Pharmacological Modulation of the Epithelial Calcium Channel ECaC1

Inaugural Dissertation presented to the High Faculty of Medicine, Ruhr-Universität Bochum, Germany, in order to get the degree of Dr. med.

by
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Copromotor:

Oral Examination:
To my dearest parents.
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ADP</td>
<td>adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>$[\text{Ca}^{2+}]_{\text{cyt}}$</td>
<td>cytosolic calcium concentration</td>
</tr>
<tr>
<td>$[\text{Ca}^{2+}]_{\text{ext}}$</td>
<td>extracellular calcium concentration</td>
</tr>
<tr>
<td>BAPTA</td>
<td>1,2-bis(2-aminophenoxy)ethane-N,N',N',N'-'tetraacetic acid</td>
</tr>
<tr>
<td>CaT</td>
<td>calcium transport protein</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary DNA</td>
</tr>
<tr>
<td>cGK</td>
<td>cGMP dependent protein kinase</td>
</tr>
<tr>
<td>cGMP</td>
<td>guanine-3',5'-cyclic monophosphate</td>
</tr>
<tr>
<td>CHO cells</td>
<td>Chinese hamster ovary cells</td>
</tr>
<tr>
<td>CRAC</td>
<td>$\text{Ca}^{2+}$ release activated $\text{Ca}^{2+}$ channel</td>
</tr>
<tr>
<td>CNG</td>
<td>cyclic-nucleotide-gated</td>
</tr>
<tr>
<td>DAG</td>
<td>diacylglycerol</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagles medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethylsulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>ECaC</td>
<td>epithelial calcium channel</td>
</tr>
<tr>
<td>EGTA</td>
<td>ethyleneglycol-di-β-aminoethylether-N,N,N',N'-tetraacetic acid</td>
</tr>
<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
</tr>
<tr>
<td>GLUT4</td>
<td>glucose transporter 4</td>
</tr>
<tr>
<td>GFP</td>
<td>green fluorescent protein</td>
</tr>
<tr>
<td>GTP</td>
<td>guanine trinucleotide phosphate</td>
</tr>
<tr>
<td>HCN</td>
<td>hyperpolarization-activated cyclic-nucleotide-gated cation channel</td>
</tr>
<tr>
<td>HEK 293 cells</td>
<td>human embryonic kidney 293 cells</td>
</tr>
<tr>
<td>HEPES</td>
<td>N-(hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)</td>
</tr>
<tr>
<td>$I_{\text{CRAC}}$</td>
<td>calcium release-activated calcium current</td>
</tr>
<tr>
<td>$I_{50}$</td>
<td>concentration for half-maximal inhibition</td>
</tr>
<tr>
<td>INAD</td>
<td>inactivation-no-afterpotential D</td>
</tr>
<tr>
<td>IP$_3$</td>
<td>inositol-(1, 4, 5)-triphosphate</td>
</tr>
<tr>
<td>IP$_3$R</td>
<td>inositol-(1, 4, 5)-triphosphate receptor</td>
</tr>
<tr>
<td>kb</td>
<td>kilobase pair(s)</td>
</tr>
<tr>
<td>kDa</td>
<td>kilodalton</td>
</tr>
<tr>
<td>LGCC</td>
<td>ligand-gated non-specific cation channel</td>
</tr>
<tr>
<td>LTB$_4$</td>
<td>leukotriene B$_4$</td>
</tr>
<tr>
<td>LTRPC</td>
<td>long chain TRP channel</td>
</tr>
<tr>
<td>MagNuM Current</td>
<td>magnesium-nucleotide-regulated metal current</td>
</tr>
<tr>
<td>$n_H$</td>
<td>Hill coefficient</td>
</tr>
<tr>
<td>NCX</td>
<td>$\text{Na}^+$/Ca$^{2+}$ exchanger</td>
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NINAC  neither-inactivation-nor-afterpotential C
NMDA   N-methyl-D-aspartate
NMDG⁺  N-methyl-D-glucamine
NPPB   5-nitro-2-(3-phenylpropylamino)benzoic acid
OMIM   online Mendelian inheritance in men
OTRPC  Osm9-like TRP channel
PAM    point accepted mutation
RBL cells  rat basophilic leukemia cells
ROC    receptor-operated cation channel
Pₓ/Pᵧ  permeability ratio
4α-PDD  4α-phorbol-12,13-didecanoate
PDGF   platelet derived growth factor
PDZ    PSD-95, DLG, zonular occludens-1
PKD    polycystic kidney disease
PKC    protein kinase C
PLA    phospholipase A enzyme
PLC    phospholipase C enzyme
PMCA   plasma membrane Ca²⁺ ATPase pump
pS     picosiemens
PSA    prostate specific antigen
RyR    ryanodine receptor
SCaMPER sphingolipid Ca²⁺-release mediating protein of endoplasmic reticulum
SEM    standard error of the mean
SERCA  sarcoplasmic and endoplasmic Ca²⁺ ATPase
SIC    stretch-inactivated channel
SOC    store-operated Ca²⁺ channel
SR     sarcoplasmic reticulum
STRPC  short TRP channel
TRP    transient receptor potential
TRPL   transient receptor potential like
TRP-PLIK transient receptor potential phospholipase C interacting kinase
VOCC   voltage operated Ca²⁺ channel
VR1    vanilloid receptor 1
VRL-1  vanilloid receptor-like 1
1. Introduction

When asked about ion channels, most biologists will probably think at first of the voltage dependent K\(^+\), Na\(^+\) and Ca\(^{2+}\) channels, or maybe the excitatory postsynaptic Na\(^+\)/K\(^+\) (and Ca\(^{2+}\)) permeable neurotransmitter-activated channels. All of these are cation-selective channels. Indeed, calcium channels offer a great field of research. The physiological importance of calcium channels is dramatically shown by their involvement in various (hereditary) diseases of different organ systems, for example the central nervous system, the renal excretion system, the cardiovascular system, just to name a few. This aspect is shortly dealt with in this introduction.

The introduction of this work initially discusses principles of calcium signaling in general, before focusing on the main subject of this work, the transient receptor potential channel family and more specific the epithelial calcium channel. Since this work deals with the pharmacological modulation of the epithelial calcium channel 1 (ECaC1) the introduction also gives an overview of calcium channelopathies.

1.1 General principles of calcium signaling

Calcium is supposed to be one of the most universal intracellular messengers, regulating a whole range of cellular functions such as gene transcription, cell differentiation, cell growth and cell proliferation, egg fertilization, embryogenesis, dorso-ventral axis formation, synaptic signal transduction, excitation-secretion coupling, excitation-contracting coupling, just to name a few (Cohen et al., 1988; Tsien et al., 1991; Hofmann et al., 1994; Zhang et al., 1994). Since it can also activate some intracellular proteases and nucleases, sustained elevated levels of cytosolic free Ca\(^{2+}\) are highly toxic for the
cell. In that sense, Ca\(^{2+}\) is also invariably involved in cell apoptosis and necrosis (Furuya et al., 1994; Waldron et al., 1995). This versatility of calcium regulated processes arises through the use of a calcium-signaling “tool kit”, whereby the Ca\(^{2+}\) ion acts in various contexts of space, time and amplitude (Berridge et al., 1998). Depending on their physiological identity, cells regulate their intracellular processes by combining a specific set of Ca\(^{2+}\) signals. Through its interactions with other signaling pathways the calcium mediated effects are very complex (Berridge et al., 2000).

At rest the cytosolic free calcium concentration ([Ca\(^{2+}\)]\(_{cyt}\)) of most cell types is around 100 nM. Upon stimulus-mediated activation [Ca\(^{2+}\)]\(_{cyt}\) can rise up to about 1-10 µM which is about 10\(^4\) fold higher than at rest. These changes of [Ca\(^{2+}\)]\(_{cyt}\) are the main mechanism for calcium signaling. To control the intracellular calcium concentration various counteracting processes are interacting in the cell. Depending on whether they cause an increase or a decrease of [Ca\(^{2+}\)]\(_{cyt}\), these regulating processes can be divided into “on” and “off” mechanisms (Berridge et al., 2000). In general, the calcium signaling network can be considered as being constituted of the four separate units, schematically represented in figure 1.

Calcium mobilizing stimuli like growth factors, hormones and neurotransmitters are always needed to trigger the Ca\(^{2+}\) signaling process (see 1 in figure 1). These stimuli act by generating Ca\(^{2+}\)-mobilizing signals (2). Thereby “on mechanisms”(3) become activated, allowing Ca\(^{2+}\) ions to flow into the cytoplasm from the extracellular space or from the intracellularly localized endoplasmic reticulum. Once in the cytosol, the Ca\(^{2+}\) ions function as a messenger for stimulation of numerous Ca\(^{2+}\)-sensitive processes (5). To restore the resting state, the “off mechanisms” (6) remove Ca\(^{2+}\) from the cytoplasm by a process mediated by pumps and exchangers.
Figure 1: The calcium signaling network.

Cell stimuli generate calcium mobilizing signals (1 and 2) that act on various ON mechanisms (3 and 4) to trigger an increase in the intracellular concentration of calcium. This calcium rise stimulates various calcium sensitive processes and thereby induces many different cellular pathways (5). The response is terminated by OFF mechanisms that restore calcium to its resting level (6 and 7). Figure adapted from Berridge et al., 2000.

