to a combined effect of insignificant water uptake and NaCl loss.

A significant increase in NaCl content was observed between G1 and S phase. NaCl uptake was followed by water, hence, there was no difference in NaCl concentration, and the cells swelled osmotically in the process. A minor volume increase between G1 and S phase has previously been described in CNE-2Z nasopharyngeal carcinoma cells, where a decreased capacity for volume regulation in the S phase compared to G1 was also observed (Wang et al. 110-19). The role of osmotic volume changes in regulation of cell proliferation is still very rudimentarily understood, but cell swelling promotes and cell shrinkage inhibits proliferation of a number of cell types (Anbari and Schultz 24-28;Dubois and Rouzaire-Dubois 227-32;Rouzaire-Dubois et al. 249-57). The functional importance could be speculated to involve cell shape as changing cell morphology involves volume regulatory channels and transporters e.g. during migration (Schneider et al. 1055-62;Schwab et al. 421-32). As the nuclear volume is strictly regulated in relation to cell morphology, in a process which involves rearrangement of the cytoskeleton and which is particularly important in the G1- S phase transition (14), such morphological changes could very well be initiated by volume changes, which strongly affect the cytoskeleton arrangement in many cell types including the ELA related cell line, Ehrlich ascites tumor cells (Pedersen, Mills, and Hoffmann 63-74;Lionetto et al. 163-78).

Substituting either Na\(^{+}\) or Cl\(^{-}\) with impermeable ions had significant effects on cell proliferation, demonstrating that these ions are important for normal cell cycle progression despite the lack of significant
concentration difference between G1 and S phases. However, there was a marked difference in the potency of Na\(^+\) and Cl\(^-\) substitution, Na\(^+\) substitution having a much stronger effect than Cl\(^-\) substitution. This could imply that the two ions regulate the cell cycle via different mechanisms, or at least that Na\(^+\) has roles in cell cycle regulation beyond its role in cell volume regulation. Our data suggest that such additional effects of Na\(^+\) may involve the Na\(^+\)/H\(^+\) exchanger NHE1. EIPA, a specific inhibitor of NHE1, dose dependently inhibited ELA cell proliferation as has also been seen in other cell types. E.G. inhibition of NHE1 activity inhibited proliferation of vascular smooth muscle cells (18) and NHE1 siRNA inhibited proliferation of CNS pericytes (9). NHE1 tended to be upregulated in G0 phase cells when compared to G1 and we recently showed that NHE1 expression is strongly upregulated, whereas its activity is posttranslationally inhibited, in G0 phase compared to interphase, in several cell types(Schneider et al. 163-76). This may indicate that NHE1 is kept in a “readied, but quiescent” state in G0, allowing for a rapid increase in NHE1 activity once the cells are released from G0(Schneider et al. 163-76). In CNS pericytes, NHE1 controlled pH oscillations which facilitated proliferation by stimulating Ca\(^{2+}\) oscillations (9). It has also been shown that NHE1 activity regulates G2/M progression by eliciting an increase in pH\(_i\) which in turn regulates Cyclin B1 expression and cdc2 activity(Putney and Barber 44645-49). Hence, we hypothesized that Na\(^+\) depletion might inhibit proliferation by reducing pH\(_i\), since, under these conditions, the cells cannot extrude acid equivalents via either NHE1 or Na\(^+\)-dependent HCO\(_3\) cotransport, the two major acid extruders in most cell types. Counter to our expectation, ELA cells grown in 0 Na\(^+\) medium for 24 h showed a significant increase in steady state pH\(_i\), measured in the same medium. Since, as noted above, cells in 0 Na\(^+\) medium cannot increase their pH\(_i\) via either NHE1 or Na\(^+\)-dependent HCO\(_3\) cotransport, this must reflect the upregulation of Na\(^+\)-independent acid extrusion mechanisms in these cells, possibly H\(^+\) ATPases. The capacity of the Na\(^+\) depleted cells for pH\(_i\) recovery in Na\(^+\) containing solutions after an acid load was also strongly increased relative to that in control cells. This could reflect either upregulation of NHE1 expression and/or the greater transmembrane Na\(^+\) gradient in depleted cells, a question not addressed here. In any event, however, it seems clear that at least at time 24 h, the effect of Na\(^+\) depletion does not reflect a decrease in pH\(_i\). It seems likely that the pH\(_i\) may have been reduced initially upon Na\(^+\) removal, possibly leading to a proliferation delay, followed by an increase of pH\(_i\) as compensatory mechanisms were upregulated. Another possibility is that the altered transmembrane Na\(^+\) gradient will cause dysregulation of [Ca\(^{2+}\)], e.g. via altered driving force for Ca\(^{2+}\) transport via NCX, however, further experiments are needed to distinguish between these possibilities.

As already noted, the very different response of Na\(^+\) and Cl\(^-\) substitution on proliferation led us speculate that Cl\(^-\) might exert its effects on cell cycle progression via Na\(^+\)-independent mechanisms. Substituting Cl\(^-\) with MSA gave a much lower effect than gluconate and especially gluconerate. This is noticeable since MSA have a medium permeability across several Cl\(^-\) channels while gluconate and gluconerate generally have very low permeability (Evans and Marty 437-60;Arreola, Melvin, and Begenisich 677-87;Bosma 67-90;Hume and Thomas 241-61;Stoddard, Steinbach, and Simchowitz C156-C165). These results strongly indicate that anion permeability rather than Cl\(^-\) concentration per se is the dominating factor for regulating ELA cell proliferation. With a Nernst potential for Cl\(^-\) of -19.3, -26.2, and 23.9 mV in G0, G1, and S phase respectively, and a measured E\(_m\) between 60 and 70 mV, changes in Cl\(^-\) permeability will regulate E\(_m\). Our measurement shows a significant decrease in Cl\(^-\) permeability in S phase compared to G1. This drop in Cl\(^-\) permeability results in a hyperpolarization of S phase cells in congruence with predictions of the GHK equation. Hence, we suggest that Cl\(^-\) channels regulate proliferation of ELA cells mainly by fine tuning E\(_m\). Membrane
potential has been broadly recognized in regulating the cell cycle, primarily by changing the driving force for Ca\textsuperscript{2+} uptake (see Kunzelmann 159-73; Berridge, Bootman, and Roderick 517-29)). Indeed the Cl\textsuperscript{-} channel inhibitor DIDS inhibited proliferation by inhibiting Ca\textsuperscript{2+} influx in T lymphocytes (Wang et al. 437-46). In this study DIDS did not inhibit cell proliferation. However, all identified channels in ELA cells except for Ca\textsuperscript{2+} activated Cl\textsuperscript{-} channels is inhibited by DIDS with an IC\textsubscript{50} > 100\textmu M (see (6)). VRAC is inhibited by DIDS but only at positive potentials (Klausen et al. C757-C771). Hence, the reason DIDS is not inhibiting ELA cell proliferation is probably due to the channels expressed.

This raises the question of which Cl\textsuperscript{-} channel(s) regulate the cell cycle. Of course any channel increasing or decreasing Cl\textsuperscript{-} permeability would also affect E\textsubscript{m}. Accordingly any Cl\textsuperscript{-} channel antagonist inhibiting any open channel would affect E\textsubscript{m}. In our hands, neither niflumic acid nor DIDS – both of which inhibit the calcium activated Cl\textsuperscript{-} channel in ELA cells (7) – affected cell proliferation. DIDS also inhibits VRAC in ELA cells, however only at positive E\textsubscript{m} which are not relevant in these studies (7). The VRAC inhibitors NS3728 and tamoxifen both potently inhibited ELA cell proliferation. We (7) and others (16) have previously shown that VRAC is active under isotonic conditions. However, neither the pattern of expression of VRAC (estimated as its maximal activity) during the cell cycle in ELA cells (7), nor the previously described isotonic VRAC activity in perfused cells (7) correspond to the pattern of changes in Cl\textsuperscript{-} conductance in the cell cycle found in the present study, suggesting that VRAC is unlikely to be the main player. Also in accordance with this view, the observed volume increase during the G1/ S phase transition is not in line with a decreased VRAC activity. Indeed, we have previously described that isotonic VRAC is increased in S phase compared to G1 (7). This would implicate that VRAC is an important participant in setting the physiological Cl\textsuperscript{-} conductance, but that it is only one of several Cl\textsuperscript{-} channels involved, and that it is probably not responsible for hyperpolarizing ELA cells during the G1/ S phase transition. Both CIC-2 and CLIC1 were down regulated in the plasma membrane in S phase, correlating with the down regulation of Cl\textsuperscript{-} permeability, suggesting that altered activity of these channels may underlie the observed changes. In accordance with this view, CIC-2 regulates proliferation of porcine arterial smooth muscle cells (Cheng et al. 198-207) and CLIC1 was found to be exclusively expressed in the plasma membrane in G2/M phase of CHO cells (17).

This leaves us with the final question of the role of VRAC upregulation during G1/S transition as previously described (7). However, this transition is a very dynamic phase with serial Ca\textsuperscript{2+} oscillations (see (15)) which according to the Donnan equilibrium (see (5)) will challenge the cell volume. Further, the S phase of course is named for synthesis and the DNA replication constitutes a major osmotic challenge. If volume is only sequentially challenged VRAC is only sequentially activated but has to be present in large number due to the severity of volume challenges during this phase of the cell cycle. Hence it is feasible to speculate, that VRAC is activated on demand and the expression of VRAC will not be reflected in the general Cl\textsuperscript{-} permeability.

In conclusion we show that proliferation of ELA cells is dependent on Na\textsuperscript{+} as well as Cl\textsuperscript{-}. Both ions are accumulated during the G1/ S phase transition, mediating cells swelling. We show that Na\textsuperscript{+} is regulating proliferation via NHE1 and possibly via pH. Cl\textsuperscript{-} channel inhibitors inhibited proliferation of ELA cells and CIC-2 and CLIC1 expression was down regulated during the S phase in congruence with the Cl\textsuperscript{-} permeability. As the inhibitory effect of Cl\textsuperscript{-} substitution was correlated to substituent permeability we conclude Cl\textsuperscript{-} is regulating ELA cell proliferation by fine- tuning E\textsubscript{m}. In addition, VRAC may, as previously discussed, also contribute to cell cycle progression by counteracting challenges to the cell volume in the S phase (7).
Table 1: Flow cytometric analysis of cell cycle distribution

Cells where serum starved for 72 h and incubated with serum as indicated in the table. Cells where harvested and labeled as described in Materials and methods and analyzed for DNA content by flow cytometry. M phase cells where recognized by phospho-Histone H3 (ser 10) alexa-fluor conjugated antibody. Results are summarized from 3 independent experiments.

Fig. 1: Cyclin D1 expression:
A: Cells where grown without serum in 0, 24, 48 and 72 h, harvested and protein fractions where analyzed for cyclin D1 expression by western blotting. Representative of 3 independent experiments. B: Cells where serum starved for 72 h and then incubated with serum. Samples were collected every hour and cell lysates where analyzed for cyclin D1 expression by western blotting and immune attaining. Results are representative of 3 independent experiments.

Fig. 2: Ionic concentrations during the cell cycle:
Cells where synchronized by 72 h serum starvation and sampled 0 h (G0) 8 h (G1) and 15 h (S) after reentry into the cell cycle by re-exposure to serum (see Fig. 1). Inserts shows total protein pr. sample (A) or cellular content of the ion in question (B-D) in pairs. Cells where sampled and analyzed as explained in materials and methods section. A: intracellular water content pr µg protein as a function of the cell cycle. Insert shows the measured protein content. B: Cellular K⁺ concentration as a function of the cell cycle phases. K⁺ was measured from the ionic fraction by flame spectrometry as described in methods and materials. C: Na⁺ concentrations measured by flame spectrometry. D: Cellular Cl⁻ concentrations. Cl⁻ was estimated by coulombic measurement from the ionic fraction. n= 8-10 for all figures. * represents significant difference to G0 values (P< 0.05).

Fig. 3: Ionic concentrations affects proliferation
Following 24 h substitution of K⁺ (A) or Na⁺ (B) by NMDG⁺, cellular proliferation was estimated by BrdU incorporation. Cl⁻ (C) was substituted by either MSA, gluconate or glucuronate. BrdU incorporation relative to the control is depicted as a function of percentage substitution. N=4-9 in all figures. * represents P< 0.05.

Fig. 4: EIPA sensitivity of ELA cell proliferation.
BrdU incorporation assays where performed as in Figure 3 with increasing concentrations of EIPA in the growth medium. (n=10 , *represents P< 0.05.)

Fig. 5: Na⁺ dependent pH regulation in ELA cells:
Intracellular pH was measured by fluometry using the ratiometric pH sensitive fluorescent dye BCECF. Cells were loaded 30 min at 37°C before measurements. A: Representative traces showing pH recovery after an NH₄Cl prepuls +/- Na⁺ depletion. Cells were incubated 24 h in Na⁺ containing or Na⁺ free growth medium before being transferred to Na+ recording medium (+/- Na⁺). pH was measured for 10 min after which the cells were subjected to a 10mM NH₄Cl prepuls followed by Na⁺ depleted medium. Reinstalling Na⁺ in the medium led to a pH regulation towards normal cellular pH levels. Notice the faster recovery rate of cells
acclimatized to Na\(^+\) free medium. Data are representative for 4-5 independent experiments. B: Resting pH in cells incubated for 24h in control or sodium free medium. n= 3-4. C: EIPA sensitivity of pH\(_i\) recovery. Relative pH\(_i\) recovery rate after acid load in medium in the presence or absence of the NHE1 inhibitor, EIPA (5 µM), in sodium containing recovery solution. (n= 4). *represents P< 0.05.

**Fig. 6: Cl\(^-\) affects the E\(_m\):**

To asses E\(_m\), cells were loaded with the anionic fluorescent dye DiBaC\(_4\)(3) which distributes Nernstian across the plasmamembrane. A: Representative experiment. Cells where continuously superfused. 500 µM DiBaC\(_4\)(3) was added two minutes after recording started. Perfusion solution was chaged as indicated in top-bars. DiBaC DiBaC\(_4\)(3) was continuously present. B: The change in E\(_m\) following Cl\(^-\) substitution with gluconate. C: Same experiments as in a showing average E\(_m\) in the different phases of the cell cycle. n= 56-62 in 3 independent experiments. *represents P< 0.05.

**Fig. 7: Anion channel antagonist inhibits cell proliferation:**

The effect of the anion channel inhibitors NS3728 (A), Tamoxifen (B), Niflumic acid (C) and DIDS (D) on cell proliferation was tested via BrdU incorporation assay as described in materials and methods. Cells where incubated 24h with the given inhibitor before BrdU incorporation was measured. Results are depicted as a function of inhibitor concentration. n= 3-8. *represents P< 0.05.