To augment $[Ca^{2+}]_{cyt}$ cells use both external and internal sources of $Ca^{2+}$. On the one hand $Ca^{2+}$ is available from internal stores within the membrane systems of the endoplasmatic reticulum (ER) or the equivalent organelle, the sarcoplasmatic reticulum (SR), typically found in muscle cells (see figure 2). A signal cascade is activated by stimulation of G-protein-coupled receptors that activate phospholipase C that generates inositol-(1,4,5)-triphosphate (IP$_3$) and diacylglycerol (DAG) from phosphatidylinositol-(4,5)-bisphosphate. The intracellular second messenger IP$_3$ binds to the IP$_3$ receptor (IP$_3R$) in the membrane of the ER. IP$_3R$ functions as a channel releasing $Ca^{2+}$ from the ER into the cytosol upon binding by IP$_3$. Calcium release from the
SR is mediated by a direct coupling between a voltage-sensitive dihydropyridine receptor (DHPR) in the skeletal muscle cell membrane and the SR ryanodine receptor (RyR), which functions as the calcium release channel in this compartment. This process is called “Ca$$^{2+}$$ release” (Berridge, 1993; Clapham, 1995).

**Figure 2: Basic concepts of calcium signaling.**
Stimulation of G-protein coupled receptors or receptor tyrosine kinase leads to stimulation of phospholipase C and production of IP$_3$ and DAG from PIP$_2$. IP$_3$ activates the IP$_3$R leading to release of stored calcium into the cytosol. Additionally several calcium influx pathways are available, including voltage-operated channels, receptor-operated channels, ligand-gated channels, mechanosensitive channels and the Na$$^+$$/Ca$$^{2+}$$ exchanger (NCX) working in reverse mode. A special type of receptor operated calcium entry channel is the store-operated calcium entry channel (SOC). DAG activates protein kinase C (PKC) and a calcium entry pathway in some cell-types. Calcium is removed from the cytosol through the action of the plasma membrane calcium ATPase (SERCA). In muscle cells the ryanodine receptor (RyR) is the predominant calcium release channel, activated by the dihydropyridine receptor (DHPR).
Other Ca\(^{2+}\) mobilizing second messengers are cyclic ADP ribose (cADPR), nicotinic acid adenine dinucleotide phosphate (NAADP), sphingolipids and leucotriene B\(_4\) (Bootman et al., 2001). The ability of calcium to activate ryanodine receptors is modulated by cADPR. A related molecule, NAADP, acts on an as yet uncharacterized channel. The mechanism by which sphingolipids increase calcium possibly involves a distinct calcium release channel named sphingolipid calcium-release mediating protein of endoplasmic reticulum (SCaMPER) (Mao et al., 1996). calcium release by LTB\(_4\) is only shown in oocytes and it is unclear whether it plays a significant role in other cell types (Striggow and Ehrlich, 1997).

On the other hand, the extracellular milieu is readily available since for calcium ions a huge concentration gradient exists across the cellular membrane. Together with the negative interior plasma-membrane potential, typically around –60 mV in a resting cell, this electrochemical calcium gradient causes an enormous driving force enabling the passive entry of calcium through various channels upon electrical or agonist-evoked stimulation of the cell. This process is called “Ca\(^{2+}\) entry” and depends on many families of Ca\(^{2+}\) entry channels with different activation mechanisms. In general four main pathways can be distinguished for this Ca\(^{2+}\) influx. First, “voltage operated Ca\(^{2+}\) channels” (VOCCs) open upon membrane depolarization, allowing Ca\(^{2+}\) to flow into the cell. Second, many channels open in response to a preceding receptor activation step and are therefore called “receptor-operated channels” (ROCs). These channels open as a result of binding of extracellular compounds like glutamate, ATP or acetylcholine to a distinct membrane receptor. Third, Ca\(^{2+}\) can flow into the cytosol through “ligand-gated non-specific cation channels”(LGCCs). These channels are directly gated by binding of an activating ligand to the channel. Fourth, a so-called “capacitative Ca\(^{2+}\) entry” can be triggered in which “store operated
Ca\(^{2+}\) channels” (SOCs) are activated by emptying the internal Ca\(^{2+}\) stores.

Magnitude, duration and location of the resulting rise in [Ca\(^{2+}\)\(_{\text{cyt}}\)] will be influenced by Ca\(^{2+}\) pumps, including on the one hand the sarcoplasmic and endoplasmic Ca\(^{2+}\) ATPases (SERCA’s) which pump calcium back into the intracellular Ca\(^{2+}\) stores and on the other hand the plasma membrane Ca\(^{2+}\) ATPases (PMCAs) and Na\(^+\)/Ca\(^{2+}\) exchangers which pump calcium across the plasma membrane to the outside milieu. Furthermore two other important “off mechanisms” include the mitochondrial Ca\(^{2+}\) transporters and channels, and the cytoplasmic and intra-organelle proteins and metabolites that bind Ca\(^{2+}\) (Berridge et al., 1997; Berridge et al., 2000; Clapham, 1995). Both cell organelles mitochondria and Golgi apparatus participate in shaping the Ca\(^{2+}\) signal in the sense of both the amplitude and the spatio-temporal pattern (Jouaville et al., 1995; Budd and Nicholls, 1996). Also, the relative importance of intra- and extracellular Ca\(^{2+}\) sources depends on the cell type and on the stimulus.

1.2 Calcium entry from the extracellular space

1.2.1 Voltage-operated calcium channels

Voltage operated calcium channels (VOCCs) are large transmembrane proteins with a critical role in the regulation of the intracellular calcium concentration. In excitable cells calcium influx is predominately mediated through this type of channel. VOCCs are multimeric channels consisting of a \(\alpha_1\)-, \(\beta\)-, \(\alpha_2\delta\)- and sometimes a \(\gamma\)-subunit and have been extensively characterized (Hofmann et al., 1999a). They mediate a calcium current upon depolarization of the cell membrane above a threshold potential, for instance during an action potential. In order to fulfill their specific voltage-dependent functions,
VOCCs are well equipped with intramolecular tools, including the voltage sensor, constituted by four positively charged membrane spanning segments. Besides the membrane potential however, VOCCs can also be modulated by various hormones, protein phosphatases, protein kinases and drugs. Different biophysical and pharmacological properties (e.g. dihydropyridine sensitivity) of VOCC have led to a classification into five classes, L- T- P/Q-, N- and R-type channels.

Figure 3: Schematic representation of the membrane topology of VOCCs.

The pore forming α1 subunit consists of four domains, each containing six transmembrane domains, S1-6. The voltage sensor of these channels consists of a group of positively charged residues and is located in the S4 domain. The pore loop is located between S5 and S6. Voltage-operated sodium channels have an analogous core structure. On the basis of hydropathy plots it is generally accepted that the TRP coding sequence contains six transmembrane domains with the pore forming region between S5 and S6. This is also the case for voltage-operated potassium channels and cyclic nucleotide-gated channels. Considering the obvious similarity with the domains of VOCCs, it was suggested that four of these TRP subunits are necessary to form a functional channel. The absence of positively
charge residues in the S4 region indicates the lack of a voltage sensor in these channels.

Ten genes have been identified that encode for the $\alpha_1$-subunit. This subunit has a typical structure consisting of four repeated domains containing six transmembrane regions and constitutes the pore-forming unit and the voltage sensor of the channel (figure 3). $\alpha_1$S, C and D encode L-type channels, $\alpha_1$G, H and I encode T-type channels and $\alpha_1$A, $\alpha_1$B and $\alpha_1$E encode P/Q-type, N-type and R-type calcium channels respectively. Furthermore multiple genes have been identified which encode for $\beta$-, $\alpha_2\delta$- and $\gamma$-subunits, adding further complexity to the family of voltage-gated calcium channels.

1.2.2 Ligand-gated cation channels

Especially in neurons ligand-gated cation channel (LGCCs) constitute an important calcium influx pathway. They become activated by direct binding of a ligand (neurotransmitter) to the channel. Among all ligands, glutamate has one of the most important roles, since it is the major excitatory neurotransmitter molecule in the mammalian brain. Glutamate acts via various receptor types, which can be divided into metabotropic and ionotropic receptors. Upon ligand binding to metabotropic glutamate receptors, intracellular signaling cascades are initiated, leading to the indirect modulation of ion channels. In contrast, ionotropic receptors are cation channels, that are directly gated by glutamate.

A notable example of a non-selective but highly calcium permeable channel is the N-methyl-D-aspartate type glutamate-gated cation channel (NMDA receptor) (Dingledine et al., 1999). Three gene families consisting of NR1, NR2A-D and NR3A encode NMDA receptor subunits. Each subunit consists of four hydrophobic transmembrane domains and the pore lining residues are in the M2 region.
Heteromeric assembly of a number of these subunits forms a channel, but it is unknown which and how many of these subunits are necessary to form functional channels in vivo. Interestingly, postsynaptic calcium influx via the NMDA receptor has been observed in a process called long-term potentiation of synaptic transmission in the hippocampus. This is the leading experimental model for the synaptic changes that may underlie learning and memory (Dingledine et al., 1999). The channel is also associated with diseases like epilepsy and neuronal cell death (Collingridge and Watkins, 1994).

1.2.3 Receptor-operated cation channels

In contrast to LGCCs receptor-operated cation channels (ROCs) are typically gated by binding of an agonist to a receptor in the plasma membrane. The receptor is always distinct from the channel protein itself. ROCs can thus be gated by many simultaneous effects resulting from agonist-binding to various cell surface receptors. The best-characterized channels in this group are the cyclic nucleotide-gated non-selective cation channels found in many cell types including mammalian photoreceptor and olfactory cells where they are involved in the transduction pathway of vision and olfaction (Barrit, 1999). One interesting of the many subtypes of ROCs is the group of store-operated channels (SOCs). These channels are activated upon “active” or “passive” depletion of the intracellular Ca$^{2+}$ stores per se, independent of the mechanism of store-depletion. Concerning the signaling mechanism between the empty store and the store operated channel several possibilities have been addressed including a diffusible signal coming from the store (Randriamampita and Tsien, 1993), a physical association between the channel and the IP$_3$R (Kiselyov et al., 1998) or exocytotic supply of channels or regulatory molecules to the plasma membrane (Yao et al., 1999). The best-
described example of these channels is the Ca\textsuperscript{2+} release activated Ca\textsuperscript{2+} channel (CRAC) which was first identified in lymphocytes and mast cells (Parekh and Penner, 1997). Characteristics of this channel include a high selectivity for Ca\textsuperscript{2+} over Na\textsuperscript{+}, feedback inhibition by cytosolic Ca\textsuperscript{2+} and potent block of the channel by di- and trivalent cations such as Zn\textsuperscript{2+}, Ni\textsuperscript{2+}, Gd\textsuperscript{3+} and La\textsuperscript{3+}.

1.2.4 Mechanosensitive cation channels

These channels gate in response to mechanical forces and are thought to form the molecular basis for touch, hearing, balance and osmosensation. They are present in cells that are specialized for detecting mechanical forces in multicellular organisms. Probably, most cell types have this kind of channel and use it to respond to stretch generated during basic cellular responses, such as changes in volume or movement (Gillespie and Walker, 2001). An important progress has been made in the molecular identification of these channel types. From the *Drosophila melanogaster* mechanosensory bristle organ a channel called NOMPC for *no mechanoreceptor potential* was cloned. This channel is needed to generate mechanoreceptor potentials and therefore most probably is a mechanosensitive channel. The insect bristle shows similarities to hair cells of the vertebrate inner ear which might indicate that NOMPC could play a role in vertebrate hearing. It was shown that calcium influx in vertebrate hair cells through a mechanosensitive calcium permeable channel in response to mechanical stimulation is important for modulation and adaptation of the hair cell response to auditory stimuli, but the identity of this channel is still elusive. NOMPC is a member of the TRPC family of ion channels (Gillespie and Walker, 2001). Indeed, other TRP family members have been shown to be mechanosensitive including an osmosensitive and stretch-inactivated channel (SIC) (Suzuki *et al.*, 18...
1999) and OTRPC4 (TRPV4 in the new nomenclature), another osmosensitive channel (Strotmann et al., 2000).

In specialized cells other calcium entry pathways can be found. A notable example is the calcium influx pathway located in the apical membrane of the distal tubule of the kidney (Freeman, 2000; Hoenderop et al., 2002). This pathway will be discussed in detail later.

1.2.5 The sodium/calcium exchanger

In some cell types calcium entry is mediated through the action of the sodium/calcium exchanger (NCX). This transporter as shown in figure 2 exchanges in the “forward mode” three extracellular sodium ions and one intracellular calcium ion. In the “reverse mode” extracellular calcium is imported into the cell in exchange for the export of three intracellular sodium ions. The switch for the forward or reverse mode depends on the electrochemical gradients for sodium and calcium across the plasma membrane. In vascular endothelium, for example, several groups have shown that a reduction of the extracellular sodium concentration increases \([\text{Ca}^{2+}]_{\text{cyt}}\) via an NCX mediated mechanism (Nilius and Droogmans, 2001).

Except for voltage gated calcium channels and several ligand-gated channels such as the NMDA and glutamate receptor, the cyclic nucleotide-gated channels and the TRP and TRPL channel in *Drosophila melanogaster* photoreceptors, very little is known about calcium influx channels. However, with the identification of TRP and TRPL channels a new starting point was set for the molecular identification of calcium influx pathways in both excitable and non-excitable cells.

1.3 Role of calcium in the human body
Calcium is the most abundant cation in our body. It is a major component of the mineral phase of bone and a well known first and second messenger in signal transduction being involved in a variety of cellular processes. Among others calcium triggers muscle contraction and transmitter release and participates in the regulation of cell proliferation, gene transcription and cell death.

Calcium homeostasis in blood and other extracellular fluids is tightly controlled through the actions of calcitropic hormones on bone, kidneys and intestine, namely parathyroid hormone (PTH), 1,25-dihydroxyvitamin D$_3$ (1,25-(OH)$_2$D$_3$) and calcitonin (Bronner, 1997). Due to this interactive process, the organism can respond to dietary fluctuations of nutritional calcium and adapt to the body's demand during processes such as growth, pregnancy, lactation and aging. Disturbances in active calcium reabsorption are most likely accompanied by significant alterations in the overall calcium homeostasis.

The availability of dietary calcium is a critical determinant of calcium homeostasis. In humans, dietary intake of calcium approximates 500-1000 mg/day, and obligatory endogenous losses in stool and urine total about 250 mg/day. In the order of 30% of calcium in the diet must be absorbed to sustain bone growth in children and to prevent postmenopausal bone loss in aging women. (Schron, 1995). To meet the body’s need for calcium, the intestine evolved specialized vitamin D-dependent and –independent mechanisms to ensure adequate intestinal calcium uptake. Intestinal absorption of calcium occurs by a saturable, transcellular process and a nonsaturable, paracellular pathway. In the kidney calcium is reabsorbed throughout its functional unit, the nephron, the principal sites being proximal tubules, thick ascending limbs and distal tubules. The magnitude and mechanisms of this process differ importantly between those segments as does the extend of hormonal regulation. Since the kidneys present
the sole route for excretion of calcium, they play a major role in extracellular calcium homeostasis.

Several diseases have been described which could partly result from disturbances in calcium reabsorption (Lopez-Nieto and Brenner, 1997). In general, these calcium-related disorders can be caused by malfunction of the production and/or action of calcitropic hormones or by disturbances of renal or intestinal calcium transporters. The latter group includes human inherited diseases resulting from mutations in these channels. These diseases are called channelopathies. For some of them the underlying pathophysiological mechanism has been defined at the molecular level.

The importance of 1,25-dihydroxyvitamin D₃ in calcium homeostasis of the body is reflected by mutations in the genes encoding 1α-hydroxylase and a renal enzyme controlling its synthesis. Hypocalcemic vitamin D-resistant rickets for example is a human genetic disease due to target organ resistance to the action of 1,25-dihydroxyvitamin D₃. (Malloy et al., 1997).

Another calcium-related human disease is Dent’s disease, a rare X-linked familial defect of proximal tubules that is characterized by hypercalciuria and in some cases rickets or progressive renal failure (Friedman, 1999). A chromosomal microdeletion led to the identification of a gene encoding a voltage-dependent chloride channel, ClC-5, that is expressed predominantly in the thick ascending limb of Henle which is the major site of paracellular calcium-reabsorption in the kidney. The role of this channel in calcium-reabsorption remains to be established.

Malfunction of ion transporters can lead to hypokalemic alkalosis as seen in Gitelman’s or Bartter’s syndrome. Gitelman’s syndrome is accompanied by hypocalciuria possibly resulting from an indirect activation of the apical NaCl channel in the distal convolute tubule (Scheinman et al., 1999).
Bartter’s syndrome is caused by inactivation mutations in either the bumetanide-sensitive $\text{Na}^+\text{-K}^+\text{-Cl}^-$ cotransporters, the ATP-regulated $\text{K}^+$ channel or chloride channels (Simon et al., 1996 a and b; Antes et al., 1998). The impaired NaCl reabsorption is supposed to result in a lowered electrochemical gradient for paracellular calcium.

Mutations in TRPM6, a new member of the TRPM gene family, are on the basis of autosomal recessive hypomagnesia with secondary hypocalcemia (HSH, OMIM 602014) (Schlingmann et al., 2002; Walder, et al., 2002). TRPM6 is expressed in intestinal epithelia and kidney tubules. The disease HSH is characterized by very low calcium serum levels coming up shortly after birth. Affected individuals show neurologic symptoms of hypomagnesemic hypocalcemia with seizures and muscle spasms during infancy. Untreated, the disorder may be fatal or may result in neurological damage. Hypocalcemia is secondary to parathyroid failure resulting from magnesium deficiency. Normocalcemia and relief of clinical symptoms can be assured by oral administration of high doses of magnesium. The pathophysiology of HSH is largely unknown, but physiological studies indicate a primary defect in intestinal magnesium transport. In some individuals, an additional renal magnesium leak, due to altered magnesium reabsorption in the distal convoluted tubule, was suspected.

In addition to these well described channelopathies it is not unlikely that pathological conditions exist that are linked to calcium channels which have not yet been determined at the molecular level. For a complete picture of calcium channelopathies, we will probably have to wait for the revelation of the molecular structures of these and perhaps other calcium channels.
1.4 The Transient Receptor Potential channel family

It has been more than three decades since the *Drosophila* transient receptor potential (trp) mutant was discovered and named based on its transient rather than maintained response to light (Cosens and Manning, 1969). In the ensuing years, the trp gene was cloned (Montell and Rubin, 1989) and shown to encode a novel receptor-operated cation channel (Montell, 1999). The transient receptor potential channel superfamily (TRP) includes more than twenty related voltage-independent cation channels that vary significantly in their selectivity and mode of activation. They play critical roles in processes ranging from sensory physiology to vasorelaxation and male fertility.

Its members can be divided into three subfamilies (TRPC, TRPV and TRPM) based on sequence homology and functional information (Clapham et al., 2001; Harteneck et al., 2000). All share significant sequence homology and predicted structural similarities, such as six membrane-spanning domains, a pore loop separating the final two transmembrane segments, and similarity in the lengths of the cytoplasmic and extracellular loops (Montell, 2001). The forth transmembrane segment S4 lacks the complete set of positively charged residues necessary for the voltage sensor in many voltage-gated ion channels. Like the nicotinic and glutamate-receptor channels, many of them show no cation selectivity (Clapham, 1995). Most of the members of the TRP superfamily have been expressed in vitro. The three subfamilies show significant similarities to the founding member of this superfamily, *Drosophila* TRP, and as it turns out, there are not only mammalian homologues of TRP. In fact, TRP-related channels are conserved in every metazoan organism that has been subjected to genome-wide sequencing efforts.

Defects in TRP channels have been associated with changes in growth control and one TRP-related protein may be a tumor
suppressor. Moreover, mutations in a member of the TRP superfamily are a common cause of polycystic kidney disease, while defects in another member lead to mucolipidosis, a neurodegenerative disease. These examples already show that TRP proteins are widely expressed in the nervous system, and also in non-excitable cells, where they may be the primary mode of calcium entry.