**Fig. 8: Cl\(^-\) channel expression in ELA cells:**

A: Cell lysates were prepared from synchronized cells and proteins separated by SDS-PAGE. Proteins were labeled by specific antibodies. B: representative expression patterns from different phases of the cell cycle. Total lysates and biotinylated proteins where prepared as in Fig. 6 and visualized by immuno-staining following SDS-PAGE. C: Densitometric quantification of Cl data shown in B. Summarized result for 4-5 independent experiments. *represents P< 0.05.

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Reference List


Figure 1

A

Cyclin D1

Serum starvation

0 h 24 h 48 h 72 h

B

Cyclin D1

Time after release

CC 0 h 1 h 2 h 3 h 4 h 5 h 6 h 7 h 8 h
Figure 2

A

![Graph A](image1)

B

![Graph B](image2)

C

![Graph C](image3)

D

![Graph D](image4)
Figure 3

A

K⁺ substitution (%)

BrdU incorporation

B

Na⁺ substitution (%)

BrdU Incorporation

C

Cl⁻ substitution (%)

BrdU Incorporation

- Gluconate
- Glucoronate
- MSA

* * *
Figure 4

Inhibition (%) vs. [EIPA] (µM)
Figure 5

A

Intracellular pH

Control | Na⁺ Free

0 mM Na⁺ | 10 mM NH₄Cl

0 Na⁺ | 10 Na⁺

Time (min)

0 5 10 15 20 25 30

B

Intercellular pH

Control | Na⁺ Free

*
Figure 7

A

B

C

D

Inhibition (%)

Inhibition (%)

Inhibition (%)

Inhibition (%)

[DIDS] (mM)

[NS3728] (µM)

[Niflumic Acid] (mM)

[Tamoxifen] (mM)
**Figure 8**

### A

- 120 kDa marker
- Bands for CLIC1, CLIC2, CLIC3, TMEM16A
- Band for CLIC1

### B

- CLIC1-total
- CLIC1-Biotin
- CLC2-total
- CLC2-Biotin
- TMEM16A-total
- TMEM16A-biotin

### C

- Band intensity for G0, G1, S
- Comparison of Biotin and Total
- Stars indicate significant difference

---

CLC1, CLC2, CLC3, and TMEM16A are proteins that show differences in their expression and modification states across different stages of the cell cycle (G0, G1, S). The diagram illustrates the band intensity for each protein in these stages, highlighting the changes induced by biotin tagging and total protein expression.
CHAPTER VI: H-RAS TRANSFORMATION SENSITIZES VOLUME-ACTIVATED ANION CHANNELS AND INCREASES MIGRATORY ACTIVITY OF NIH3T3 FIBROBLASTS


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H-ras transformation sensitizes volume-activated anion channels and increases migratory activity of NIH3T3 fibroblasts

Linda Schneider · Thomas K. Klausen · Christian Stock · Sabine Mally · Søren T. Christensen · Stine Falsig Pedersen · Else K. Hoffmann · Albrecht Schwab

Abstract The expression of the H-ras oncogene increases the migratory activity of many cell types and thereby contributes to the metastatic behavior of tumor cells. Other studies point to an involvement of volume-activated anion channels (VRAC) in (tumor) cell migration. In this paper, we tested whether VRACs are required for the stimulation of cell migration upon expression of the H-ras oncogene. We compared VRAC activation and migration of wild-type and H-ras-transformed NIH3T3 fibroblasts by means of patch-clamp techniques and time-lapse video microscopy. Both cell types achieve the same degree of VRAC activation upon maximal stimulation, induced by reducing extracellular osmolarity from 300 to 190 mOsm/l. However, upon physiologically relevant reductions in extracellular osmolarity (275 mOsm/l), the level of VRAC activation is almost three times higher in H-ras-transformed compared to wild-type fibroblasts. This increase in VRAC sensitivity is accompanied by increased migratory activity of H-ras fibroblasts. Moreover, the high-affinity VRAC blocker NS3728 inhibits migration of H-ras fibroblasts dose-dependently by up to about 60%, whereas migration of wild-type fibroblasts is reduced by only about 35%. Consistent with higher VRAC activity in H-ras than in wild-type fibroblasts, more VRAC blocker is needed to achieve a comparable degree of inhibition of migration. We suggest that H-ras modulates the volume set point of VRAC and thus facilitates transient changes of cell volume required for faster cell migration.

Keywords Migration · Cell volume · Cl⁻ channel · Ras oncogene

Introduction

Mutated forms of ras proto-oncogenes are found in 70% of neoplasias [20]. The enhanced expression of these oncogenes is associated with an increase in the metastatic potential of tumor cells, which includes an increase in migratory activity. Thus, H-ras-dependent stimulation of tumor cell migration has been observed, among others, in fibroblasts, keratinocytes as well as in endothelial, melanoma, and mammary epithelial cells [1, 2, 7, 12, 13, 23, 36–38]. Several mechanisms such as increased collagenase IV production, synergy with epidermal growth factor receptor activity, down-regulation of α3β1-integrins, and activation of the extracellular signal-regulated kinase and p38 mitogen-activated protein kinase (MAPK) pathway or of matrix metalloprotease 2 were proposed to account for the enhanced migratory and invasive activity after H-ras expression.

Most migrating cells undergo profound morphological changes while crawling. Lamellipodial protrusion and uropodial retraction often occur at a different pace. Thus, the extension of the lamellipodium may prevail before the lagging
edge eventually “catches up,” causing the cell to gain volume during the protrusive phase and to lose it during lagging edge retraction [30, 32]. This concept is supported experimentally in that transport proteins mediating an osmotic regulatory volume increase (RVI) such as the Na+/H+ exchanger NHE1, the anion exchanger AE2, and aquaporins are predominantly active at the front of migrating cells [4, 10, 16, 18, 27]. Moreover, the combined activity of Ca2+-sensitive K+ channels (IK1) and volume-activated anion channels (VRACs) that mediate an osmotic regulatory volume decrease (RVD) is also required for optimal cell migration [21, 22, 25, 39]. The spatially localized activity of IK1 channels restricts RVD to the rear part of migrating cells [29, 31, 35]. Consequently, isosmotic RVI and RVD support the protrusion of the lamellipodium and the retraction of the rear part of migrating cells, respectively.

While it is well established that ion channels and transporters are important components of the cellular migration machinery [32], it is not yet known whether VRACs contribute to the increased migratory activity after transformation with the H-ras oncogene. We tested this hypothesis by comparing the effects of the high affinity VRAC blocker NS3728 on the migratory behavior of wild-type (wt) and H-ras-transformed (H-ras) NIH3T3 fibroblasts and by relating migratory behavior to the volume sensitivity of VRAC in both wt and H-ras cells.

Materials and methods

Cells and chemicals

Experiments were performed on wt and v-H-ras NIH3T3 mouse fibroblast cell lines. The v-H-ras oncogene contains two activating mutations, G12R and A59T. These mutations increase Ras activity, whereas GTPase activity is lowered [40]. The NIH3T3 mouse fibroblast cell line was stably transfected to express constitutively active v-H-ras, as previously described, using the plasmid pBW 1423 [41]. The NIH3T3 mouse fibroblast cell line was stably transfected to express constitutively active v-H-ras, as previously described, using the plasmid pBW 1423 [41]. The NIH3T3 mouse fibroblast cell line was stably transfected to express constitutively active v-H-ras, as previously described, using the plasmid pBW 1423 [41]. The NIH3T3 mouse fibroblast cell line was stably transfected to express constitutively active v-H-ras, as previously described, using the plasmid pBW 1423 [41].

The outlines of individual cells were marked semiautomatically at each time step throughout the entire image stacks with the Amira software (Mercury Computer Systems, Düsseldorf, Germany) as described previously [6]. These segmentation data were used for further processing. Migration was quantified as the movement of the cell centre. The x- and y-coordinates of the cell center (μm) were determined as the geometric mean of equally weighted pixel positions within the cell outlines. We used two parameters to reveal the inhibitory effect of Cl—channel blockade on fibroblast migration. The velocity of migrating cells (μm/min) was calculated for each time interval by applying a three-point difference quotient. The displacement (μm) is the distance between the position of cells at the beginning and at the end of the experiment.

Cell viability assays

The cell viability was measured by using a MTT Cell Proliferation Assay (Molecular Probes, Taastrup, Denmark). The MTT assay involves the conversion of the water soluble MTT (3,4,5-dimethylthiazole-2-yi)2,5-diphenyle-tetrazolium bromide) to a colored insoluble formazan by dehydrogenase in the metabolically active mitochondria of living cells. The formazan concentration is determined by optical density at 570 nm.

Cells were plated in 96-well plates and grown in normal culture medium to 80% confluency. Before the experiment, the medium was replaced by a serum-free medium, supplemented with NS3728 at concentrations of 400, 500, 800, 1,000, 1,500, 2,000, 2,500, or 3,000 nmol/l. Cells treated with vehicle (DMSO) alone or medium alone without cells served as positive and negative controls, respectively. Cells were exposed to NS3728 for 5 h. Thereafter, 25 μl MTT (5 mg/ml) was added to each well. The reaction was stopped after 1.5 h by the addition of 100 μl lysis buffer (10% SDS in 10 mM HCl). The 96-well plates were kept in the dark at room temperature overnight before the absorbance (ABS) was measured in a Fluostar OPTIMA Plate Reader (Ramcon A/S DK) at 570 nm. Cell viability was assessed by calculating the ratio ABSSample−ABSNegative control/ABSPositive control−ABSNegative control. All experiments were performed in triplicate.
Whole cell recording

For patch-clamp experiments, the cells were grown on coverslips in 40-mm Petri dishes. Membrane currents were recorded from single cells using the fast whole-cell mode of the patch-clamp technique. The isotonic bath solution (300 mOsm/l) contained (in mmol/l) 90 NMDG-Cl, 0 KCl, 1 MgCl₂, 1 CaCl₂, 10 hydroxyethyl piperazineethanesulfonic acid (HEPES), and 110 mannitol, pH 7.4 (pH adjusted with Tris in all solutions for electrophysiology). In the hypotonic bath solution, osmolarity was decreased to 275 or 190 mOsm/l by adjusting the amount of mannitol. The intracellular pipette solution (295 mOsm/l) contained (in mmol/l) 90 CsCl, 2 MgCl₂, 10 ethylene glycol tetraacetic acid, 10 HEPES, 1.5 Na₂-adenosine triphosphate (ATP), 0.1 Na₂-guanosine triphosphate, and 80 mannitol, pH 7.4. The osmolarity of solutions was routinely checked by a freezing-point osmometer (Knaur; Berlin, Germany).

Patch pipettes had resistances of 5–7.5 MΩ, and an Ag⁺/AgCl wire served as the reference electrode. Pipette offsets, series resistances (≤11 MΩ), and capacitive transients were compensated by the patch-clamp amplifier (model EPC7, List Electronic, Darmstadt, Germany). Currents were digitized with an analog-to-digital converter (model 1401+, Cambridge Electronic Design) at 500 Hz and filtered with the built-in four-pole Bessel filter at 3 kHz. Data acquisition and analysis were done with the Cambridge Electronic Design patch- and voltage-clamp software (version 6.41).

The holding potential was −40 mV, and voltage ramps from −80 to +80 mV of 2.6-s duration were applied every 15 s, with a 500-ms prepulse at −80 mV preceding each ramp. Cells were continuously perfused at a rate of 2 ml/min with a complete exchange of the bath solution every 0.5 min. All anion channel experiments were performed at room temperature (18–21°C).

Statistics

Data are presented as representative original experiments or as means±standard error of the mean (SEM). The statistical significance of differences was assessed with paired or unpaired Student’s t test as appropriate. The level of significance was set to p<0.05.

Results

Effect of the VRAC channel inhibitor NS3728 on VRAC current and cell migration

We assessed the possible role of VRACs during cell migration with the acidic di-aryl-urea derivative NS3728, which is a high-affinity VRAC blocker in HEK293 and in Ehrlich Lettre Ascites (ELA) cells [11, 14]. First, we verified that NS3728, when present in the bath solution, likewise inhibits VRAC in both wt and H-ras fibroblasts. The block is concentration dependent. In a single experiment in wt cells as well as H-ras cells, it was confirmed that the IC₅₀ value is similar to the value reported for HEK293 and ELA cells, which is estimated at about 400 nmol/l [11, 14]. In addition, we found in three separate patch clamp experiments 1.4±0.2 and 15±11% inhibition at 100 nmol/l NS3728 and 14±2 and 30±15% inhibition at 200 nmol/l NS3728 in wt and H-ras cells, respectively. We next performed additional control experiments to assess the potential toxicity of NS3728. Viability is unaffected by this inhibitor, even at 2.5 μmol/l for 5 h (Fig. 1). This concentration is several fold higher than the maximal concentration used in the migration and patch-clamp experiments. Thus, NS3728 could be used to evaluate the role of VRAC in the H-ras transformation-induced facilitation of migration, without confounding toxic effects.

H-ras transformation of NIH3T3 fibroblasts enhances the migratory activity in wound-healing assays (see Figs. 2 and 3). Under control conditions, speed and displacement of H-ras fibroblasts are 0.24±0.02 μm/min and 60.5±6.3 μm (n=42), while they are only 0.18±0.01 μm/min and 41.0±4.0 μm (n=39) in wt cells, respectively (see also Fig. 3).

Both H-ras and wt fibroblasts respond with a dose-dependent inhibition of migration when volume-regulated anion channels are blocked by NS3728. The speed of...
Migration and the displacement are reduced when VRACs are inhibited. However, there are distinct differences between the behavior of wt and H-ras cells. Figure 2 shows the trajectories of both cell types under control conditions and in the presence of 400 nmol/l NS3728. The speed is reduced to a similar extent in both cell types: by up to 30% in wt cells and up to 37% in H-ras cells. However, the relative reduction in the displacement of H-ras fibroblasts is much more pronounced than that of wt fibroblasts. The corresponding values are 32 and 60%, respectively. The fact that the displacement is reduced more strongly than the speed of migration indicates that VRAC inhibition also impairs the ability for directed migration of H-ras cells. Moreover, closer inspection of the dose–response curves shown in Fig. 3 indicates that the migration of wt fibroblasts is more sensitive to Cl⁻ channel blockade than that of H-ras fibroblasts. The estimated IC₅₀ values are ~250 and ~350 nmol/l for wt and H-ras fibroblasts, respectively. Thus, more NS3728 is needed to inhibit migration of H-ras cells to a similar degree as wt fibroblasts. Taken together, our results clearly show the necessity of VRAC activity for optimal migration of fibroblasts in a wound-healing assay.