The TRP-Canonical (TRPC) subfamily (formerly short TRPs or STRPs referring to the relatively short proteins of its members) is comprised of those proteins that are the most highly related to Drosophila TRP. The TRPV subfamily is so named based on the original designation, Vanilloid Receptor 1 (TRPV1), for the first mammalian member of this subfamily (now TRPV1). Formerly the name of this subfamily was OTRPC for Osm-9 like TRPC after the structural and functional similarities with a C. elegans gene (Harteneck. et al., 2000). The name for the TRPM subfamily (formerly long-TRPs or LTRPs describing the relatively long transcripts) is derived from the first letter of Melastatin, the former name (now TRPM1) of the founding member of this third subfamily of TRP-related proteins (Clapham et al., 2001; Harteneck. et al., 2000) To better understand the new nomenclature old and new names of all members of the mammalian TRP superfamily are mentioned in table 1. The phylogenetic tree of the TRP superfamily in figure 5 clearly illustrates the relationship between all known family members (Montell et al., 2002b).
<table>
<thead>
<tr>
<th>Name</th>
<th>Former Names</th>
<th>Proposed Regulation</th>
<th>Tissue Distribution</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>TRPC Subfamily</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TRPC1</td>
<td>TRP1 TRPC1</td>
<td>Store operated?</td>
<td>Heart, brain, testis, ovaries</td>
</tr>
<tr>
<td>TRPC2</td>
<td>TRP2 TRPC2</td>
<td>Store operated?</td>
<td>Vomeronasal organ, testis, heart, brain, sperm</td>
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<td>TRP3 TRPC3</td>
<td>Store operated, DAG</td>
<td>Brain</td>
</tr>
<tr>
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<td>TRP4 TRPC4</td>
<td>Store operated?</td>
<td>Brain, endothel, adrenal gland, retina, testis</td>
</tr>
<tr>
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<td>Brain</td>
</tr>
<tr>
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<td>TRP6 TRPC6</td>
<td>DAG</td>
<td>Lung, brain</td>
</tr>
<tr>
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<td>TRP7</td>
<td>Store operated, DAG</td>
<td>Eye, heart, lung</td>
</tr>
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<td></td>
<td></td>
</tr>
<tr>
<td>TRPV1</td>
<td>VR1 OTRPC1</td>
<td>Heat (43°C), vanilloids, anadamide, PIP$_2$, H$^+$</td>
<td>Trigeminal (TG) and dorsal root ganglia (DRG)</td>
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<td>Heat (52°C), translocation, growth factors</td>
<td>DRG, spinal cord, brain, spleen, small and large intestine</td>
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<td>Skin (keratinocytes), tongue, DRG, TG, spinal cord, brain</td>
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<td>ECaC1 CaT2</td>
<td>Low [Ca$^{2+}$]$_{ovl}$, hyperpolarization</td>
<td>Kidney, duodenum, jejenum, placenta, pancreas</td>
</tr>
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<td>Similar to TRPV5, store-operated (CRAC?)</td>
<td>Small intestine, pancreas, placenta, prostate cancer</td>
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<td></td>
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</tr>
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<td>Mela-statin</td>
<td>Translocation?</td>
<td>Eye, melanocytes</td>
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<td>?</td>
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<td>TRP-p8</td>
<td>Menthol, cold (&lt;25°C), icilin</td>
<td>Prostate, TG, DRG</td>
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</table>
1.4.1 Drosophila melanogaster TRP

The first receptor stimulated cation channels identified at the molecular level were the TRP and TRP-like (TRPL) visual transduction channels in the fruitfly Drosophila melanogaster.

In contrast to vertebrate photoreceptors, visual transduction in the dipterian system involves rhodopsin and is mediated via phospholipase-C (PLC) coupled to a G-protein. Whereas in vertebrates light induces the closure of cyclic nucleotide-gated channels and a subsequent hyperpolarizing membrane potential in photoreceptor cells, PLC-mediated activation of membrane channels evokes a depolarizing membrane current in Drosophila (Hofmann et al., 1999).

Analyzing the components of the light-induced current led to the identification of a Drosophila mutant which showed a transient response to light, compared to the sustained response in wild-type flies. Further investigation showed that these mutants lack a calcium channel named “TRP” for “transient receptor potential”.

In addition to TRP, there exist two other TRP-related proteins in Drosophila, TRP-like (TRPL) and TRPγ, which are also involved in this process (Xu et al., 2000; Phillips et al., 1992). They share 45 to 50% amino acid identity with TRP over the NH₂-terminal 800 to 900 amino acids. Flies devoid of TRPL were originally reported to be indistinguishable from wild type (Niemeyer et al., 1996). However, the light response in trpl mutant flies shows several differences from wild type, including changes in the permeability ratios for several cations (Reuss et al., 1997) and a reduced response to a light stimulus of long duration (Leung et al., 2000). Whole-cell voltage clamp recordings on photoreceptors suggested that TRP is a calcium selective channel while the TRP-like channel is a non-selective calcium permeable channel (Hardie and Minke, 1992; Peretz et al., 1994). Double mutants
lacking both TRP and TRPL are completely unresponsive to light indicating that TRPγ cannot function independently from TRP and TRPL. TRPγ may function in combination with TRPL because the light response is nearly eliminated in trp mutant flies expressing a dominant negative form of TRPγ (Xu et al., 2000).

Until now it is still unclear how TRP, TRPL and TRPγ are activated but genetic analyses have confirmed the importance of the PLC pathway. Initially it was proposed that these channels were gated by the depletion of intracellular calcium stores. However, Drosophila mutants that lack IP3 receptors show a normal response to light which indicates that calcium release is not involved in this process (Raghu et al., 2000; Acharya et al., 1997).

Drosophila visual transduction channels take part in the formation of a spatially well-organized supramolecular signaling complex called transducisome. Proteins active in phototransduction such as TRP, TRPL, rhodopsin, PLC, protein kinase C calmodulin and the NINAC (neither-inactivation-nor-afterpotential C) myosin III are assembled by the scaffolding protein INAD (inactivation-no-afterpotential D). (Huber et al., 1996; Tsunoda et al., 1997). This is a protein consisting of five ~90-amino-acid protein domains referred to as PDZ (PSD-95, DLG, zonular occludens-1). The tight physical coupling of diverse components of the light-induced signaltransduction into a supramolecular assembly is supposed to increase sensitivity of the photoreponse and speed of the signaling process (Tsunoda and Zuker, 1999).
Figure 4: Proposed structure of the TRP Superfamily. TRPs form three groups. All have six transmembrane segments and a pore-forming loop between the fifth and sixth segment. Adapted from Harteneck et al., 2000.

A steadily increasing number of TRP homologues have been cloned from Caenorhabditis elegans, Drosophila, chicken and from mammalian genomes over the past few years. All isoforms share a predicted topology of six transmembrane segments (S1-S6) with a short hydrophobic stretch between transmembrane segment 5 and 6, supposed to be the pore-forming region (figure 4). This six transmembrane topology is similar to the core structure of the pore-forming subunits of voltage-gated Na\(^+\), K\(^+\) and Ca\(^{2+}\) channels, and also those of cyclic-nucleotide-gated (CNG) cation channels and hyperpolarization-activated cyclic-nucleotide-gated (HCN) cation channels and the polycystins (PKD) (Harteneck et al., 2000). By analogy with voltage-gated Na\(^+\) and Ca\(^{2+}\) channels, which have four linked domains of six transmembrane segments, it is likely but not yet shown that TRP channels are also formed of four single subunits.
Figure 5: Phylogenetic relationship between members of the TRP superfamily. The tree was calculated using the neighbor-joining method and human, rat, and mouse sequences. Adapted from Clapham et al., 2001.
1.4.2 The TRP-Canonical subfamily - TRPC

This subfamily consists of two *Drosophila* homologues (TRP and TRPL) mentioned before, seven mammalian TRP channels named TRPC1-7 and four *C. elegans* homologues (CeSTRPC1-4). Members of this subfamily are the most highly related to *Drosophila* TRP (Montell et al., 2002b). The tissue localization of TRPCs differs greatly among family members. Some are present in a wide range of tissues (TRPC1), whereas others are highly localized (TRPC2, TRPC3 and TRPC5). TRPC3, TRPC4 and TRPC5 are almost exclusively or strongly localized to the brain (Harteneck et al., 2000; Montell et al., 2002a). In their C-terminal tail these channels show a similar sequence in the highly conserved 25-amino acid segment of unknown function called the TRP domain with its invariant TRP box (Glu-Trp-Lys-Phe-Ala-Arg) (Montell, 2001). The activation mechanism after PLC stimulation for many of the TRPC channels remains unclear and controversial. Both store-depletion dependent and store-depletion independent mechanisms have been described, often for the same channel (see table 1). In contrast to the channels from *Drosophila*, data for the mammalian TRPCs come almost exclusively from heterologous expression systems. Within the mammalian TRPC subfamily, there is close similarity between TRPC3, TRPC6 and TRPC7, and between TRPC4 and TRPC5. Several TRPC proteins, such as TRPC1, -2, -4 and -5, appear to be activated through a store operated mechanism because application of IP₃ or thapsigargin results in increases on cation influx in tissue culture cells expressing any one of these proteins (Zhu et al., 1996; Philipp et al., 1996; Vannier et al., 1999; Zitt et al., 1996). The first knockout-mouse model involving mammalian TRP channels was published for TRPC4 (Freichel et al., 2001). In contrast to the wildtype, mouse vascular endothelial cells (MAECs) from
homozygous TRPC4 knockout mice lacked store-operated calcium currents. The absence of the SOC function by TRPC4 markedly reduced agonist-induced calcium entry resulting in a significant decrease of endothelium-dependent vasorelaxation of blood vessels. This observation shows that TRPC4 is an indispensable component of store-operated channels in native endothelial cells essentially contributing to the regulation of blood vessel tone.