Maximal activation of VRAC currents

Figure 4 shows the maximal activation of VRAC upon reducing the extracellular osmolarity from 300 to 190 mOsm/l. After exposure to a hypotonic medium, a Ca²⁺-independent Cl⁻ current exhibiting moderate outward rectification and minor time-dependent inactivation at depolarized potentials is activated in both H-ras and wt cells. These current properties are characteristic of the swelling-activated Cl⁻...
current found in many cell types and in good agreement with some earlier studies on VRAC in NIH3T3 fibroblasts [9, 14, 19, 24]. The reversal potentials $V_{\text{rev}}$ in both H-ras and wt cells are significantly different from the Nernst potential of $\text{Cl}^-$ for the given solutions. This discrepancy between measured and theoretical values is, as discussed in Riquelme et al. [26], likely to reflect dilution of the intracellular solution as water flow across the plasma membrane exceeds the rate of intracellular exchange with the pipette solution. Such dilution is setup specific and has previously been reported with the used setup [15]. $\text{Cl}^-$ currents were measured over time in both cell types, and the osmolarity of the extracellular solution was changed from 300 to 190 mOsm/l as indicated. The activation kinetics and the maximal amplitude of VRAC currents after severe cell swelling are not different in wt and H-ras fibroblasts. A steady state of current activation is reached approximately 7 min after hyposmotic exposure. Outward current density rises from $2.0\pm0.8$ and $4.8\pm1.7$ to $137\pm10.0$ and $152\pm2.7$ pA/pF ($n=3$) in H-ras and wt cells, respectively.

Activation of VRAC currents by slightly hypotonic solutions

Figure 5 shows VRAC currents in wt and H-ras fibroblasts under isotonic control conditions and after addition of a slightly hypotonic solution (275 mOsm/l; 8.3% hypotonic). It can be seen that after hypotonic exposure, VRAC current rises within 10 min to an approximately 2.5-fold higher level in H-ras than in wt cells. Thus, the increase in migratory activity of H-ras fibroblasts is correlated with a more pronounced VRAC activation upon physiologically relevant changes of cell volume.

Discussion

The main findings of this study are the observations that (1) H-ras NIH3T3 fibroblasts move significantly faster than their wt counterparts, (2) the VRAC inhibitor NS3728 affects migration of H-ras and wt fibroblasts cells differ-

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**Fig. 4** Activation of VRAC by reducing the extracellular osmolality to 190 mOsm/l. **a** $\text{Cl}^-$ currents were measured over time in the whole-cell patch-clamp configuration in wild-type and H-ras-expressing NIH3T3 fibroblasts ($n=3$). The osmolarity of the extracellular solution was reduced after 2 min from 300 to 190 mOsm/l. We plotted the currents measured at holding potentials of +40 and $-55$ mV, respectively. **b**, **c** Original recordings of VRAC currents monitored after maximal activation. The clamp protocol was such that the membrane voltage was clamped in 20-mV steps from $-80$ to $+80$ mV with a holding potential of $-40$ mV. **d**, **e** Original recordings of VRAC currents monitored at 190 and 300 mOsm/l in the absence and presence of the indicated concentrations of NS3728. Voltage ramps of 2.6 s duration were applied from $-80$ to $+80$ mV.
ently, and (3) the volume activation threshold of VRAC in H-ras cells is lower than that in wt cells. The strong correlation between increased migratory activity of H-ras cells and their reduced volume activation threshold of VRAC is consistent with the notion that VRAC is an important component of the cellular migration machinery. The effectiveness of the VRAC blocker NS3728 in inhibiting cell migration suggests that fluctuations of the cell volume play a critical role during migration. When VRAC is inhibited, such fluctuations are no longer possible, and cell volume is clamped at an elevated value. So far, we have not measured the volume of wt and H-ras fibroblasts during migration. This is technically extremely demanding and will be the focus of future studies. However, we determined the projected cell areas of wt and H-ras cells under control conditions and in the presence of 800 nmol/l of the VRAC blocker NS3728. We are aware that these data provide at best only a rough estimation of relative volume changes. NS3728 increased the projected cell area in both cell lines, the effect reaching significance only in H-ras cells (data not shown). With the abovementioned reservations in mind, this would be consistent with cell swelling induced by NS3728.

In slowly migrating cells like fibroblasts, such volume fluctuations probably have a low frequency. Therefore, the delayed activation of VRACs after a slight reduction of the extracellular osmolarity is not in contradiction with an important role of VRACs in cell migration. We and others previously showed that inhibition of Ca^{2+}-sensitive K⁺ channels, the counterpart of VRAC in mediating RVD in many cell types, results in an impairment of cell migration [3, 28, 34, 35] and that this is accompanied by a significant volume increase of migrating cells [29]. If the VRAC blocker affected migration by perturbing the capability of cell volume regulation, NS3728 should inhibit migration to a similar degree as blockers of Ca^{2+}-sensitive K⁺ channels. This is indeed the case, as IK1 channel blockade also reduces migration of fibroblasts by approximately 50% [33].

In the present study, we have demonstrated a clear difference in the ability of NS3728 to impair migration in H-ras and wt cells, such that the VRAC-dependent component of the displacement in H-ras cells amounts to more than 36 μm compared to 14 μm in wt cells. A similar relation between H-ras and wt fibroblasts is also observed for the speed of migration. Importantly, the level of the basal and VRAC-independent component of migration is the same in both H-ras and wt cells. This raises the question of why the VRAC-dependent component of migration is larger in H-ras fibroblasts. We suggest that the increased volume sensitivity of VRAC in H-ras fibroblasts accounts for this difference. Small and physiologically relevant changes of cell volume, as those seen after the 8.3% decrease in the extracellular osmolarity, elicit a much stronger activation of VRAC in H-ras cells than in wt cells. Migration of fibroblasts is dependent on the activity of the Na⁺/H⁺ exchanger NHE1 at the leading edge of the lamellipodium [5, 8, 17]. NHE1 mediates the cellular uptake of solute and water in concert with the anion exchanger AE2 [16] as well as aquaporins [27]. The resulting gain in volume is balanced by the combined action of VRAC and IK1 channels, which restricts isosmotic RVD to the rear part and facilitates the retraction of this cell pole as shown schematically in Fig. 6 [29, 31, 35]. In this scenario, the lowered volume threshold of VRAC in H-ras cells would ease the retraction of the rear part of the migrating fibroblasts.

The fact that the IC₅₀ value for VRAC-mediated inhibition of migration is lower in wt than in H-ras fibroblast is consistent with the model outlined above. The lowered volume threshold of VRAC in H-ras fibroblasts leads to a stronger activation of this channel during migration. Hence, more channel blocker is required to bring

**Fig. 6** Schematic representation of a mechanism by which the combined action of VRACs and IK1 channels supports the retraction of the rear part of migrating fibroblasts.
down VRAC activity to a level that is suboptimal for migration. It is interesting to note that this basal level of migration is very similar in both cell strains, which lends further support to the important role played by VRAC in the process of cell migration. Once VRAC is blocked, further factors like H-ras oncogene-mediated downregulation of integrins [37] and reduced substrate adhesion are no longer effective in promoting the acceleration of migration. Future studies will focus on possible mechanisms by which the expression of the H-ras oncogene leads to a shift of the volume activation threshold of VRAC. Expression of constitutively active RhoA (RhoAV14) also potentiates VRAC activation in NIH3T3 fibroblasts in response to physiological levels of osmotic swelling [24]. Moreover, the fact that VRAC is regulated in an F-actin-dependent manner [15] constitutes another link between the cytoskeletal migration machinery and VRAC-mediated volume changes during migration.

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CHAPTER VII: REACTIVE OXYGEN SPECIES ACTIVATE K, CL CO-TRANSPORT IN NON-ADHERENT EHRLICH ASCITES TUMOR CELLS BUT K+ AND CL- CHANNELS IN ADHERENT EHRLICH LETTRÉ CELLS AND NIH3T3 CELLS


ROS activate KCl cotransport in nonadherent Ehrlich ascites cells but K+ and Cl- channels in adherent Ehrlich Lettre and NIH3T3 cells

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ROS activate KCl cotransport in nonadherent Ehrlich ascites cells but K\(^+\) and Cl\(^-\) channels in adherent Ehrlich Lettré and NIH3T3 cells

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ROS activate KCl cotransport in nonadherent Ehrlich ascites cells but K\(^+\) and Cl\(^-\) channels in adherent Ehrlich Lettré and NIH3T3 cells. Am J Physiol Cell Physiol 297: C198–C206, 2009. First published May 6, 2009; doi:10.1152/ajpcell.00613.2008.—Addition of H\(_2\)O\(_2\) (0.5 mM) to Ehrlich ascites tumor cells under isotonic conditions results in a substantial (22 ± 1%) reduction in cell volume within 25 min. The cell shrinkage is paralleled by net loss of K\(^+\) and Cl\(^-\) channels. Addition of H\(_2\)O\(_2\) to hypotonically exposed cells accelerates the regulatory volume decrease and the concomitant net loss of K\(^+\), whereas no additional increase in the K\(^+\) or Cl\(^-\) conductances could be observed. The H\(_2\)O\(_2\)-induced cell shrinkage was unaffected by the presence of clofilium and clotrimazole, which blocks volume-sensitive and Ca\(^2+\)-activated K\(^+\) channels, respectively, and is unaffected by a raise in extracellular K\(^+\) concentration to a value that eliminates the electrochemical driving force for K\(^+\). On the other hand, the H\(_2\)O\(_2\)-induced cell shrinkage was impaired in the presence of the KCl cotransport inhibitor (dihydro-indenyl)oxyalkanoic acid (DIOA), following substitution of NO\(_3\) for Cl\(^-\), and when the driving force for KCl cotransport was omitted. It is suggested that H\(_2\)O\(_2\) activates electroneutral KCl cotransport in Ehrlich ascites tumor cells and not K\(^+\) and Cl\(^-\) channels. Addition of H\(_2\)O\(_2\) to hypotonically exposed cells accelerates the regulatory volume decrease and the concomitant net loss of K\(^+\), whereas no additional increase in the K\(^+\) and Cl\(^-\) conductance was observed. The effect of H\(_2\)O\(_2\) on cell volume was blocked by the serine-threonine phosphatase inhibitor calyculin A, indicating an important role of serine-threonine phosphorylation in the H\(_2\)O\(_2\)-mediated activation of KCl cotransport in Ehrlich cells. In contrast, addition of H\(_2\)O\(_2\) to adherent cells, e.g., Ehrlich Lettré ascites cells, a subtype of the Ehrlich ascites tumor cells, and NIH3T3 mouse fibroblasts increased the K\(^+\) and Cl\(^-\) conductances after hypotonic cell swelling. Hence, H\(_2\)O\(_2\) induces KCl cotransport or K\(^+\) and Cl\(^-\) channels in nonadherent and adherent cells, respectively.

MAMMALIAN CELLS swell as almost perfect osmometers following exposure to a hypotonic solution, where after they release KCl and organic osmolytes with concomitant cell water to regain the original cell volume and cell function. Dependent of the cell type, swelling-induced KCl loss occurs via separate K\(^+\) and Cl\(^-\) channels, electroneutral KCl cotransport, or coupled Cl\(^-\)/HCO\(_3\) and K\(^+\)/H\(^+\) exchange, whereas the organic osmolytes leave the cell via an efflux pathway which in many cells differ from the swelling-induced Cl\(^-\) channel (12). The fundamental, biophysical, and pharmacological characteristics of the volume-sensitive transporters have recently been extensively reviewed (12, 13, 19, 22, 24).

The production of reactive species (ROS) in various cell types is increased within the first minute following hypotonic exposure (8, 18, 20, 29, 39), and it has turned out that the swelling-induced increase in the ROS production in, for example, HTC (39) and NIH3T3 cells (8), is reduced in the presence of diphenyl iodonium, revealing a role for the NADPH oxidase. ROS are also generated upon hypertonic stress in, for example, HEK293 (44), collecting duct cells (42), and cardiomyocytes (6). ROS are involved in the swelling-induced activation and inactivation of the volume-sensitive release pathway for the organic osmolyte taurine in, for example, NIH3T3 fibroblasts (18, 20) as well as in the activity of the volume-sensitive, outwardly rectifying Cl\(^-\) channel in liver cells (39), HeLa cells (36), and ventricular myocytes (3). Similarly, oxidants have been associated with modulation of the activity of the KCl cotransporter (see Ref. 1), and it has, in the case of NIH3T3 cells, been demonstrated that H\(_2\)O\(_2\) reduces taurine accumulation via TauT (40).

The present investigation was initiated to test whether ROS are general modulators of all volume-sensitive transporters and channels. As ROS-induced anion current has been related to osmotic stretching of \(\beta_1\)-integrin in, e.g., rabbit cardiac myocytes (3), an additional question raised in this paper is whether ROS play an equivalent role in adherent/nonadherent cells. We have used the nonadherent Ehrlich ascites tumor cells because they are well characterized with respect to volume-sensitive transporters as well as the intracellular signaling cascade (12) and its adherent subtype Ehrlich Lettré cells. It is demonstrated that exogenous H\(_2\)O\(_2\) induces KCl loss in Ehrlich ascites tumor cells and that the KCl loss involves an electroneutral, (dihydriodinloyxalkanoic acid (DIOA)-sensitive KCl cotransporter in nonadherent Ehrlich cells but electrogenic K\(^+\) and Cl\(^-\) channels in the adherent subtype of the Ehrlich cells, e.g., Ehrlich Lettré cells and in adherent NIH3T3 mouse fibroblasts.

MATERIALS AND METHODS

Chemicals. Antibiotics (penicillin, streptomycin), DMEM (high glucose, \(\alpha\)-glutamine; Gibco), fetal calf serum (Gibco) and trypsin (10×, Gibco) were from Invitrogen. 5-(and-6)-Carboxy-2',7'-di-chlorodihydrofluorescein diacetate (carboxy-H\(_2\)DCFDA) was from Molecular Probes (Leiden, The Netherlands). \(^{[3]}\)H]polyethylene glycol (PEG) and \(^{[14]}\)Cl]taurine were from NEN, Life Science Products. RPMI-1640 (\(\alpha\)-glutamine) and other chemicals were from Sigma Chemical (St. Louis, MO). The following stock solutions were prepared: carboxy-H\(_2\)DCFDA (50 mM, solvent DMSO), DIOA (100 mM, solvent DMSO), calyculin A (20 \(\mu\)M, solvent ethanol), genistein (4 mg/ml, solvent ethanol), clofilium toxylate (10 mM, solvent water), vanadate (Na\(_2\)VO\(_4\), 20 mM, solvent water), H\(_2\)O\(_2\) (1 M, solvent water), niflumic acid (500 mM, solvent DMSO), and clotrimazole (5 mM, solvent DMSO).
**Cell lines.** Ehrlich ascites tumor cells, grown either in the abdominal cavity of female NMRI mice (Naval Medical Research Institute, propagation of ascites cells was approved by “Dyreforsøgstilsynet” 2007/561-1313) or in RPMI-1640 medium supplemented with 10% serum and 100 U/ml penicillin-streptomycin. In the first case cells were maintained in female NMRI mice by weekly intraperitoneal transplantation (1.5 × 10⁷ cells/mouse) and harvested 6–7 days after transplantation in standard NaCl medium containing heparin (2.5 IU/ml, added to prevent cell cloting), washed twice by centrifugation (700 g, 45 s), and resuspended in standard NaCl medium before incubation at 37°C. In the second case Ehrlich ascites tumor cells were maintained by transfer of 8.5 × 10⁶ cells to 10 ml fresh RPMI-1640 every 3–4 days and grown at 37°C, 5% CO₂, 100% humidity. Ehrlich Lettré ascites cells (ATCC), an adherent subtype of the Ehrlich ascites tumor cells, and NIH3T3 mouse fibroblasts (clone 7) were grown in 75-cm² culture flasks as monolayer cultures in RPMI-1640 and DMEM, respectively, containing heat-inactivated fetal bovine serum (10%) and antibiotics (1% penicillin-streptomycin). Both adherent cell lines were kept at 37°C/5% CO₂/100% humidity and split every 3–5 days by using 0.5% trypsin in phosphate-buffered saline (PBS) to detach the cells.

**Inorganic media.** The PBS contained (in mM) 137 NaCl, 2.6 KCl, 6.5 Na₂HPO₄, and 1.5 KH₂PO₄. Standard NaCl medium used for the Ehrlich ascites tumor cells contained (in mM) 143 NaCl, 5 KCl, 1 Na₂HPO₄, 1 CaCl₂, 1 MgSO₄, and 5 3-(N-morpholino) propane sulfonic acid, 5 N-tris(hydroxymethyl)methyl-2-aminoethane sulfonic acid, and 5 HEPES. NaCl medium for the adherent cell lines contained 10 mM HEPES as buffer system. Hypotonic NaCl medium was prepared by 50% dilution of the isotonic medium with distilled water containing the buffer alone. K⁺-channel equilibrium medium contained 128 mM NaCl and 27 mM KCl. For estimation of the extracellular K⁺ concentration, we used previously published values for the membrane potential (−61 mV) (21). KCl-cotransporter equilibrium medium where the driving force for KCl cotransport is omitted, i.e., the product of the K⁺ and Cl⁻ concentration in the intracellular compartment equaled the product of the K⁺ and Cl⁻ concentration in the extracellular compartment, contained 87 mM NaCl and 68 mM KCl. NaNO₃ medium was prepared by substituting the Na⁺ by using 88 mM NaNO₃ for NaCl and KCl. pH was in all solutions adjusted to 7.4. Media for electrophysiological experiments are specified below.

**Cellular potassium and taurine content.** Ehrlich ascites tumor cells (cell density 4%) were incubated with [¹⁴C]taurine (0.925 kBq/ml) and [²H]polyethylene glycol (PEG) (46.7 kBq/ml) at 37°C. PEG was used for estimation of and correction for extracellular trapped medium. Briefly, aliquots of the cell suspension (1 ml) were transferred to preweighed Eppendorf vials, and the cells were separated from the medium by centrifugation (14,000 g, 45 s). The supernatant (100 µl) was diluted 10 times with perchloric acid (final concentration 7%) and saved for estimation of [¹⁴C]taurine- [²H]PEG activities and potassium content in the extracellular compartment. Excess supernatant was removed by suction, and the Eppendorf vials were reweighed for estimation of the wet weight of the cell pellet. The cell pellet was lyzed in distilled water, vortexed to ensure homogenization, deproteinized by addition of perchloric acid (final concentration 7%), and finally centrifuged (14,000 g, 45 s). The supernatant was used for estimation of [¹⁴C]taurine-[²H]PEG activities and potassium content in the cell pellet. The perchloric acid precipitate was dried (90°C, 48 h) and used for determination of the cell dry weight (see Ref. 21).

[¹⁴C]taurine-[²H]PEG activities were measured in a liquid scintillation spectrometer (Packard, TRI-CARP) using Ultima Gold as scintillation fluid. Potassium was determined by atomic absorption flame photometry (model 2380, Perkin Elmer atomic absorptions spectrophotometer) after 100-fold dilution of samples and potassium standards (0 to 10 mM) with 1 mM CsCl to eliminate sodium interference in the potassium measurements. Cellular water content (ml/mg cell dry wt), cellular taurine activity (cpm/g cell dry wt), and potassium content (µmol/g cell dry wt) were calculated and corrected for trapped extracellular medium using [²H]PEG as marker (see Ref. 21).

**Estimation of ROS production.** Ehrlich ascites tumor cells, harvested from mice were transferred to culture and grown as suspension cells in RPMI 1640 medium with 10% serum and 100 U/ml penicillin and streptomycin at 37°C and 5% CO₂. For estimation of ROS, cells were washed twice with PBS and subsequently incubated in serum-free growth medium containing the ROS-sensitive, fluorescent probe carboxy-H₂DCFDA (25 µM, 1 h). The cells were subsequently washed with isotonic NaCl medium and resuspended in isotonic-hypotonic NaCl medium. ROS estimation was performed on a PTI Ratio Master spectrophotometer, and the experimental solution in the cuvette was continuously stirred by use of a Teflon-coated magnet, driven by a motor attached below the cuvette house. The excitation and emission wavelengths were 490 and 515 nm, respectively, and data were collected every 2 s for 200 s.

**Cell volume measurements.** Absolute cell volumes of Ehrlich ascites tumor cells were estimated by electronic cell sizing in a Coulter Counter MultiCizer 3 using too medium of cell volume distribution curves and latex beads (diameter 15 µm) for calibration (for details see Ref. 21). Volume restoration following hypotonic cell swelling was estimated from the absolute cell volumes as: (Vol_max – Vol)/Vol_max – Vol_iso), where Vol_max, Vol, and Vol_iso are the cell volumes under hypotonic conditions at the time of maximal cell swelling, at time t, and under isotonic conditions, respectively.

**Electrophysiology.** Ehrlich ascites tumor cells were transferred to poly-L-lysine-coated coverslips (25 mm) and mounted on a perfusion chamber, installed on an inverted microscope. All experiments were performed at room temperature with continuous perfusing using a combined gravity-fed and pump suction mechanism effectively exchanging bath solution every 30 s. Bathing solution contained (in mM) 90 NaCl, 1 MgCl₂, 1 CaCl₂, and 10 HEPES. The osmolality was adjusted to 300 using d-mannitol and pH was adjusted to 7.4 with Tris-base. Hypotonic solution was obtained by omission of d-mannitol from the bath solution. Pipettes of 4–7 MΩ in asymmetric patch solutions were pulled from 1.7 OD borosilicate glass capillaries. Pipette solution contained (in mM) 90 CsCl, 2 MgCl₂, 1 EGTA, 1.5 Na2ATP, and 0.1 Na2GTP. pH was adjusted to 7.4 with Tris-base, and osmolarity was adjusted to 295 mosmol using d-mannitol. With the use of the whole cell configuration of the patch-clamp technique current, measurements were performed during linear voltage ramps between −100 mV and 100 mV applied every 15 s. Voltage clamp was performed using an Axopatch 200B amplifier (Axon Instruments, Foster City, CA) with a Ag/AgCl electrode in the bath as reference. Currents were filtered at 1 kHz with the built-in Bessel filter, and capacitance, series resistance, and pipette offset were compensated on the amplifier. Voltage-clamp command pulses were generated and data digitized and stored via Digidata 1200 interface and pClamp 8 software. Ehrlich ascites Lettré cells and NIH3T3 mouse fibroblasts were treated as previously described (16, 31); i.e., cells were allowed to adhere to glass coverslips and subsequently placed on the microchamber of a heat-generating module (Brook, IL) mounted in an inverted microscope, thus granting a constant temperature of 37°C. The gravity-fed in-flow and a peristaltic pump-suctioned drain system were used for total exchange of the solution chamber volume. Patch pipettes were filled with a solution including (in mM) 2 NaCl, 40 KCl, 76 K-gluconate, 1.2 MgCl₂, 10 EGTA, 10 HEPES, 1 ATP, and 0.1 GTP. Osmolarity in the pipette solution was adjusted to 7.4 with Tris-base and d-mannitol, and the pH was set to 7.4 at 37°C using Tris-base. The bath solution contained (in mM) 28 NaCl, 62 Na-gluconate, 5 KCl, 1 MgCl₂, 1 CaCl₂, 10 HEPES, and the mixture was titrated to pH 7.4 with Tris-base. To obtain hypotonic osmolality, the bath solution was left as reported above, giving a value of 190 mosmol/L. For isotonic solutions the same solutions were adjusted to 300 mosmol/L with d-mannitol. To determine K⁺ and Cl⁻ currents following cell swelling the protocol from Riquelme and coworkers (34) was used. Briefly the membrane potential was clamped at either the K⁺ or the Cl⁻ equilibrium potential to isolate the individual fluxes. Record-
ings, at 500 Hz and filtered with a four-pole Bessel filter at 2 kHz, according to the standard whole cell configuration, were compensated for pipette offsets, capacitive transients, and series resistances on the Axopatch 200B amplifier. The acquisition and analysis of data were done using pClamp7 software.

Statistical analysis. Data are presented either as individual experiments that are representative of at least three independent sets of experiments or as mean values ± SE. Statistical significance was estimated by Student’s t-test and one-way analysis of variance (ANOVA)/Tukey-Kramer Multiple Comparisons Test. For all statistical evaluations P values < 0.05 were taken to indicate a significant difference.

RESULTS

ROS induces electroneutral K\(^+\) loss and cell shrinkage in nonadherent cells. Ehrlich ascites tumor cells, suspended in isotonic NaCl medium, shrink more than 20% within 25 min following addition of H\(_2\)O\(_2\) (0.5 mM) to the extracellular compartment (Fig. 1A, Table 1). The H\(_2\)O\(_2\)-induced cell shrinkage is parallel to a net loss of potassium, which is significant within 8 min following addition of H\(_2\)O\(_2\) (Fig. 1B). We have previously demonstrated that exposure to the thiol-alkylating agent NEM induces net loss of KCl and a concomitant reduction in cell volume in the Ehrlich cells (17). From Fig. 1, C and D, it is seen that the H\(_2\)O\(_2\)-induced cell shrinkage is impaired and the concomitant net loss of potassium reduced in the presence of DIOA, which blocks KCl cotransport (35). Furthermore, H\(_2\)O\(_2\) has no effect on cell volume when added to Ehrlich ascites cells suspended in a KCl-cotransport equilibrium medium where the driving force for KCl cotransport is omitted; i.e., the product of the extracellular K\(^+\) and Cl\(^-\) concentrations equals the product of the intracellular K\(^+\) and Cl\(^-\) concentrations (Table 1), or when all cellular and extracellular Cl\(^-\) is substituted by NO\(_3\)^- (data not shown). On the
Table 1. H$_2$O$_2$-activated cell shrinkage in nonadherent Ehrlich ascites tumor cells

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Cell Shrinkage, %</th>
<th>n</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (no H$_2$O$_2$)</td>
<td>2 ± 1</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>H$_2$O$_2$</td>
<td>22 ± 1</td>
<td>13</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>DIOA + H$_2$O$_2$</td>
<td>5 ± 1</td>
<td>3</td>
<td>&lt;0.01†</td>
</tr>
<tr>
<td>Calyculin A + H$_2$O$_2$</td>
<td>−3 ± 2</td>
<td>3</td>
<td>&lt;0.01†</td>
</tr>
<tr>
<td>Vanadate + H$_2$O$_2$</td>
<td>21 ± 1</td>
<td>3</td>
<td></td>
</tr>
</tbody>
</table>

Electrochemical K$^+$ gradient omitted

| H$_2$O$_2$                 | 16 ± 1           | 3 | <0.001*|
| KCl gradient omitted       | −1 ± 1           | 3 |        |

Cell volumes were estimated by electronic cell sizing for Ehrlich ascites cells suspended in standard NaCl medium, K$^+$-channel equilibrium medium (no driving force for K$^+$ through channels), or KCl-cotransport equilibrium medium (no driving force for K$^+$ through electro neutral KCl transporters) before and after 10 min exposure to 0.5 mM H$_2$O$_2$. The K$^+$-channel equilibrium medium contained 128 mM NaCl and 27 mM KCl, and we used previously published values for the membrane potential (−61 mV) (21) for estimation of the extracellular K$^+$ concentration. KCl-cotransport equilibrium medium contained 87 mM NaCl and 68 mM KCl; i.e., the product of the K$^+$ and Cl$^-$ concentration in the intracellular compartment equaled the product of the K$^+$ and Cl$^-$ concentration in the extracellular compartment. Control cells were not exposed to H$_2$O$_2$. Inhibitors dihydro-indenyl)oxyalkanoic acid (DIOA), vanadate, and calyculin A, when present, were added at a final concentration of 100 μM, 200 μM, and 100 nM, respectively. Calyculin A and vanadate had no significant effect on cell volume; i.e., the cell volume after 10 min exposure to 200 μM vanadate and 100 nM calyculin A relative to nontreated control cells 1.04 ± 0.02 (n = 4) and 1.05 (n = 2), respectively. Percent values for cell shrinkage were of the initial values at time 25 min. n indicates the number of experiments. * and †Significantly different from control (no H$_2$O$_2$) and H$_2$O$_2$-treated cells, respectively.