TRPC1 was the first member of the mammalian TRP family purported to form an ion channel. In the initial study, expression of TRPC1 gave rise to a linear non-selective cationic current activated by the depletion of calcium stores (Zitt et al., 1996). In a more recent study, (Lintschinger et al., 2000) found no evidence of store-dependent regulation of TRPC1, but reported activation of the channel by DAG in calcium-free solution.

The TRPC subfamily most closely related to TRPC1 comprises TRPC4 and TRPC5. These three channels can be found in mammalian brain where they represent subunits of heteromeric neuronal channels. With co-immunoprecipitation analysis it was shown that TRPC1 is capable of forming functional heteromultimers with either TRPC4 or TRPC5 similar to the transducisome of Drosophila photoreceptors. Co-expression of either TRPC4 or TRPC5 with TRPC1 in tissue culture cells results in the production of nonselective cation conductances distinct from those generated by expression of the individual proteins (Strübing et al., 2001). In these experiments expression of TRPC1 cells in HEK 293 alone did not elicit any current. Currents in TRPC5 and TRPC1/TRPC5 expressing HEK293 cells could be activated by receptors that engage G\textsubscript{q} proteins and not by store-depletion. The current properties were however significantly different between TRPC5 and TRPC1/TRPC5 expressing cells (Strübing et al., 2001). These observations raise the question whether
the heterologously expressed channels are the channels that function in vivo.

In contrast to some TRPC channels that may be SOCs (store operated channels), other TRPC proteins, notably TRPC6 and –7, are activated in vitro by diacyl glycerol (DAG) (Hofmann et al., 1999; Okada et al., 1999). Like TRPC3 these channels are inwardly and outwardly rectifying and have a relatively low selectivity for calcium and sodium (Hofmann et al., 1999; Okada et al., 1999). Their relatively high expression levels in smooth muscle and heart cells make them promising candidates for the as yet unidentified non-selective cationic channels in these muscle cells. The fact that TRPC6 is an essential part of the α1-adrenoreceptor-activated cation channel in rabbit portal vein myocytes supports this idea (Inoue et al., 2001).

Most information is available about TRPC3. According to (Hofmann et al., 1999) activation of TRPC3 depends on production of DAG, whereas another study by (Zhu et al., 1996) indicates that TRPC3 is store-operated. Evidence has also been presented that calcium release via another calcium release channel, the ryanodine receptor (RyR), can also lead to activation of TRPC3 since distinct TRPC3 channels appeared to be functionally coupled to either the ryanodine receptor or the IP3R, (Kieselyov et al., 2000). A similar functional assembly could be observed for TRPC4. This channel is linked to a PLC isoenzyme by a protein called “regulatory factor of the Na+/H+ exchanger” (NHERF). NHERF contains two PDZ domains and is itself linked to the actin cytoskeleton. (Tang et al., 2000). In this case however there is no evidence that this interaction might have a functional impact on the channel. The disparate observations that TRPC3 may be store operated in some studies and gated by DAG in others may reflect differences in the cell types used for the expression studies: human embryonic kidney (HEK)- and Chinese hamster ovary (CHO)-derived cell lines respectively. Different cell lines may express
distinct sets of endogenous proteins that interact with TRPC3 and affect its mode of regulation. Therefore it is critical to characterize the modes of regulation controlling TRPC proteins in vivo.

TRPC2 shares ~30% sequence identity with the TRPC3, TRPC6 and TRPC7 subfamilies. In mouse and rat tissue several amino terminal splice variants have been found but in humans TRPC2 seems to be a pseudogene. TRPC2 protein has been localized to neuronal microvilli in rat vomeronasal organ (Liman et al., 1999) and in the head of mouse sperm (Jungnickel et al., 2001). Heterologous expression of TRPC2 in HEK 293 cells showed that the channel could be activated by store depletion (Vannier et al., 1999). The functional role of TRPC2 could be elucidated in male mice (Stowers et al., 2002). In mammals the vomeronasal organ is thought to mediate social behaviors and neuroendocrine changes elicited by pheromonal cues. Pheromones are a discrete class of chemical cues that signal the sex and the social status of an individual and promote changes essential for breeding and aggression among conspecifics. Genetic ablation of TRPC2 in male mice eliminated the sensory activation of vomeronasal organ neurons by pheromones leading to loss of sex discrimination and male-male aggression in TRPC2−/− male mice. These observations proposed that TRPC2 is involved in mammalian pheromone signaling (Stowers et al., 2002).

1.4.3 The TRP-Vanilloid subfamily - TRPV

The name of this subfamily is based on the original designation, Vanilloid Receptor 1 (VR1), for the first mammalian member of this subfamily (now TRPV1) (Montell et al., 2002b). At present, this group has six mammalian members grouped into two subfamilies. One subfamily includes the temperature sensitive channels TRPV1, 2 and 4 and the recently identified TRPV3 (Smith et al., 2002; Xu et al., 2002).
The calcium selective channels TRPV5 and TRPV6, also referred to as ECaC1 (or CAT2) and CaT1(or ECaC2) respectively, are members of the other subfamily (see figure 5) and will be discussed later. A summary of functional properties of these channels is given in table 1. The proteins of the TRPV subfamily typically contain three ankyrin repeats and share ~25% amino acid identity to TRPC proteins over a span that includes transmembrane segments 5 and 6 and the TRP box.

The first identified member is osm-9, a C. elegans protein (Colbert et al., 1997), which is expressed in a subset of sensory neurons. Behavioral studies indicate that osm-9 is involved in responses to odorants, high osmotic strength and mechanical stimulation. When expressed in mammalian cells this non-selective cation channel is activated by a decrease in osmolarity (Liedke et al., 2000). But unfortunately, there are neither functional studies of the channel in C. elegans neurons nor successful measurements of channel activity following expression in heterologous systems (Harteneck et al., 2000).

Mammalian homologues that belong to this family include the vanilloid receptor 1 (TRPV1) and vanilloid receptor-like 1 (TRPV2) that are both involved in the transduction of peripheral tissue injury and/or pain-producing heat in sensory nerves (Caterina et al., 1997; Caterina et al., 1999; Tominaga et al., 1998).

TRPV1 is a cation channel with significant preference for divalent cations such as calcium and magnesium (Caterina et al., 1997) and is expressed in dorsal root, in sensory ganglia and in the dorsal horn of the spinal chord. A special characteristic of TRPV1 is that it can be activated through a surprisingly broad range of stimuli. The channel was identified through an expression cloning strategy using vanilloid compounds such as capsaicin that are present in spicy foods (i. e., hot chili peppers) to activate the channel resulting in
increases in intracellular free calcium (Caterina et al., 1997). In addition, moderate heat (≥43°C) and low extracellular pH (≤5.9) can activate TRPV1. Since the channel is expressed in nociceptors it was strongly suggested that it is involved in nociception. Indeed, analysis of vanilloid receptor knock-out mice confirmed that the channel contributes to the detection and integration of painful chemical and thermal stimuli that could cause tissue damage (Caterina et al., 2000). Tissue injury generates endogenous proalgesic factors that heighten our sense of pain by increasing the response of sensory nerve endings to noxious stimuli. Therefore it was interesting to observe in vivo that the sensitivity of TRPV1 to capsaicin or heat is potentiated by agents that change in concentration at sites of inflammation or ischemia as protons, bradykinin and nerve growth factor (Chuang et al., 2001). The effects of bradykinin and nerve growth factor on TRPV1 appear to occur through activation of PLCγ, which in turn relieves phosphatidylinositol-4,5-bisphosphate (PIP2)-mediated inhibition of TRPV1 (Chuang et al., 2001). This is an intriguing observation as it may explain the mechanism through which bradykinin and nerve growth factor promote sensitization to pain. Furthermore, these studies raise the possibility that similar mechanisms involving release of channel inhibition by PIP2 may account for the activation of other members of the TRP superfamily that are gated in a PLC-dependent manner. In vitro studies indicate that Drosophila TRPL is inhibited by PIP2. Whether TRPL is inhibited by PIP2 in vivo and whether PIP2 binds directly to members of the TRP superfamily remains to be determined (Runnels et al., 2001). One interesting observation was done by Shin et al. It was shown that bradykinin excites sensory nerve endings by activating TRPV1 via production of 12-lipoxygenase metabolites of arachidonic acid (Shin et al., 2002). Therefore it seems likely that interactions between the protein kinase C and PLA2/lipoxygenase pathways could play a role in bradykinin-induced
effects on TRPV1 (Shin et al., 2002). The observation that a single molecular entity, TRPV1, responds to each of these factors indicates that the integration of highly diverse chemical and physical stimuli that elicit pain occurs through one common pathway. In addition to playing a role in nociception, the high permeability exhibited by TRPV1 strongly desensitizes capsaicin-operated responses (Szallasi and Blumberg, 1999). This property partially accounts for the antinociceptive activity exhibited by vanilloids. Several studies suggest that arginine-rich peptides are channel blockers of TRPV1 with in vivo analgesic activity and may constitute a novel family of analgesic molecules (Planells-Cases et al., 2000).

The vanilloid receptor-like channel 1 (TRPV2) is 50% identical to TRPV1. Next to its expression in neurons of sensory ganglia TRPV2 is present in brain, spinal cord, spleen and lung. The channel is not activated by capsaicin, but by noxious heat with a threshold of >52°C (Caterina et al., 2000). Furthermore, TRPV2 can also be activated, at least in vitro, through regulated translocation of the protein from an intracellular compartment to the plasma membrane in response to either insulin-like growth factor-1, PDGF, or the neuropeptide head activator (Boels et al., 2001). Though the mechanisms underlying the regulated translocation of TRPV2 remain unclear, it appears to be dependent on the activity of phosphatidylinositol-3-kinase, as is the translocation of GLUT4.