H$_2$O$_2$ is reported to activate volume-sensitive anion channels (VRAC) in numerous cells (3, 36, 37, 39). To test whether H$_2$O$_2$ induces electrogenic K$^+$ and Cl$^-$ ion transport in parallel to the electroneutral KCl cotransporter, we performed current measurements using patch-clamp technique (whole cell configuration). From Fig. 3 it is seen in accordance with previously published data (27) that hypotonic exposure activates Cl$^-$ currents at both positive (open symbols) and negative (filled symbols) potentials. It is noted that the current density at +100 mV is larger than the current density at −100 mV, which reflect outwardly rectification; i.e., VRAC activity (16). However, whereas exposure to H$_2$O$_2$ (0.5 mM) induces a significant reduction in cell volume and K$^+$ content within 8 min in Ehrlich ascites tumor cells under isotonic conditions (Fig. 1B), the H$_2$O$_2$ exposure has no effect on the Cl$^-$ currents (Fig. 3). Similarly, H$_2$O$_2$ does not enhance the Cl$^-$ currents under hypotonic conditions (Fig. 3). Hence, H$_2$O$_2$ induces electro-neutral net loss of KCl in Ehrlich ascites tumor cells. To test whether the Ehrlich ascites tumor cells buffered the H$_2$O$_2$, we measured the ROS production under isotonic and hypotonic conditions in the absence and presence of 0.5 mM H$_2$O$_2$ using a ROS-sensitive fluorescent probe. From Fig. 4 it is seen that the ROS production is not increased following hypotonic exposure. On the other hand, exogenous H$_2$O$_2$ induces a significant increase in the intracellular availability of ROS under isotonic as well as hypotonic conditions (Fig. 4). Hence, the intracellular availability of ROS increases following exogenous H$_2$O$_2$ application, whereas hypotonic exposure in itself does not induce any detectable increase in the cellular ROS production in the nonadherent Ehrlich ascites tumor cells.

ROS induces K$^+$ and Cl$^-$ current in adherent cells. The nonadherent Ehrlich ascites tumor cells are grown and maintained in free suspension. To test whether the previously published ability of H$_2$O$_2$ to evoke VRAC activity could be associated with cells being adherent, we repeated the current measurements on two adherent cell types; i.e., the Ehrlich Lettré cells, which are an adherent subtype of the Ehrlich ascites tumor cells, and the NIH3T3 mouse fibroblasts. From Fig. 5 it is seen that osmotic cell swelling evokes K$^+$ and Cl$^-$...
current in both cell lines, confirming previously published data (16, 31), and that H2O2 potentiates the volume-sensitive Cl\textsuperscript{−}/H\textsubscript{11002} current as well as the volume-sensitive K\textsuperscript{+}/H\textsubscript{11001} current under hypotonic conditions. Furthermore, H2O2 also induces Cl\textsuperscript{−}/H\textsubscript{11002} current under isotonic conditions in Ehrlich Lettré cells; i.e., addition of 1 mM H\textsubscript{2}O\textsubscript{2} increased the Cl\textsuperscript{−}/H\textsubscript{11002} current by 3.0 ± 1.5 pA/pF within 6 min \((n = 3\) independent sets of experiments). Hence, H\textsubscript{2}O\textsubscript{2} activates K\textsuperscript{+} and Cl\textsuperscript{−} channels in adherent but not in free, nonadherent Ehrlich ascites tumor cells. It is noted that the KCl cotransport inhibitor DIOA (100 \(\mu\)M) reduces the regulatory volume decrease (RVD) response in hypotonic NaCl medium in the absence and presence of H\textsubscript{2}O\textsubscript{2} (0.1 mM/0.5 mM). Values are given relative to the isotonic control. The curves represent mean values ± SE of 3 independent sets of experiments. \#Significantly reduced compared with the isotonic control. \*Significantly reduced by H\textsubscript{2}O\textsubscript{2} compared with the hypotonic control with no H\textsubscript{2}O\textsubscript{2}.

**DISCUSSION**

**ROS-mediated KCl transport: adherent versus nonadherent cell lines.** Cell volume restoration in Ehrlich ascites tumor cells following osmotic cell swelling normally involves net loss of KCl via Ca\textsuperscript{2+}-independent K\textsuperscript{+} and Cl\textsuperscript{−} channels and net loss of organic osmolytes via a transport volume-sensitive transport pathway, separate from the Cl\textsuperscript{−} efflux pathway (see Refs. 12 and 19). However, we have previously demonstrated that at pH 7.4 and in the presence of Ca\textsuperscript{2+} in the extracellular compartment a minor fraction (≈8%) of the volume regulatory response is Cl\textsuperscript{−}-dependent in nonadherent Ehrlich cells and that...
activated by exogenous H$_2$O$_2$ in a process that seems to involve ROS, and the thiol-alkylating agent NEM, as well as modulation of protein phosphatase 1 activity (see Refs. 1, 2, 10). The volume-sensitive anion channel in human epithelia HeLa cells during staurosporine-induced apoptosis (36). H$_2$O$_2$ stimulates ion currents and taurine transport in the adherent Ehrlich Lettré tumor cells and NIH3T3 fibroblasts, whereas H$_2$O$_2$ has no detectable effect on electrogenic K$^+$ and Cl$^-$ transport or taurine release in the nonadherent Ehrlich ascites tumor cells. To see whether the preferential reliance on KCl cotransport versus K$^+$ and Cl$^-$ channels in nonadherent (Ehrlich ascites tumor cells) and adherent (Ehrlich Lettré cells) reflects a dramatic downregulation of the expression of KCl in Ehrlich Lettré cells, we have used microarray analysis (2 sets of experiments). Preliminary results (T. Littman and E. K. Hoffmann, unpublished data) indicate that nonadherent as well as adherent Ehrlich cells express mRNA coding for the cotransporters KCC1, 3, and 4 as well as for the K$^+$ channel TASK2 (K2p5.1), which is assumed to represent the volume-sensitive K$^+$ channel in Ehrlich cells (12). We know from previous experiments that TASK2 is highly expressed and active in the nonadherent Ehrlich ascites tumor cells (34), and we therefore assume that transduction of cell volume and hence activation of electroneutral and electrogenic ion transporting pathways differs between the adherent and nonadherent Ehrlich cells. There is evidence that integrins are implicated in cell volume sensing (see Ref. 12), and an interaction among ROS, Rac, focal adhesion kinase (FAK), integrins, and VRAC has previously been suggested (3), we are currently investigating whether the ability of H$_2$O$_2$ to provoke K$^+$ and Cl$^-$ currents in adherent cells is associated with integrins, FAK, and Src kinase signaling.

Fig. 3. Effect of H$_2$O$_2$ on Cl$^-$ current ($I_{Cl}$) in nonadherent Ehrlich ascites tumor cells. Current measurements were performed on Ehrlich ascites tumor cells using patch-clamp technique in the whole cell configuration and linear voltage ramps between −100 mV (closed circles) and 100 mV (open circles) applied every 15 s. The bath solution contained 90 mM NaCl and adjusted to 300 mosmol using d-mannitol. Hypotonic condition, indicated by the bar, was obtained by omitting d-mannitol from the bath solution. H$_2$O$_2$ (0.5 mM) was added as indicated by the bars. Traces are representative of at least 4 sets of experiments.

this fraction increases following reduction of extracellular pH and buffering of Ca$^{2+}$ (17). KCl cotransporters also contribute to the RVD response in adherent Ehrlich cells as evidenced by its sensitivity toward DIOA (see RESULTS). Thus both KCl cotransporters and K$^+$ and Cl$^-$ channels contribute to the volume regulatory process following osmotic swelling, but their fractional contribution depends on the experimental setup. Four KCl cotransporters have been cloned; e.g., KCC1, KCC2, KCC3, and KCC4 (=SCL12A4...A7) and KCl cotransport is reported to be evoked not only by cell swelling (9, 25, 26, 33) but also by high oxygen pressure, acidification, exposure to ROS, and the thiol-alkylating agent NEM, as well as modulation of protein phosphatase 1 activity (see Refs. 1, 2, 10). The KCl cotransporters in the nonadherent Ehrlich ascites tumor cell, which is activated following exposure to thiol-alkylating agent NEM (17), are in the present work demonstrated to be activated by exogenous H$_2$O$_2$ in a process that seems to involve a shift in the serine-threonine directed phosphorylation of the transporter. The serine-threonine protein phosphatases 1, 2A, and 2B control KCl cotransporters; i.e., KCC transport activity is stimulated and attenuated by phosphatase activation and inhibition, respectively (see Ref. 12), and the With-No-Lysine Kinases (WNK) have been suggested to control KCl cotransport activity through regulation of a KCC-regulatory phosphatase activity (4, 11). However, even though consensus sites for phosphorylation by both serine-threonine and tyrosine kinases have been identified in the COOH-terminal domain of the KCCs (43), and two- and three-state models for regulation of KCl cotransport have been proposed (5, 14), direct phosphorylation/dephosphorylation of KCCs after cell volume perturbations has yet to be demonstrated.

H$_2$O$_2$ when added to adherent cell types under hypotonic conditions enhances the volume-sensitive anion current in HTC cells (37) and Ehrlich Lettré/NIH3T3 fibroblasts (Fig. 5), the volume-sensitive taurine release in Ehrlich Lettré cells/NIH3T3 cells (20), as well the volume-sensitive K$^+$ current in Ehrlich Lettré/NIH3T3 fibroblasts (Fig. 5). Furthermore, H$_2$O$_2$ also provokes anion current under isotonic conditions in HTC cells (37, 39) and Ehrlich Lettré cells (see RESULTS), and ROS have been assigned a role in the activation of the volume-sensitive anion channel in human epithelia HeLa cells during staurosporine-induced apoptosis (36). We know from previous experiments that TASK2 is highly expressed and active in the nonadherent Ehrlich ascites tumor cells (34), and we therefore assume that transduction of cell volume and hence activation of electroneutral and electrogenic ion transporting pathways differs between the adherent and nonadherent Ehrlich cells. There is evidence that integrins are implicated in cell volume sensing (see Ref. 12), and an interaction among ROS, Rac, focal adhesion kinase (FAK), integrins, and VRAC has previously been suggested (3), we are currently investigating whether the ability of H$_2$O$_2$ to provoke K$^+$ and Cl$^-$ currents in adherent cells is associated with integrins, FAK, and Src kinase signaling.

Fig. 3. Effect of H$_2$O$_2$ on Cl$^-$ current ($I_{Cl}$) in nonadherent Ehrlich ascites tumor cells. Current measurements were performed on Ehrlich ascites tumor cells using patch-clamp technique in the whole cell configuration and linear voltage ramps between −100 mV (closed circles) and 100 mV (open circles) applied every 15 s. The bath solution contained 90 mM NaCl and adjusted to 300 mosmol using d-mannitol. Hypotonic condition, indicated by the bar, was obtained by omitting d-mannitol from the bath solution. H$_2$O$_2$ (0.5 mM) was added as indicated by the bars. Traces are representative of at least 4 sets of experiments.

Fig. 4. Reactive oxygen species (ROS) production in nonadherent Ehrlich ascites tumor cells. Ehrlich ascites tumor cells were washed two times with phosphate-buffered saline and subsequently incubated in serum-free growth medium containing the ROS-sensitive, fluorescent probe carboxy-H$_2$DCFDA (20 μM, 1 h). The cells were subsequently washed with isotonic NaCl medium and resuspended in either isotonic or hypotonic NaCl medium in the absence or presence of 0.5 mM H$_2$O$_2$. Emission was followed with time 200 s. In all cases the fluorescence increased linearly within the experimental time period, and the slope of the time traces was used as an estimate of the ROS production. ROS production under hypotonic conditions in the absence of H$_2$O$_2$ is given relative to the isotonic control (open bars). ROS production in the presence of H$_2$O$_2$ is given relative to control with the same tonicity but no H$_2$O$_2$ added (closed bars). All values are given as mean values ± SE of 4 sets of paired experiments.

processing of RVD and taurine transport in the adherent Ehrlich Ascites tumor cells and NIH3T3 fibroblasts, whereas H$_2$O$_2$ has no detectable effect on electrogenic K$^+$ and Cl$^-$ transport or taurine release in the nonadherent Ehrlich ascites tumor cells. To see whether the preferential reliance on KCl cotransport versus K$^+$ and Cl$^-$ channels in nonadherent (Ehrlich ascites tumor cells) and adherent (Ehrlich Lettre cells) reflects a dramatic downregulation of the expression of KCl in Ehrlich Lettre cells, we have used microarray analysis (2 sets of experiments). Preliminary results (T. Littman and E. K. Hoffmann, unpublished data) indicate that nonadherent as well as adherent Ehrlich cells express mRNA coding for the cotransporters KCC1, 3, and 4 as well as for the K$^+$ channel TASK2 (K2p5.1), which is assumed to represent the volume-sensitive K$^+$ channel in Ehrlich cells (12). We know from previous experiments that TASK2 is highly expressed and active in the nonadherent Ehrlich ascites tumor cells (34), and we therefore assume that transduction of cell volume and hence activation of electroneutral and electrogenic ion transporting pathways differs between the adherent and nonadherent Ehrlich cells. There is evidence that integrins are implicated in cell volume sensing (see Ref. 12), and an interaction among ROS, Rac, focal adhesion kinase (FAK), integrins, and VRAC has previously been suggested (3), we are currently investigating whether the ability of H$_2$O$_2$ to provoke K$^+$ and Cl$^-$ currents in adherent cells is associated with integrins, FAK, and Src kinase signaling.
Role of ROS in swelling-induced KCl loss. Osmotic cell swelling in Ehrlich ascites tumor cells and NIH3T3 cells elicits sequential activation of a phospholipase A2 [Ca\(^{2+}\)-dependent cPLA\(_2\) in Ehrlich ascites tumor cells (23, 32)/Ca\(^{2+}\)-independent iPLA\(_2\) in NIH3T3 cells (30)], mobilization of arachidonic acid from the nuclear envelope, oxidation of the fatty acid via the 5-lipoxygenase system (5-LO), and subsequently activation of the volume-sensitive leak pathways for the organic osmolytes and K\(^{+}\)/Cl\(^{-}\) by a 5-LO-derived second messenger (12, 19).

ROS, including the nonradical oxygen species H\(_2\)O\(_2\), play an important role in intracellular signaling in nonphagocytes (7, 15, 38), and it has been demonstrated that the ROS production increases within the first minutes following hypotonic exposure in various adherent cell lines, including NIH3T3 fibroblasts (8, 18, 20), Ehrlich Lettré cells (20), skeletal muscle (29), and HTC cells (39). The swelling-induced ROS production involves a NADPH oxidase complex, which in the case of NIH3T3 is constituted by a catalytic NOX4 isotype/p22phox that is activated at a step downstream to the iPLA\(_2\) activation and regulated by protein tyrosine phosphatases, protein kinase and lysophosphatidic acid (8, 18). In the case of rabbit cardiac myocytes it has been proposed by Browe and Baumgarten (3) that osmotic stretching of β\(_1\)-integrin activates the NADPH oxidase and that ROS subsequently triggers the volume-sensitive anion current. It has previously been demonstrated that the swelling-induced increase in the ROS production in the Ehrlich Lettré cells is significantly lower compared with NIH3T3 fibroblasts (20), and in the present work we find that the ROS production in the nonadherent Ehrlich ascites tumor cells is unaffected by osmotic cell swelling. As exogenous H\(_2\)O\(_2\) leads to a detectable increase in the cellular ROS load, we assume that the lack of ROS response in osmotic exposed Ehrlich ascites tumor cells reflects variation in the expression and/or regulation of the NADPH oxidase in this cell type compared with the NIH3T3 cells. Browe and Baumgarten (3) indicated that the volume-sensitive anion current in rabbit cardiac myocytes required NADPH oxidase-derived ROS. However, taking into account that net loss of KCl in Ehrlich ascites tumor cells is almost entirely mediated by separate K\(^{+}\) and Cl\(^{-}\) channels (TASK2, VRAC) (12), that H\(_2\)O\(_2\) provokes no electrogenic K\(^+\)...
or Cl\(^{-}\) transport in these cells, and that no detectable increase in the ROS production occurs following osmotic exposure, it is suggested that ROS are not required for normal volume restoration via K\(^{+}\) and Cl\(^{-}\) channels in the nonadherent Ehrlich ascites tumor cells.