Another related TRPV protein is a truncated isoform of TRPV1, supposed to be a stretch-inactivated channel (SIC) and activated by cell shrinkage in hypertonic conditions (Suzuki et al., 1999).

TRPV4 is a human non-selective cation channel which can be activated by changes in volume, a feature which was not expected to be associated with TRP channels (Liedke et al., 2000; Strotmann et al., 2000, Wissenbach et al., 2000). Recently it was shown that this channel can be activated by heat as well however lower temperatures
are needed than those required to activate TRPV1 (Güler et al., 2002). Activation of the channel enables large inward currents and calcium entry in HEK 293 cells. Heat evoked TRPV4-mediated responses are greater in hypo-osmotic solutions and reduced in hyperosmotic solutions. Since TRPV4 immunoreactivity could be observed in anterior hypothalamic structures involved in temperature sensation and the integration of thermal and osmotic information these functional properties might implicate TRPV4 as a possible transducer of warm stimuli within the hypothalamus. (Güler et al., 2002). It could also be shown that TRPV4 channels are activated by 4\(\alpha\)-PDD, a phorbol derivative, independently from protein kinase C, in a manner consistent with direct agonist gating of the channel (Watanabe et al., 2002). This lipophilic ligand will be a valuable pharmacological tool to characterize endogenous swelling-activated currents that may be carried by native VRL2/TRP12 (TRPV4) channels.

Very recently, a novel TRP channel called vanilloid receptor-like protein 3 (VRL3, also known as TRPV3 in the newly defined TRP gene nomenclature) was identified (Peier et al., 2002; Smith et al., 2002; Xu et al., 2002) (figure 6).
Together with TRPV1 to which it shows a high degree of structural as well as functional homology this channel colocalizes on the human chromosome 17 a feature that was previously noted for TRPV5 and TRPV6 and helped in the search of this heat-sensitive channel (Smith et al., 2002). The observations of co-expression of TRPV3 and TRPV1 in individual sensory neurons an the biochemical evidence for association between the two proteins, gives rise to a hypothesis that TRPV3 may contribute subunits to vanilloid receptor-like ion channels. Heteromerization may allow sensory neurons to express a range of receptors with a spectrum of response characteristics wider than those of the individual homomeric receptors.

Figure 6: TRPV3. Rooted tree showing protein sequence relationship of different members of the TRPV ion channel family. Adapted from Peier et al., 2002.
Therefore the identification of TRPV3 with its different threshold and kinetic characteristics compared to TRPV1 may implicate that members of the TRPV family constitute a family of thermal sensors that code for a dynamic range of thermal sensitivity in sensory neurons (Smith et al., 2002). As a highly thermo-sensitive but capsaicin-insensitive nonselective ion channel TRPV3 is uniquely sensitive around the physiological 37°C temperature point in mammals. The temperature dependence of hTRP3 (between 25 and 49 °C) fills a gap between the cool-sensing TRPM8 (25 to 15°C, as will be mentioned later) and the heat sensing TRPV1 (>43°C) channels. Human TRPV3 (hTRPV3) may report graded responses to warm stimuli in peripheral tissues that are normally below 17 to 22°C, the channel's thermal threshold but responds robustly to small perturbances around core body temperature (36 to 38°C). The calcium-permeable channel is expressed in a wide range of tissues going from skin and tongue to dorsal root ganglia, trigeminal ganglia, spinal cord and brain suggesting that hTRPV3 may have additional, as yet unidentified functions (Xu et al., 2002). It could be observed that TRPV3 is expressed in keratinocytes of the skin and indeed colocalizes with epidermal endings of temperature-sensing neurons (Peier et al., 2002).

The calcium-selective channels ECaC1/CaT2 (epithelial calcium channel/calcium transporter) (Hoenderop et al., 1999) and ECaC2/CaT1 (Peng et al., 1999) are the main subject of this thesis and will be discussed in detail later.

1.4.4 The TRP-Melastatin subfamily - TRPM

About the TRPM group the least is known. They form the subfamily with extremely long coding sequences (up to 1600 amino acids) as their former name LTRPC suggests, consisting of eight
members divided into four groups (figure 5). They lack ankyrin-like repeats at the N-terminal end but contain a C-terminal proline-rich P region. The major properties are demonstrated in table 1.

The founding member melastatin (LTRPC1 or TRPM1) is found in melanocytes and as a tumor suppressor (as the name melastatin already might suggest this function) its levels are decreased in metastatic cells indicating its function in cell growth and differentiation (Duncan et al., 1998). This feature makes TRPM1 a prognostic marker for metastasis in patients with localized malignant melanoma. In addition, expression of TRPM8 (Trp-p8) and TRPV6 appears to be upregulated in prostate cancers and may represent new markers for prostate cancer (Wissenbach et al., 2001).

TRPM7 (also known as TRP-PLIK) was identified as a protein interacting with PLC-ß1 and is the first member of this subfamily to be expressed as a functional ion channel (Runnels et al., 2001). TRPM7 is a widely expressed ion channel that conducts calcium and also magnesium at negative membrane potentials. Unique among ion channels, it is controlled by its carboxy terminal kinase thus it can be regarded a protein being both an ion channel for divalent cations as calcium and magnesium and a protein kinase. An atypical serine/threonine kinase lacking primary amino acid sequence homology with classical protein kinases displays a three dimensional structure similar to typical protein kinases (Yamaguchi et al., 2001). TRPM7 is capable of autophosphorylation in vitro, and mutations that interfere with the protein kinase binding to ATP in TRPM7 greatly reduced channel activity (Runnels et al., 2001). According to these authors, TRPM7 requires protein kinase activity for channel function, although the detailed mechanism is not known. Another study did not invoke a requirement for the kinase domain in channel activity. Rather, TRPM7 may be regulated by Mg\textsuperscript{2+}-ATP which could provide a mechanism for coupling channel activity with the metabolic state of the
cell (Nadler et al., 2001). From these different observations it was concluded that ATP contributed to channel activation by reducing the free magnesium which inhibits TRPM7 (Nadler et al., 2001). When Mg\(^{2+}\)-complexed ATP falls below 1 mM the current is strongly activated. This current is designated MagNuM for magnesium-nucleotide-regulated metal current (Hermosura et al., 2002). Since both \(I_{\text{CRAC}}\) and MagNuM current are activated in RBL cells when using divalent-free solutions differential suppression of \(I_{\text{CRAC}}\) and MagNuM current based on their differences in activation mechanism, magnesium dependence and pharmacology was applied to assess the relative contributions of each. Thereby it could be observed that MagNuM current contributes significantly to membrane currents under experimental conditions previously thought to enhance \(I_{\text{CRAC}}\) carried by monovalent ions alone (Hermosura et al., 2001).

TRPM2 is a 1503-amino-acid protein highly expressed in fetal and adult brain (Nagamine et al., 1998). Similar to TRPM7 the C-terminus contains an ADP-ribose pyrophosphatase domain suggesting that the channel might be regulated by nucleoside diphosphates and indeed perfusion of HEK 293 cells expressing LRPC2 with ADP-ribose increased the cationic current indicating that ADP-ribose and not NAD or other nucleotides such as ATP, acts as a second messenger through its ability to gate TRPM2 and leading to increased calcium entry (Perraud et al., 2001). However, in two other studies, TRPM2 was also stimulated by NAD though the basis of the discrepancy with NAD remains unclear (Sano et al., 2001; Hara et al., 2002). In their experiments Sano et al. used immunocytes where TRPM2 is highly expressed contributing to the calcium influx that acts as a trigger for the immune responses in immunocytes.

TRPM6 (also known as CHAK2) is a new member of the long transient receptor potential family and is highly similar to TRPM2. Until now the channel has not been functionally expressed. TRPM6 is
expressed in intestinal epithelia and kidney tubules indicating its crucial role in magnesium homeostasis (Schlingmann et al., 2002; Walder et al., 2002). Mutation of TRPM6 causes hypomagnesemia with secondary hypocalcemia as can be seen in individuals with abnormal renal magnesium excretion (Walder et al., 2002). Therefore it is supposed to be a calcium or magnesium channel.

TRPM8 has already been mentioned together with the heat receptors TRPV1 and TRPV2 as it is implicated in thermosensation as well showing that TRP channels detect temperatures over a wide range and are the principal sensors of thermal stimuli in the mammalian peripheral nervous system (McKemy et al., 2002). Previously the protein of 1104 amino acids in length but with little homology to other TRPM class members was identified in prostate cancer cells (Tsavaler et al., 2001; Clapham, 2002). TRPM8 is an excitatory nonselective ion channel expressed in prostate and also in trigeminal and dorsal root ganglia where specialized neurons detect sensory stimuli. According to its activation mechanism it is also called cold- and menthol-sensitive receptor, CMR1 (McKemy et al., 2002; Peier et al., 2002). The observation that the menthol sensitive channel is also activated by cold (8-28°C) indicates that menthol elicits a sensation of cool by serving as a chemical agonist of a thermally responsive receptor (McKemy et al., 2002). Furthermore, while the cold-activated currents are maximal at 8°C, in the presence of sub-activating concentrations of menthol, the threshold and saturation temperatures increase by approximately 5°C (Peier et al., 2002). As there are multiple conditions that potentiate the sensitivity of TRPV4 to heat, it will be of interest to determine whether there are additional stimuli that influence the response of TRPM8 to cold. Physiological roles for TRP channels in the prostate are currently unknown but expression or repression of several TRP genes in tumor cells suggests
that these proteins influence cell proliferation possibly through their ability to regulate intracellular calcium levels (Clapham et al., 2001).

Very recently TRPM4 has been cloned and characterized (Launay et al., 2002). This channel has the distinct properties of a CAN (calcium-activated nonselective) cation channel as it is directly activated by increased cytosolic calcium levels and carries monovalent cations such as Na\(^+\) and K\(^+\) without significant permeation of calcium (Siemen, 1993). As in other ion channels, the pore region of TRPM4 is thought to reside between transmembrane-spanning domains five and six. One remarkable feature of TRPM4 and other CAN channels is that they do not seem to undergo voltage-or calcium-dependent inactivation. Therefore TRPM4 is capable of maintaining membrane depolarization.

### 1.5 The epithelial calcium channels ECaC1 and 2

#### 1.5.1 Structural features and expression pattern of ECaC 1

Until 1999, the molecular entity responsible for the apical influx of calcium was unknown. Recently (Hoenderop et al., 1999) described the channel that is responsible for the passive apical calcium influx originally identified from rabbit kidney cortex and called ECaC1, for Epithelial Calcium Channel (or TRPV5 in the new nomenclature (Montell et al., 2002b)). In this thesis and according to most publications its original name ECaC1 will be used. ECaC1 is the first member of a new subfamily of calcium channels that includes numerous channel proteins (see figure 5) and to date it has also been cloned from rat and human tissues (Suzuki et al., 2000). The genomic structure of the human ECaC1 gene was recently elucidated (Müller et al., 2000). The gene spans 25 kb on chromosome 7q35 and consists of 15 exons. Comparison of the ECaC sequence revealed amino acid
sequence similarity with several ion channel-like proteins (figure 7).

Being a member of the TRPV family (or formerly called OTRPC family),

ECaC has low but significant homology (~30%) with other members of

this family suggesting that ECaC1 is the first member of a new class of

ion channels.
Figure 7: Multiple alignment of amino acid sequence (GenBank accession numbers are indicated in brackets) of hECaC1 (AJ271207), rbECaC (AJ133128), rEcaC1 (AB032019), rCaT2 (AF209196), rCaT1 (AF160798) and mCaT1 (AB037373). Identical residues are indicated in gray, conserved amino acids in black and non-conserved amino acids in white boxes. The putative pore-forming region, transmembrane domains (TM) and ankyrin repeats (ANK) are indicated above the sequence alignment. Potential PKA, CaMKII and PKC sites are indicated in light-gray. Potential PDZ binding domain are indicated in boxes. See text for further details.
1.5.1.1 Molecular characterization of ECaC 1

The ECaC1 cDNA encodes a protein of 729 amino acids with a predicted relative molecular mass of 83 kDa. The predicted secondary structure of this protein contains three structural domains: a large hydrophilic amino-terminal domain with three ankyrin binding repeats and several potential protein kinase C phosphorylation sites, suggesting an intracellular location; a six transmembrane-spanning domain with two potential N-linked glycosylation sites and an additional short hydrophobic stretch between transmembrane segments 5 and 6 predicted to be the pore-forming region; and a hydrophilic 151-amino acid carboxyl terminus containing potential protein kinase A and C phosphorylation sites. The channel also includes two classical PDZ domains. (figure 8) (Hoenderop et al., 1999). The physiological impact of these however is unclear.

Figure 8: Schematic representation of the membrane topology and domain structure of EcaC1. Structural and regulatory elements are indicated. Adapted from Hoenderop et al., 1999.
1.5.1.2 Tissue distribution

Screening various human tissues detected ECaC1 highly expressed in kidney, small intestine and pancreas and less intensely expressed in testis, prostate, placenta, brain, colon and rectum. These ECaC1 positive tissues also express the 1,25-dihydroxyvitamin D₃-sensitive calcium-binding proteins and calbindin (Müller et al., 2000). This conspicuous colocalization in organs that are not prime regulators of plasma calcium levels could illustrate new pathways in cellular calcium homeostasis.

In rabbit kidney and intestine the localization of ECaC1 was investigated using immunohistochemical staining methods showing that ECaC was predominantly found along the apical membrane of the connecting tubule, the distal tubular segments. These findings strongly support the concept of (Friedman, 2000) that the connecting tubule is the main site for transcellular calcium transport in the rabbit kidney. Staining was not observed in glomeruli, proximal tubular segments, or the loop of Henle. In the small intestine staining was only observed in brush-border membranes of the duodenal absorptive epithelial cells but not in ileum (Hoenderop et al., 2000).

Furthermore, double immunofluorescence labeling experiments revealed colocalization of ECaC1 with calbindin-28k, the Na⁺/Ca²⁺ exchanger (NCX) and the plasma membrane calcium ATPase (PMCA) in the distal nephron of rabbit kidney (Hoenderop et al., 2000). In rabbit intestine, ECaC was coexpressed with PMCA and calbindin-9k whereas NCX could not be detected in doudenal tissue. The fact that in both rabbit kidney and intestine, ECaC1 colocalizes with the other calcium transporting proteins known to be involved in transcellular calcium transport confirm that ECaC indeed is the gatekeeper in transepithelial calcium transport.

1.5.2 Functional features of ECaC 1

Calcium currents through ECaC1 channel show a large inward rectification pattern which was strongly dependent on extracellular calcium (Vennekens et al., 2000a). The current rapidly decays during long-term calcium permeation. This effect was significantly delayed if barium was the
charge carrier. Therefore, ECaC1 activity was thought to be negatively regulated by a calcium dependent feedback mechanism which is controlled by the calcium concentration in a micro-domain near the inner mouth of the channel (Nilius et al., 2001a). Furthermore, ECaC1 is highly permeable to monovalent cations in the absence of extracellular calcium (Vennekens et al., 2000a). These results suggest similarities between ECaC channels and voltage-operated calcium channels, which might be reflected in analogous permeation mechanisms. Mutation of one specific negatively charged aspartate residue (Asp542) in the channel pore abolished calcium permeation (Nilius et al., 2001b). The magnesium block was affected as well according to the proposed mechanism for calcium selectivity constituted by negatively charged aspartate or glutamate pore residues (Tsien et al., 1987). In this model the negative charged amino acids function as a binding site for the divalent permeated cations and both the charge and the side-chain length are thought to influence calcium binding. This negatively charged binding site is not present in the low calcium selective TRPV1 channel. Studies showed that the permeation of monovalent and divalent cations through ECaC1 resembles the permeation properties of L-type voltage-operated calcium channels (Vennekens et al., 2000a). The main difference was a stronger calcium affinity and a significantly higher current density for ECaC1 in micromolar calcium concentrations. Until now no store-dependent activation of ECaC1 has been described.

1.5.3 ECaC2: a homologue of ECaC1

Just as ECaC1, ECaC2 (CaT1 or TRPV6 in the new nomenclature (Montell et al., 2002b)) is widely expressed throughout the body. The channels show almost identical pore regions but can be discriminated by their sensitivity for ruthenium red and differences in calcium-dependent regulation (Hoenderop et al., 2001) ECaC2 has been shown to exhibit some I_{CRAC} properties when expressed in vitro, however this is contestable (Yue et al., 2001). These include activation of ECaC2 through a store-operated mechanism as the channel becomes activated by a low
intracellular calcium concentration and inactivates by higher cytoplasmic calcium concentrations, and a high degree of calcium selectivity in the presence of divalent cations. Though some common features between TRPV6 and CRAC channels have been confirmed in an independent study (Voets et al., 2001), there are several differences between $I_{CRAC}$ detected in rat basophilic leukemia (RBL) cells and $I_{TRPV6}$ (Voets et al., 2001). For example, ECaC2 displays a mode of voltage-dependent gating that is fully absent in CRAC and originates form the voltage-dependent un-/binding of magnesium inside the channel pore, indicating that ECaC2 is a magnesium gated channel not directly related to CRAC (Voets et al., 2001). Furthermore, initial reports on ECaC2 did not mention any store depletion dependency, a typical feature of CRAC, as mentioned by Yue et al. In this view it seems that other unidentified features will be needed for proper constitution of $I_{CRAC}$. 
2. Rationale and aims

With the molecular cloning of the epithelial calcium channel, ECaC1, from rabbit kidney connecting tubule and cortical collecting duct cells an important step was taken to elucidate the molecular mechanism of active calcium reabsorption in the kidney and intestine. With electrophysiological techniques the functional significance of ECaC could be shown which demands an intensive search for compounds that modulate currents through these channels.

Therefore the objective of this work was a pharmacological study of ECaC1 by investigating a number of pharmacological tools that inhibit ECaC1 currents carried out by monovalent cations. This approach circumvents the fast inactivation of currents through ECaC1 carried by calcium, which even occurs if intracellular calcium is buffered by BAPTA or EGTA. Here a variety of inorganic and organic compounds were used that inhibit ECaC1.
3. Material and Methods

3.1 Cell culture and transfection

In order to analyze its pharmacological properties rabbit ECaC1 was
overexpressed in HEK 293 (human embryonic kidney) cells. HEK 293 cells
were grown in DMEM (Dulbecco’s modified Eagles medium) containing
10% (v/v) human serum, 2 mM L-glutamine, 2U/ml penicillin and 2 mg/ml
streptomycin at 37°C in a humidity controlled incubator with 10% CO₂. HEK
293 cells were transiently transfected with the pCINeo/IRES-GFP/rbECaC
vector (Vennekens, et al., 1999) which contains the entire open reading
frame of rabbit ECaC1 (rbECaC1) cloned as a PvuII-BamHI fragment in the
pCINeo/IRES-GFP (green fluorescent protein) vector, a so-called
bicistronic expression vector (Hoenderop et al., 1999; Trouet et al., 1997).
Cells were then transferred to poly-L-lysin-coated coverslips 24 hours after
transfection and electrophysiological measurements were done during 2 to
4 days after transfection. Incorporation of ECaC1 in the bicistronic unit
allows coupled expression of the channel and GFP. Transfected cells,
positive for GFP, could be visually identified in the patch-clamp set up.
GFP was excited at a wavelength between 450 and 490 nm and the
emitted light was passed through a 520 nm long pass filter. The ECaC1-
expressing cells were identified by their green fluorescence and GFP
negative cells from the same batch were used as controls.