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GRANTS

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CHAPTER VIII: MODULATION OF THE TRANSIENT RECEPTOR POTENTIAL VANILLOID 4 CHANNEL BY 4A-PHORBOL ESTERS. A STRUCTURAL STUDY

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Modulation of the Transient Receptor Potential Vanilloid Channel TRPV4 by 4α-Phorbol Esters: A Structure—Activity Study

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The mechanism of activation of the transient receptor potential vanilloid 4 (TRPV4) channel by 4α-phorbol esters was investigated by combining information from chemical modification of 4α-phorbol-didecanoate (4α-PDD, 2a), site-directed mutagenesis, Ca2⁺ imaging, and electrophysiology. Binding of 4α-phorbol esters occurs in a loop in the TM3–TM4 domain of TRPV4 that is analogous to the capsaicin binding site of TRPV1, and the ester decoration of ring C and the A,B ring junction are critical for activity. The lipophilic ester groups on ring C serve mainly as a steering element, affecting the orientation of the diterpenoid core into the ligand binding pocket, while the nature of the A,B ring junction plays an essential role in the Ca2⁺-dependence of the TRPV4 response. Taken together, our results show that 4α-phorbol is a useful template to investigate the molecular details of TRPV4 activation by small molecules and obtain information for the rational design of structurally simpler ligands for this ion channel.

Introduction

The transient receptor potential (TRP) channel proteins form cation-permeable structures of low evolutionary relation, grouped into seven subfamilies structurally characterized by six transmembrane (TM) domains and a pore region between TM5 and TM6.1–5 Most TRP channels are Ca2⁺ permeable and sense multiple physical stimuli (cold, heat, osmolarity, voltage) as well as a plethora of noxious compounds and intracellular signaling molecules like endocannabinoids and phospholipase C (PLC)- and phospholipase A2 (PLA2)-derived products.1 Recently, evidence has been mounting that TRP channels, and especially those of the TRP vanilloid (TRPV)-type, are involved in a host of human inflammatory pathologies.6 Thus, compounds capable of preventing activation of TRPV1 by classic receptor antagonism or by desensitization have been extensively investigated, and several capsaicinoid agonists and a host of synthetic TRPV1 antagonists have reached clinical studies.7 The large number of TRPV1 ligands from the natural products pool has undoubtedly contributed to the wealth of studies on this channel; while much less is known regarding ligand interactions of related TRP channels (TRPV2–4).8

TRPV4 is widely expressed in epithelial cells and has also been found in the brain, endothelium, liver, and trachea.9 Several lines of evidence have pointed out the involvement of TRPV4 in important pathological conditions such as hypotonic hyperalgesia, thermal hyperalgesia, asthma, and neuropathic pain.10 Currently, the best described small molecule TRPV4 ligands are bisandrogropholide A (BAA, Figure 1), a plant dimeric diterpenoid,11 4α-phorbol-12,13-didecanoate (4α-PDD, 2a), a semisynthetic nontumor promoter phorbol ester,12,13 and GSK1016790A (3), a synthetic peptide.14,15 No recognized structural relationship exists between these compounds, and limited information is available on their structure–activity relationships. 4α-Phorbol esters have been extensively employed in biomedical research as negative controls for the activity of their corresponding phorbol esters, a class of ultrapotent biological analogues of the secondary messenger 2-arachidonylglycerol (2-AG) for the activation of protein kinase C (PKC).16 The discovery that 4α-PDD (2a) strongly interacts with TRPV4 has further emphasized that the phorbol core, a per se biologically inert structure, is a pleiotropic framework for the induction of bioactivity by acylative modification.17

TRPV1 and TRPV4 show some degree of similarity (60% homology, 40% identity) in the TM3–4 region, an element involved in vanilloid binding and activation of TRPV1,18 and site-directed mutagenesis experiments have indeed confirmed the location of a ligand-binding element in this region. Thus, mutation of L584A and of W586A in TM4 of TRPV4 specifically inhibited activation by heat and 4α-phorbol-esters,19 while the mutations Y591A and R594A inhibited activation by all stimuli, suggesting a role in gating rather than in ligand binding.13 The YS motif at the N-terminal end of TM3 in TRPV4 (Y556 and S557) is strongly reminiscent of the capsaicin domain of TRPV1,20 and mutating Y556 to alanine strongly inhibited the activation of TRPV4 by 4α-phorbol-esters.21 This effect correlated with the length of the aliphatic side chains of the 4α-phorbol esters, pointing to the involvement of Y556 in binding to the aliphatic side chain of these compounds.13 Binding of 4α-phorbol-12-myristate-13-acetate (2b), an analogue of 4α-PDD (2a) with potent TRPV4 agonistic activity (EC50 ∼ 3 μM), was strongly affected by the Y556A mutation, while binding of 4α-phorbol diacetate (2c) was unaffected by this mutation,19 suggesting that the long acyl moiety at C-12 is critically involved in binding. Conversely, mutation of S557 to
aliphatic chains, with a substantial dissociation of TRPV1 activation. This was, however, clearly the case because: (i) 2f did not affect [Ca^{2+}]_{i}, Figure 4A). Given the poor activity of the diacetoate 2c, the 12,13-diestearate (2j) turned out to be too lipophilic and insoluble to allow a reliable measurement of activity. These observations show that the relationship between the length of the side chain and the activity is not linear, with two maxima of activity at C-6 and C-10 carbon length, respectively. This differs markedly from the bell-shape curve of potency vs acyl moiety length observed for the activation of PKC by phorbol diesters.16 A bell-shaped relationship also holds for the pungency of vanillamides, an indirect measurement of TRPV1 activation,25 but this relationship is presumably more complex because the introduction of unsaturations has different effects in vanillamides with long (C-18) and medium (C9) length aliphatic chains, with a substantial dissociation of TRPV1 affinity and pungency.25,26

Previous site-selective mutagenesis data have suggested that the binding of 4α-phorbol-diesters to TRPV4-Y556 involves only the C-12 ester groups.19 However, the diester 4α-PDD (2a) and its 13-monoester (2k) showed similar EC_{50} values (370 nM vs 450 nM, Table 1), although the maximal increase in [Ca^{2+}]_{i} was lower than that of the diester 4α-PDD (2a) (Figure 3). The relationship between location and length of the acyl moieties and TRPV4 activation by 4α-phorbol-esters is complex and better accommodated by a model of interaction where the ring C ester groups eventually afforded the 12,13-diesters (Scheme 1), while methanolation of the primary allylic 20-ester groups eventually afforded the 12,13-diesters 2e–2j and the 13-monoester 2k from their corresponding triesters and the diesters.24 In a similar way, the 4-deoxy analogue of 4α-PDD (4c) was obtained from 4α-4-deoxophorbol (4b), a byproduct from the isolation of phorbol from croton oil.22 Finally, lumis-4α-PDD (5) was prepared from 4α-PDD by photochemical [2π + 2π] intramolecular photocycloaddition.22

**Biological Evaluation**

The activation of TRPV4 by 4α-phorbol deriviatives was evaluated by measuring their capacity to elicit an increase in the free intracellular Ca^{2+} concentration ([Ca^{2+}]_{i}) in mTRPV4 transfected HEK 293 cells, while electrophysiological studies were performed in the whole cell patch clamp mode. Because TRPV4 is activated by cell swelling, the osmolarity of this solution was changed by omitting mannitol from an isotonic solution (see Experimental Section).19

**Results and Discussion**

**Ring C Acylation Pattern.** Activity was strongly affected by the acylation pattern of the ring C hydroxyls. Within the series of esters investigated, the most potent compound was the 12,13-dihexanoate (2f) (4α-PDH, EC_{50} = 70 nM, Table 1, Figure 2). The closely related diacetate (2c), diocanote (2g), and dinonanoate (2h) showed only marginal activity at the maximal concentration assayed (50 μM), insufficient to estimate EC_{50} values (Table 1, Figure 2). On the other hand, while the 12,13-dimyristate (2i) showed decreased activity compared to 4α-PDD (2a), the 12,13-distearate (2j) turned out to be too lipophilic and insoluble to allow a reliable measurement of activity. These observations show that the relationship between the length of the side chain and the activity is not linear, with two maxima of activity at C-6 and C-10 carbon length, respectively. This differs markedly from the bell-shape curve of potency vs acyl moiety length observed for the activation of PKC by phorbol diesters.16 A bell-shaped relationship also holds for the pungency of vanillamides, an indirect measurement of TRPV1 activation,25 but this relationship is presumably more complex because the introduction of unsaturations has different effects in vanillamides with long (C-18) and medium (C9) length aliphatic chains, with a substantial dissociation of TRPV1 affinity and pungency.25,26

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We next focused on the characterization of the TRPV4 response by the most potent compound of the series, 4α-PDH (2f) (EC_{50} value = 70 nM, Figure 4A). Given the poor activity of the diacetoate 2g and the dinonanoate 2h, the almost 5-fold-higher potency of the dihexanoate 2f compared to 4α-PDD 2a raised the question whether the observed [Ca^{2+}]_{i} increase indeed reflected TRPV4 activation. This was, however, clearly the case because: (i) 2f did not affect [Ca^{2+}]_{i}, in nontransfected HEK293
cells (data not shown), (ii) the response was dependent on extracellular Ca\(^{2+}\) (Figure 3B), and (iii) the increase in [Ca\(^{2+}\)]\(_i\) was inhibited by the TRP antagonist ruthenium red (RR) (Figure 4C). In whole cell patch clamp studies of mTRPV4 transfected cells, 2f activated currents with outward-inward rectification (Figure 5A) with less pronounced rectification in Ca\(^{2+}\)-free solution (Figure 5B). The current could be reactivated, but exhibited substantial run-down, suggesting washout of an internal factor necessary for TRPV4 activation (Figure 5B).

Site directed mutagenesis has highlighted the relevance of residues L586, W586, and Y556 in 4\(\alpha\)-phorbol ester binding to TRPV4. To assess whether 2f activates TRPV4 by interaction with the same binding pocket as 4\(\alpha\)-PDD (2a), dose–response experiments were performed on wild type, W586A, S557A, and Y556A mTRPV4 constructs expressed in HEK293 cells. Each mutation shifted the dose–response curves to higher concentrations, indicating that, indeed, 2f activates TRPV4 by binding

**Table 1. Affinity Responses for Compounds 2a,e–k, 4c, and 5**

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<th>compd</th>
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<th>WT</th>
<th>Y556A</th>
<th>S557A</th>
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<td>2.79±1.15</td>
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*EC\(_{50}\) values and Hill coefficients where calculated via eq 1 (See Experimental Section). **Values taken from ref 19.*

**Figure 2.** Relationship between EC\(_{50}\) values and side chain length of 4\(\alpha\)-phorbol-diesters. EC\(_{50}\) values were calculated using eq 1. Results for each compound are based on >15 individual cells from at least three independent transfections.

**Figure 3.** Comparison of the effects of 4\(\alpha\)-PDD (2a) and the monoester 4\(\alpha\)-PD (2k) on [Ca\(^{2+}\)]\(_i\) in HEK293 cells. The relative increase in [Ca\(^{2+}\)]\(_i\) (shown as the increase in 340/380 nm fura-2 fluorescence ratio after agonist addition, ∆Fura-2 ratio) was estimated as a function of the bath concentration of 4\(\alpha\)-PDD (2a, black circles) and 4\(\alpha\)-PD (2k, gray triangles). Solid lines represent a fit to the Hill equation. Points are ∆Fura-2 ratio for 18–32 individual cells from three independent transfactions. * indicates significant differences at the level \(p<0.05\).
R binding of capsaicin to TRPV1, its mutation to alanine had TRPV1 equivalent (S512) to S557 in TRPV4 is important for

\[ \text{Ca}^{2+} \]

could still elicit a significantly greater increase in \[ \text{Ca}^{2+} \] activation of TRPV4 by -response curve for the S557A mutation shifted the dose response relationship for HEK 293 cells. (A) Whole cell mTRPV4 current development as a function of time. In \[ \text{Ca}^{2+} \] containing solutions, currents in mTRPV4 transfected cells were monitored via ramp protocols from \(-100 \text{ mV} \) to \(+100 \text{ mV} \). Compound 2f was included in the extracellular solution as indicated by the top bar. The traces are representative of five individual cells in \( >2 \) transfections. Right: I/V relationship of the 2f activated currents. Same experiments as in Figure 4A. The numbers refer to ramps obtained at the times indicated by numbers in left panel. (B) Left: Experiment as in A, except for the absence of \[ \text{Ca}^{2+} \] in the intra- and extracellular solutions. The traces are representative of five individual cells in \( >2 \) transfections. Right: I/V relationship of the 2f activated currents in the absence of \[ \text{Ca}^{2+} \]. Same experiments as in Figure 4C. The numbers refer to ramps indicated by numbers in Figure 4C.

Changes to the A,B Ring Junction.

A,B ring junction is a critical structural feature of the phorboid TRPV4 pharmacophore. To further explore this dependence, 4α-PDD (2a) was subjected to photocyclization, generating its corresponding cage-like lumihorabol derivative (5), while the 4-deoxygenated 4α-PDD analogue (4c) was prepared from 4α-4-deoxyphorbol (4b). Compound 4c was unable to activate TRPV4 (Table 1), suggesting that the 4-hydroxyl is crucial for agonist activation of TRPV4. Conversely, 4α-LPDD (5) outperformed 4α-PDD (2a) in terms of TRPV4 activation, with an almost 2-fold lower EC50 value (175 ± 0.07 vs 370 nM, Table 1 and Figure 7A for 5). At 0.1 \( \mu \text{M} \) concentration, 5 could still elicit a significantly greater increase in \[ \text{Ca}^{2+} \] in mTRPV4 transfected HEK293 cells than did 2a (data not shown, \( p < 0.01 \)). On the other hand, at concentrations above 10 \( \mu \text{M} \), 5 elicited \[ \text{Ca}^{2+} \] responses also in nontransfected cells (not shown). These \[ \text{Ca}^{2+} \] responses were not increased by the expression of TRPV1-3, indicating that 4α-LPDD (5) did not affect related TRP channels (data not shown). However, at less than 10 \( \mu \text{M} \), there was no effect of 5 in
nontransfected cells, neither in Ca\(^{2+}\) imaging experiments (Figure 7B,C) nor in patch clamp studies (data not shown). Furthermore, the [Ca\(^{2+}\)], response to 5 was dependent on extracellular Ca\(^{2+}\), compound 5 (500 nM) and extracellular Ca\(^{2+}\) were present as indicated by the top bars. The figure is representative of three independent experiments. (C) The [Ca\(^{2+}\)], response to 5 (500 nM) is inhibited by ruthenium red (RR, 1 µM). The presence of RR and 5 in the extracellular solution is indicated by the bars. The figure is representative of three independent experiments.

To gain information on the requirements for binding of 5 to TRPV4, dose–response experiments for the 4α-PDD (2a) were carried out on wild type TRPV4 and on W586A, S557A, and Y556A mutant TRPV4. The W586A and Y556A mutations resulted in rightward shifts of the dose–response curve (Figure 9), whereas the S557A mutation had no effect. These observations are in agreement with previously published results for 4α-PDD (2a)\(^{21}\) (Table 1). Because 2a differs from 5 in the headgroup and from 2f only in the length of the acyl groups, the fact that the S557A mutation attenuates binding of 2f but not of 2a and 5 must be related to the acyl decoration of the terpenoid core, seemingly due to a suboptimal orientation of the terpenoid core with S557. Because 4α-PDD (2a) and its lumiphorbol derivative (5) apparently bind the same pocket of TRPV4, the substantially different features of the currents elicited by these ligands suggest, in fact, that...
their terpenoid cores interact in a different way with the ligand binding site of the receptor.

Conclusions

We have provided evidence that 4α-phorbol esters are useful tools to investigate the mechanism of activation of TRPV4 by small molecules. Our findings strongly indicate that the lipophilic side chains on the C-ring, although critical for TRPV4 activation, serve mainly as a steering element for a correct positioning of the terpenoid core into the phorboid binding pocket of TRPV4, where a critical role has been identified for the TM3 YS motif. Moreover, we have demonstrated that the nature of the A,B ring junction plays a major role in binding of 4α-phorbol esters to TRPV4. Finally, the marked difference between 4α-phorbol- and 4α-lumiphoribol derivatives in their effects on TRPV4 activity imply the existence of multiple mechanisms to translate affinity into functional activity for this class of compounds. In this context, the possibility of producing continuous rather than transient TRPV4 activation could have major implications for the therapeutic exploitation of TRPV4 agonists (e.g., bladder dysfunction\textsuperscript{14,28}). Taken together, these results illustrate the potential of combining structure–activity studies and site-directed mutagenesis to map binding pockets of “difficult” proteins, providing information useful to design synthetic small molecule agonists for these proteins.

Experimental Section

Materials. Gravity column chromatography: Merck silica gel (70–230 mesh). IR: Shimadzu DR 8001 spectrophotometer. NMR: Jeol Eclipse (300 and 75 MHz for 1H and 13C, respectively). For 1H NMR, CDCl\textsubscript{3} as solvent, CDCl\textsubscript{3} at 293 K (5 mmol). For 13C NMR, CDCl\textsubscript{3} as solvent, CHCl\textsubscript{3} at 293 K (5 mmol).

Synthesis of 4α-Phorbol Diesters (2e–j) and the 4α-4-Deoxyphorbol Diester (4c): Synthesis of 4α-Phorbol Diesters (2e–j).

Synthesis of 4α-Phorbol Diesters (2e–j) and the 4α-4-Deoxyphorbol Diester (4c). Synthesis of 4α-Phorbol Diesters (2e–j) and the 4α-4-Deoxyphorbol Diester (4c).

Synthesis of 4α-Phorbol Monoster (2k). To a solution of 4α-phorbol (2d, 200 mg, 0.55 mmol) in CH\textsubscript{2}Cl\textsubscript{2}–THF (1:1, 10 mL), hexanoic acid (858 mg, 7.11 mmol, 5 mol equiv), EDC (1.58 g, 8.12 mmol, 6 mol equiv), and DMAP (251 mg, 2.11 mmol, 1.5 mol equiv) were added. After stirring at room temperature for 4 h, the reaction was worked up by the addition of brine and extraction with CH\textsubscript{2}Cl\textsubscript{2}. The organic phase was filtered over neutral alumina (5 g) to remove unreacted hexanoic acid, and the filtrate was evaporated to afford 870 mg of crude 4α-phorbol 12,20-didecanoate. The latter was dissolved in methanol (7 mL), and the solution was brought to pH 2  by the addition of few drops of 65% HClO\textsubscript{4}. After stirring overnight at room temperature, the reaction was worked up by the addition of solid NaHCO\textsubscript{3}, filtration, and evaporation. The residue was purified by gravity column chromatography on silica gel (6 g, petroleum ether–EtOAc 7:3 as eluant) to afford 425 mg (30% from 4α-phorbol) (2f) as a colorless oil.

Synthesis of 4α-Phorbol Diester (2a). To a solution of 4α-phorbol (2d, 200 mg, 0.55 mmol) in CH\textsubscript{2}Cl\textsubscript{2}–THF (1:1, 10 mL), 2,6-dimethoxybenzoic acid (870 mg, 3.14 mmol, 10 mol equiv), EDC (1.58 g, 8.12 mmol, 6 mol equiv), and DMAP (251 mg, 2.11 mmol, 1.5 mol equiv) were added. After stirring at room temperature for 4 h, the reaction was worked up by the addition of brine and extraction with CH\textsubscript{2}Cl\textsubscript{2}. The organic phase was filtered over neutral alumina (2 g) to remove unreacted 2,6-dimethoxybenzoic acid, and the filtrate was evaporated to afford 870 mg of crude 4α-phorbol 12,20-didecanoate. The latter was dissolved in methanol (7 mL), and the solution was brought to pH 2 by the addition of few drops of 65% HClO\textsubscript{4}. After stirring overnight at room temperature, the reaction was worked up by the addition of solid NaHCO\textsubscript{3}, filtration, and evaporation. The residue was purified by gravity column chromatography on silica gel (6 g, petroleum ether–EtOAc 7:3 as eluant) to afford 425 mg (30% from 4α-phorbol) (2f) as a colorless oil.

Synthesis of LUMI 4α-PDD (5). A solution of 4α-PDD (2a) in ethanol was degassed in a flow of nitrogen for 40 min and then irradiated in an immersion well photoreactor with a low-pressure mercury lamp. After 24 h of irradiation, the reaction was evaporated and the residue was purified by gravity column chromatography on silica gel (5 g, petroleum ether–EtOAc 8:2 as eluant) to afford 190 mg (95%) 5 as a colorless oil.

Cell Culture and Transfection. Human embryonic kidney cells, HEK293, were grown in DMEM containing 10% human serum, 2 mM L-glutamine, 2 units/mL penicillin and 2 mg/mL streptomycin, in a 37°C/10% CO\textsubscript{2} incubator. Transient transfection of HEK293 cells with mTRPV4 (accession number Q9EPK8) in the pCAGGS/ires-FGP vector\textsuperscript{29} was performed using Mirus TransIT-293 transfection agent (Mirus Corporation; Madison, WI) and cells were incubated overnight before experiments. For both [Ca\textsuperscript{2+}]-measurements and electrophysiological experiments, the cells were trypsinized and seeded on poly-L-lysine coated coverslips for the day of the experiment. The mTRPV4 expressing cells were identified by GFP expression. GFP-negative cells from the same coverslips were used as untransfected controls.

Solutions. For [Ca\textsuperscript{2+}]-measurements, solutions were constantly perfused with Krebs solution containing (in mM): 150 NaCl, 6 KCl, 1 MgCl\textsubscript{2}, 1.5 CaCl\textsubscript{2}, 10 HEPES, 10 glucose, pH 7.4. Where indicated, CaCl\textsubscript{2} was omitted from the solution. For electrophysiological studies, the extracellular solution contained (in mM): 150 NaCl, 6 CsCl, 1 MgCl\textsubscript{2}, 5 CaCl\textsubscript{2}, 10 glucose, and 10 HEPES, pH 7.4. For Ca\textsuperscript{2+} free experiments, CaCl\textsubscript{2} was omitted from the solution. The pipet solution contained (in mM): 20 CsCl, 100 aspartate, 1 MgCl\textsubscript{2}, 10 HEPES, 4 Na-ATP, 10 K-1,2-bis(2-aminophenoxy)ethane N,N,N′,N′-tetraacetate (BAPTA), pH 7.4. Ca\textsuperscript{2+} was buffered to a free concentration of 200 nM with CaCl\textsubscript{2} as calculated using the software Calcufluo (G. Droogmans) or omitted for Ca\textsuperscript{2+} free pipet solutions.

Electrophysiological Recording. All electrophysiological data were obtained using 400 ms linear ramp protocols between −100 mV and 100 mV after a 50 ms prepulse at −100 mV as previously described.29 Ramps were performed every 2 s, and the cells were kept at a holding potential of −30 mV between ramps. Data were recorded with 2 kHz sample rate using an EPC10 amplifier controlled by PatchMaster software (Heka, Lambrecht, Germany) and filtered at 2.9 kHz using the built-in Bessel filter. Pipets had resistances of 2–4 MΩ in asymmetrical recording solutions and an Ag-AgCl wire was used as reference electrode. Capacitance and 70–80% of the series resistance were electronically compensated.

[Ca\textsuperscript{2+}], Measurements. Cells were preincubated for 30 min at 37 °C with 4 μM Fura-2 AM (TefLab, Austin, TX) in normal growth medium and mounted on the stage of an IX81 inverted microscope (Olympus, Tokyo, Japan). The imaging system consisted of a MT10 filter-based illumination system (Olympus) and a CCD Camera (Olympus). Illumination and imaging was controlled by CellM software (Olympus). [Ca\textsuperscript{2+}], was estimated as the ratio of the emitted fluorescence at 510 nm after excitation at 340 and 380 nm, respectively (fura-2 ratio). The background fluorescence was digitally subtracted from individual experiments before the ratio was calculated.

Data Analysis and Statistics. Electrophysiological data were analyzed using Patchmaster software (HEKA, Lambrecht, Germany), WinACD (Guy Droogmans, ftp://ftp.cc.kuleuven.ac.be/pub/droogmans/winascd.zip) and Origin (OriginLab, Northampton, MA). Fura-2 AM fluorescence ratios were calculated in CellM Software (Olympus) and Origin (OriginLab, Northampton, MA).
ΔFura-2 ratio was calculated as \((R_t - R_{eq})/R_{eq}\), and maximal response plus time of maximal response were analyzed for individual cells. EC_{50} values were calculated using the Hill equation:

\[
R_{\text{max}} = \frac{V_{\text{max}} C^n}{E_{\text{50}}^n + C^n}
\]

where \(R_{\text{max}}\) is the maximal ratio at the given concentration \((C)\), \(V_{\text{max}}\) is the maximal response, \(n\) is the Hill coefficient, and \(E_{\text{50}}\) denotes the concentration eliciting half-maximal response.

Data are summarized as mean ± standard error of the mean (SEM). Individual experiments shown are representative of at least three independent experiments.

**Acknowledgment.** We thank all the members of the Ion Channel Research Laboratory at the KU Leuven for helpful discussions. This work was supported by grants from Interuniversity Attraction Poles Program—Belgian State—Belgian Science Policy (P6/28), the Research Council of the KU Leuven (GOA 2004/07), and the Flemish Government (Excellentsfincence Policy (P6/28), the Research Council of the KU Leuven, F.; Voets, T.; De Ridder, D.; Nilius, B. Deletion of the C-Terminal Calmodulin Binding Site.

**Supporting Information Available:** Full spectroscopic data for all final products. This material is available free of charge via the Internet at http://pubs.acs.org.

**References**


CHAPTER IX: TYROSINE RESIDUES IN TM5 AND 6 ARE INSTRUMENTAL FOR HTRPV4 ACTIVATION


Manuscript in preparation
Tyrosine residues in TM5 and 6 are instrumental for hTRPV4 activation
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Introduction

The Transient Receptor Potential (TRP) channel proteins form cation-permeable structures of low evolutionary relation, grouped into seven subfamilies structurally characterized by six transmembrane (TM) domains and a pore region between TM5 and TM6. Most TRP channels are Ca\textsuperscript{2+} permeable and sense multiple physical stimuli (cold, heat, osmolarity, voltage) as well as a plethora of noxious compounds and intracellular signaling molecules like endocannabinoids and phospholipase C (PLC-) and phospholipase A2 (PLA\textsubscript{2})-derived products. Recently, evidence has been mounting that TRP channels, and especially those of the TRP vanilloid (TRPV)-type, are involved in a host of human pathologies. The multiple stimuli activating TRP channels makes channel-gating a fascinating topic of research. A number of TRP channels have been shown to integrate different activating stimuli (temperature and agonists) into voltage gating of the channel via charged residues in Transmembrane (TM) domains 3-4. Voltage gating is however not recognized as a ubiquitous signal regarding TRP channel gating and e.g. TRPV4 channels have been described as voltage insensitive.

To gain better understanding of TRP channel gating Kung’s group performed a forward mutation study in the yeast ortholog (Yvc1p/ TRPY1) and identified a number of gain of function mutations in TM5 and 6. Interestingly the majority of these gain of function mutations involved aromatic residues which were conserved in several human TRP channels. Although none of these aromatic residues were identified, Myers and coworkers found numerous other gain of function TM5-6 mutations in TRPV1 in a unbiased genetic screen exemplifying the importance of this region in the mammalian TRPV channel family.

Gating of the widely expressed TRPV4 channel is still elusive. However, a number of residues in TRPV4 TM3-4 have been identified as important for activating the channel. We hypothesized aromatic residues in the TM5 and 6 domains might be important for hTRPV4 gating, by analogy to TRPY1. Aromatic residues conserved between TRPV family members were mutated and functionally tested. None of the mutants changed the basal activity but the Y621L mutant showed decreased sensitivity to the TRPV4 agonist 4\textalpha-PDD. Moreover Y702L mutants failed to activate in respect to either 4\textalpha-PDD, heating and cell swelling. These data confirm the importance of the TM5-6 region for gating of TRP channels.

Materials and Methods

Molecular biology: All sequences where retrieved from the NCBI database and transmembrane domains were identified using the online resource Sosui (http://bp.nuap.nagoya-u.ac.jp/sosui/). Aligning TM 5-6 regions was performed using ClustalW2 (http://www.ebi.ac.uk/Tools/clustalw2/) multi allignment program. Site directed mutagenesis where performed by the standard PCR overlap extension method using hTRPV4 from the pCAGGS/IRES-GFP vector.

Cell Culture and Transfection. Human Embryonic Kidney cells, HEK293, were grown in DMEM containing 10% human serum, 2 mM L-Glutamine, 2 units/ml penicillin and 2 mg/ml streptomycin, in a 37 °C/10 % CO\textsubscript{2} incubator. Transient transfection of HEK293 cells with wt hTRPV4 (acc. number Q9EPK8) and mutant DNA (2 μg DNA/ 2 ml medium) in the pCAGGS/Ires-GFP vector was performed using Mirus TransIT-293 transfection agent (Mirus Corporation; Madison, WI) and cells were incubated overnight after transfection.
before experiments. For both $[\text{Ca}^{2+}]_i$ measurements and electrophysiological experiments, the cells were trypsinized and seeded on poly-L-lysine coated coverslips on the day of the experiment. The mTRPV4 expressing cells were identified by GFP expression. GFP-negative cells from the same coverslips were used as untransfected controls.

**Electrophysiological recording.** All electrophysiological data were obtained using 400 ms linear ramp protocols between -100 mV and 100 mV after a 50 ms prepulse at -100 mV as previously described\(^\text{19}\). Ramps were performed every 2 s and the cells were kept at a holding potential of -30 mV between ramps. Data were recorded with 2 kHz sample rate using an EPC10 amplifier controlled by PatchMaster software (Heka, Lambrecht, Germany) and filtered at 2.9 kHz using the built-in Bessel filter. Pipettes had resistances of 2-4 MΩ in asymmetrical recording solutions and an Ag-AgCl wire was used as reference electrode. Capacitance and 70-80% of the series resistance were electronically compensated.

$[\text{Ca}^{2+}]_i$ measurements. Cells were preincubated for 30 min at 37 °C with 4 µM Fura-2-AM (TefLab, Austin, TX) in normal growth medium and mounted on the stage of an Nikon Eclipse Ti inverted microscope (Nikon, Tokyo, Japan). Fura-2 fluorescence was monitored using an EasyRatioPro imaging station (PTI, Seefeld, Germany) and $[\text{Ca}^{2+}]_i$ was estimated as the ratio of the emitted fluorescence at 510 nm after excitation at 340 and 380 nm, respectively ($\text{fura-2 ratio}$). The background fluorescence was digitally subtracted from individual experiments before the ratio was calculated.

**Solutions.**

*Electrophysiology:* For temperature and 4α-PDD sensitivity studies the extracellular solution contained (in mM): 150 NaCl, 6 CsCl, 1MgCl\(_2\), 5 CaCl\(_2\), 10 glucose and 10 HEPES, pH 7.4. For swelling activated currents the extracellular solution contained (in mM): 105 NaCl, 6 CsCl, 5 CaCl\(_2\), 1 MgCl\(_2\), 10 HEPES, 90 D-mannitol, 10 glucose, buffered pH 7.4 with NaOH (320 mOsmol). Cell swelling was induced by excluding manitol (240 mOsmol). The pipette solution contained (in mM): 20 CsCl, 100 aspartate, 1 MgCl\(_2\), 10 HEPES, 4 Na-ATP, 10 K-1,2-Bis(2-aminophenoxy)ethane N,N,N',N'-tetraacetate (BAPTA), pH 7.4. Ca\(^{2+}\) was buffered to a free concentration of 200 nM with CaCl\(_2\) as calculated using the software CaBuf (G. Droogmans). Bathing temperature was controlled using an SC-20 inline solution heater/ cooler (Warner Inst., Hamden, CT). if not otherwise stated experiments were performed at room temperature.

$[\text{Ca}^{2+}]_i$ measurements: Cells were constantly perfused with Krebs solution containing (in mM): 150 NaCl, 6 KCl, 1 MgCl\(_2\), 1.5 CaCl\(_2\), 10 HEPES, 10 Glucose, pH 7.4.

**Results and discussion**

**Aromatic residues are conserved**

To investigate whether aromatic residues in TMS5 and TM6 are important for gating of channels from the TRPV channels family we looked for aromatic residues in TMS5-6 that were conserved between members of the TRP channel family. Alignment of the region TMS5- TM6 for all members of the hTRPV family revealed five conserved aromatic residues, three in TMS5 and two in TM6 (Fig. 1B): hTRPV4-F617 was conserved between all hTRPV channels and hTRPV4-F702 was conserved in all aligned channels as an aromatic residue (Y or F) while hTRPV4-Y621, F624 and F707 were only conserved between HTRPV1-4 but not in the more...
distantly related channels hTRPV5-6. Although Y702 was conserved even in the yeast channel it did not correspond to previously described gain-of-function mutants in yeast\textsuperscript{12, 13} and must represent different importance. Interesting the three conserved residues in TM5 stacked in a helical wheel projection (Fig.1C). π-π interaction between aromatic residues significantly contributes to the stability of \(\alpha\)-helixes\textsuperscript{21, 22} and such structural conservation through hTRPV1-4 could suggest a structural rather than functional effect of these residues. Such a pattern was not seen for TM 6 (Fig. D) and a structural effect of these residues within the domain seems unlikely.

Aromatic residues does not affect basal activity of hTRPV4:

As mutation of residues in TM 5 and 6 in both yeast YVC1\textsuperscript{12, 13} and TRPV1\textsuperscript{14} resulted in gain-of-function mutations we speculated whether mutating identified aromatic residues would affect the basal hTRPV4 activity. hTRPV4 is a constitutively active channel at room temperature due to its temperature sensitive nature\textsuperscript{23, 24}. To investigate the role of aromatic residues in these domains we mutated identified conserved residues to the hydrophobic amino acid leucine and measured the basal current in transfected HEK293 cells at room temperature (20-25 °C). However no significant differences from WT transfected cells were detected with any of the mutated channels (Fig. 2). It must be emphasized, though, that the Y617L mutant tended to have an increased activity. This difference was not significant (P= 0.08m n= 5-7) but it cannot be excluded that further experiments would prove this mutant have increased activity.

Y621L and Y702L mutants are insensitive to 4α-PDD:

To better understand possible consequences of mutating aromatic residues for leucine we activated hTRPV with 2 μM of the archetypical TRPV4 agonist, 4α-PDD (a concentration well above the EC\textsubscript{50} value of 0.2μM\textsuperscript{25}). WT, F617L, F624L and F707L all activated currents (Fig. 3A and D) with the characteristic outward rectification (not shown). However, practically no channel activity was measured in Y621L and Y702L mutants (Fig. 3B-D).

Binding sites for 4α-PDD have previously been described in TM3 and TM4\textsuperscript{18, 19} and hTRPV4-Y621L and Y702L could potentially be activated by higher concentration of 4α-PDD. In [Ca\textsuperscript{2+}], imaging studies using FURA-2, neither cells transfected with Y621L nor Y702L mutants responded to 4α-PDD in concentrations up to 100 μM (not shown). 4α-PDD has a strongly electrophilic headgroup and two hydrophobic sidechains. It has been described to interact with polar residues (Y556 and S557) in TM3\textsuperscript{18, 19} and hydrophobic residues (L584 and W586) in TM4. However, it is hard to imagine 4α-PDD interacting with four TM domains (3-6) and it is feasible that either or both residues (Y621 and Y702) may serve structural and/ or gating purposes as discussed above rather than having a 4α-PDD binding function.

Both Y621L and Y702L are heat sensitive:

To distinguish between 4α-PDD binding and channel gating in Y621L and Y702L mutants we sought to activate hTRPV4 by alternative means. hTRPV4 is a temperature sensitive channel\textsuperscript{23} and heat activation in the absence of 4α-PDD activation would suggest a specific role for the mutated tyrosines in 4α-PDD activation. Previous mutation studies have found a strong link between 4α-PDD activation and temperature sensitivity\textsuperscript{24} and no mutation have yet been described affecting solely 4α-PDD activation. Heat activation was explored by changing the perfusion solution temperature from 14 to 43 °C (Fig. 4A). Both WT and
mutants activated outwardly rectifying currents upon heat stimulation (not shown). This shows that both mutants constitute functional channels in the plasma membrane. Y621L activated currents no different from WT currents. Hence, the mutation has not merely distorted α-helix stability of TM 5 affecting channel gating in general. These results strongly suggest this residue to be involved solely in 4α-PDD activation and possibly binding. This could potentially reveal fascinating insight into the structure of hTRPV4 when combined with information about other 4α-PDD binding sites. However further studies will have to be conducted in order to reveal whether head-group or side-chains bind Y621. Y702L mutants on the other hand activated a current which was significantly reduced at negative potentials when compared to WT currents (Fig. 4B). This suggests Y702L to have a much more profound role in channel gating in general. The importance of an aromatic residue in this position might also be seen from the extreme conservation of aromatic residues in this position as seen in Fig. 1B. Aromatic residues are found in this same position even in TRP channels of other subfamilies like hTRPC1, hTRPA1, hTRPP2 and hTRPML (not shown). Given the low general conservation of TRP channels this bodes for importance.

**Cell swelling**

In addition to agonist and heat activation, hTRPV4 is sensitive to cell swelling via arachidonic acid metabolites. Hence we investigated whether our mutations affected osmotic activation. Cells were exposed to an 80 mOsmol reduction of extracellular osmolarity leading to cell swelling. In WT cells, this leads to significant current activation (Fig. 5A). The same was seen for F617L, Y621L, F624L and F707L mutants (Fig. 5B). The swelling sensitive nature of hTRPV4 Y621L underscores that this residue is solely important for 4α-PDD activation, while other stimuli shows no phenotype resulting from the mutation. As 4α-PDD did not activate 

$[\text{Ca}^{2+}]_i$ signals even at very high concentration in Y621L transfected cells, Y621 must be absolutely essential for 4α-PDD activation.

In two initial experiments with Y702L transfected cells, practically no current activation was seen following hypotonic stimuli (Fig. 5C). If this can be confirmed by additional experiments the Y702L mutations affects all stimuli. This will imply a major importance of this very well conserved aromatic residue for the gating of TRPV4 channels. However this is yet to be confirmed and the activation by heat stresses Y702 is important yet not essential for gating of hTRPV4.

**Conclusion:**

In this study we investigated the role of aromatic residues in the TM 5 and 6 domains. We identified five conserved aromatic residues and mutated these into leucine. Y621L and Y702L mutations showed no sensitivity to TRPV4 agonist 4α-PDD even at very high concentration. While Y621L mutants responded as WT to other stimuli, Y702L mutants shoved diminished activity following heat stimulation and in preliminary studies with cell swelling. We conclude that Y621 is important for 4α-PDD binding while Y702 more generally seems important for TRPV4 gating.
Legends:

**Figure 1:** A: Topology of TRPV4 channels. Six TM domains where predicted from the Sosui resource with N and C terminals facing the cytosol. Location of mutated residues have been highlighted by stars and the pore-loop by red shadow. B: Alignment of the hTRPV channels family TM5-6 regions including yeast TRPY1. Gray shading visualizes suggested TM domains. Yellow shading identifies conserved aromatic residues mutated in this study. C and D: Helical wheel projection of TM 5 (C) and 6 (D) residue position in the transmembrane α-helix. Conserved aromatic residues are shown in yellow as above.

**Figure 2:** Basal currents in cells transfected with hTRPV4 WT or the respective mutant. Currents where obtained at room temperature (n= 4-7 from ≥2 transfections).

**Figure 3:** 4α-PDD activation of hTRPV4. A- C: Current activation in hTRPV4-WT (A), Y621L (B) and Y702L (C) transfected cells upon exposure to 2 µM 4α-PDD. D: Summarized figure of peak current with 2 µM 4α-PDD in the bathing solution. * P< 0.05 , compared to the wild type.

**Figure 4:** Heat activation of hTRPV4: A: Example of heat activation of WT hTRPV4. Cells where initially cooled to 14 °C to close all channels followed by heating to 45 °C via a 45 s heating ramp. Currents where followed continuously. B: Summarized peak currents for heat activated currents in hTRPV4-WT, Y621L and Y702L transfected cells. * P< 0.05 (n= 5 from >2 transfections)

**Figure 5:** Osmotic activation of TRPV4: A: Example of hypotonic current activation in hTRPV4 WT cells. Osmolarity of the extracellular solution where changed from 320 to 240 mOsmol by omitting manitol. B: Summarized peak currents activated by hypertonicity for WT and mutants (n= 4-6 from >2 transfections). C: Experiment representative of 2 independent tries for hypotonic activation of Y702L mutants.

References

Figure 1

A

B

TM 5

hTRPV1 (576): RCMVFYIVLFG-FSTAVTLEDGKNDS (656): KAVFIILLLAYVILTYILLLLLMLIALM---

hTRPV2 (528): QKVILRLDLLIFLLFLYLFLG-FAVALVLSQEMARPE (611): RGMVLLLASYVILTYILLLLLMLIALM---

hTRPV3 (580): QKVILHDVLKLFVYIVFLGL-FGVALASLIECKPKN (643): PILFLLITYVILFLLLLMLIALM---

hTRPV4 (608): KILFKLDFRLYLMLMEIG-YASLVLLNPCANMK (685): PVVFTILLVYLTVILLLLLMLIALM---

hTRPV5 (483): KMKIFGDLMFCHLAVAQILG-FASAFYIIIFQEDPTS (544): PFMFSIVNAFAAFIALMLLLNLFA---

hTRPV6 (484): KMKIFGDLMFCHLAVAQILG-FASAFYIIIFQEDPEE (544): PFMFSITYAFIAATMLMLLLNLIA---

YVC1 (435): AAILYGYFIVSVILILLIALYSTAYQKVIDNADDEY (502): LPPKRAKDLSTVMTVYSPFLLLISVKT---

TM 6

C

D

Arg

Val

Thr

Phe

Leu

Lys

Met

Tyr

Phe

Leu

17

6

13

3

10

19

11

4

15

2

12

5

16

9

8

1

7

14

18

617

621

624

702

707
Figure 2

The figure shows a bar graph representing the normalized current (I/pA) across different genotypes (WT, F617L, Y621L, F624L, Y702L, F707L) at two voltage levels (+80 mV and -80 mV). The graph illustrates the comparison of currents under these conditions, with error bars indicating the standard deviation.
Figure 3

A

WT

4α-PDD

B

Y621L

4α-PDD

C

Y702L

4α-PDD

D

WT

F617L

Y621L

F624L

Y702L

F707L

I (pA)
Figure 4

A

B

WT

Y621L

Y702L

+80 mV

-80 mV

I (pA)

Time (min)