3.1.1 Transfection

1. Add 6 µl of Mirus transIT 293® reagent to 100 µl of medium (OPTIMEM I®).
2. After vortexing, shortly centrifugate the sample and leave at room
temperature for 5 à 20 minutes.
3. Add 2 µg ECaC WT-DNA and mix 5 times with a sterile tip of a pipette.
4. Incubate the DNA suspension at room temperature for about 10 minutes.
Cell cultures in HEK medium were maintained at 37°C in a fully humidified atmosphere of 10 % CO₂ in air. About 300000 HEK 293 cells were seeded per six well.

1. Before transfection replace the HEK medium by 2 ml new medium.
2. Then add the DNA suspension to the medium and gently spread by shaking.
3. Incubate the transfected cells for 24 à 72 hours at 37°C and then seed.

### 3.1.2 Trypsinization

In order to measure the endogenous currents, cells were detached by exposure to 0.05% trypsin in a Ca²⁺ and Mg²⁺ free solution and seeded on poly-L-lysine coated cover slips.

1. Carefully remove the old medium from the cells.
2. Clean the incubated cells by adding 1 ml 5 mM EDTA solution (versene buffer) and remove shortly after.
3. Add 500 µl of 0.05% trypsin preheated to 37°C to detach the cells and incubate for 10 minutes at 37°C.
4. Add 2 ml HEK medium to the cell culture to stop trypsinization and mix the contents by pipetting the suspension 5 times to further detach cells.
5. Transfer the cell suspension with a sterile pipette tip to a sterile FALKON® tube and use a fine needle syringe to detach cells.

### 3.1.3 Cell counting and seeding

To count the viable cells in the Burker chamber trypan blue was used to differentiate viable cells (which excluded trypan blue) from dead cells.

1. Mix 50 µl of cell suspension with 7 µl trypan blue.
2. Transfer 10 µl of the blue suspension to the Burker chamber and count cells.
3. To calculate the cell concentration (cells/ml) count 16 fields and multiply with the factor 1979.

Cells were plated at a density of 20000 to 40000 viable cells/well of a 12-well plate adding 2 ml HEK medium per well. After approximately two hours of incubation at 37°C the cells were ready to be used for measuring currents.

3.2 Solutions and drugs

Rapid solution exchange and extracellular application of drugs occurred via a multi-barreled pipette connected to solution reservoirs, and was controlled by a set of magnetic valves.

3.2.1 Solutions for patch-clamp experiments

3.2.1.1 Standard extracellular modified “Krebs” solution:

NaCl 150 mM  
KCl 6 mM  
MgCl$_2$ 1 mM  
CaCl$_2$ 1.5 mM  
HEPES 10 mM  
D-glucose 10 mM  

Titrated to pH 7.4 with 1 mM NaOH, with an osmolarity of 320 ± 5 mosmol.l$^{-1}$.

3.2.1.2 Nominally free calcium solution

NaCl 150 mM  
CsCl 6 mM  
HEPES 10 mM  
D-glucose 10 mM  
Mannitol 45 mM  

Titrated to pH 7.4 with 1 mM NaOH.
3.2.1.3 **Standard internal pipette solution**

To exclude any effects of calcium on ECaC1 channel activity the internal pipette solution was buffered with 10 mmol.l\(^{-1}\) BAPTA.

- CsCl 20 mM
- Cs-aspartate 100 mM
- MgCl\(_2\) 1 mM
- Na\(_2\)ATP 4 mM
- HEPES 10 mM
- BAPTA 10 mM

Titrated to pH 7.2 with 1 mM CsOH.

3.2.1.4 **NMDG**

- NMDG 0.15 mM
- HEPES 10 mM
- D-glucose 10 mM
- Mannitol 45 mM

Titrated to pH 7.4 with 37% HCl.

All chemicals used for the solutions were purchased from Sigma.

3.2.2 **Chemicals and drugs**

The following compounds were used: econazol (Sigma), 5-nitro-2-(3-phenylpropylamino)benzoic acid (NPPB) (Cookson Chemicals, Southampton, UK), ZnCl\(_2\), CoCl\(_2\), FeCl\(_2\), FeCl\(_3\), CuCl\(_2\) all purchased from Merck.

The compounds were daily dissolved in the appropriate measuring solution and the final concentrations used were obtained with appropriate dilution of the stock solutions. The stock solutions were made in dimethylsulfoxide (DMSO) for the organic compounds econazol and NPPB and in water for the inorganic compounds.
3.3 Electrophysiological recordings

Whole-cell membrane currents were measured in ruptured patches. All experiments were performed at room temperature (20-23°C). Cover slips containing the seeded cells were placed in a recording chamber mounted on the stage of an Axiovert10 inverted microscope (Zeiss). Patch electrodes were pulled from Vitrex capillary tubes (Modulohm, Herlev, Denmark) on a DMZ-Universal puller (Zeitz-instruments, Augsburg, Germany). When filled with the pipette solution “BAPTA” electrode resistances were between 2 and 5 MΩ. An Ag-AgCl wire was used as reference electrode. Ag-AgCl electrodes of sintered pellets (IVM Systems, Healdsburg, CA, USA) were used to avoid contamination of the bath and pipette solutions. Membrane currents were recorded using an EPC-9 (HEKA Elektronik, Lambrecht, Germany) or an L/M-EPC-7 patch-clamp amplifier (List Electronics, Darmstadt, Germany) and filtered with an eight-pole Bessel filter, 2,9 kHz (Kemo, Bekenham, UK). For control of voltage-clamp protocols and data acquisition, we used the pCLAMP 6 software (Axon Instruments) run on an IBM-compatible PC which was connected to the amplifier via a TL-1DMA interface (Axon Instruments). Membrane currents were measured in the whole-cell mode of the patch-clamp technique (Hamill et al., 1981). The cell capacitance and series resistance were assessed using the analogue compensation circuit of the EPC-7 amplifier. Approximately 50% of the series resistance was electronically compensated to minimize voltage errors. The ramp protocol consisted of linear voltage ramps changing from –100 mV to +100 mV within 400 ms, applied every 2 s from a holding potential of +20 mV. The step protocol consisted of a series of 60 ms long voltage steps applied from a holding potential of +20 mV to voltages between +100 and –140 mV with a decrement of 40 mV. Membrane potential was held at +20 mV. Current densities, expressed per unit membrane capacitance, were measured from the current at –80 mV during the linear voltage ramp protocols. In all experiments, time zero corresponds to the rupture of the membrane.

3.5 Statistical analysis
In all experiments the analysis of electrophysiological data was performed using the Winascd package (G. Droogmans, Laboratorium voor Fysiologie, Katholieke Universiteit Leuven). Pooled data are expressed as the mean ± S.E.M. from \( n \) cells. Overall statistical significance was determined by analysis of variance (ANOVA). In case of significance (\( P<0.01 \)), individual groups were compared by Student’s t-test.

Dose-inhibition data were described by the equation [1]

\[
I_{\text{norm}} = \left[1 + (C/IC_{50})^n\right]^{-1}
\]

where \( I_{\text{norm}} \) is the current amplitude in the presence of the inhibitor normalized to that in control conditions, \( C \) the concentration of inhibitor, \( IC_{50} \) the concentration of half-maximal inhibition, and \( n \) the Hill coefficient. Data were fitted to this dose response function using Origin software v 6.0 (Microcal Software, Northhampton, MA, USA).
4. Results and conclusions

4.1 Modulation of ECaC1 by inorganic cations

In order to functionally characterize ECaC1 channel properties, rabbit ECaC1 was overexpressed in human embryonic kidney cells (HEK 293), and channel events were analyzed using the patch-clamp technique. Evidence that ECaC1 forms a constitutively active calcium channel was obtained from experiments showing a close correlation between the level of intracellular calcium concentration and the electrochemical calcium gradient in ECaC1 expressing HEK 293 cells (Vennekens et al., 2000a). It has been described that extracellular divalent cations effectively inhibit currents through ECaC1. The IC$_{50}$ value for block by magnesium at -80 mV was 62 µM in the absence of extracellular calcium and 328 µM at 100 µM [Ca$^{2+}$]$_{ext}$, respectively. Barium currents were blocked by lanthanum and gadolinium at –80 mV with IC$_{50}$ values of 4.6 and 1.1 µM respectively. Currents through ECaC1 in the presence of 100 µM [Ca$^{2+}$]$_{ext}$ were blocked by cadmium with an IC$_{50}$ of 2.5 µM (Vennekens et al., 2000a).

The present study investigates the effect of organic and inorganic modulators on ECaC1 currents carried by monovalent cations. This approach circumvents the fast inactivation of currents through ECaC1 carried by calcium, which even occurs if intracellular calcium is buffered by BAPTA or EGTA. Figure 9 shows the protocol applied for these studies. Seal formation and rupture of the patch membrane occur in the presence of 1.5 mM [Ca$^{2+}$]$_{ext}$, after which the extracellular solution is changed to a divalent cation free solution. The monovalent cation current through ECaC1 is therefore blocked immediately after breaking into the cell, and develops with a certain delay in the divalent cation free solution because dialysis of the cell with the 10 mM BAPTA buffered pipette solution removes the Ca$^{2+}$-dependent inactivation of the channel induced by cytosolic calcium (Nilius et al., 2001; 2000). This current was then stable over a relatively long time period (usually more than 30 minutes). This allowed testing of modulator compounds in the same cell.
Figure 9 A and B: Effect of Fe$^{2+}$ on monovalent cation currents through ECaC1.

(A) Currents were recorded after seal formation and membrane rupture in a calcium free solution. Monovalent cation currents developed after removal of extracellular calcium and dialyzing the cell with the pipette solution containing 10 mM BAPTA. The amplitude of the monovalent cation current at –80 mV was measured during repetitive voltage ramps and is displayed as a function of time. (B) Current voltage relationships measured at the corresponding times indicated in figure A.
Figure 9C: Dose-response relationships of the current inhibition by Fe$^{2+}$:
Current amplitudes at –80 mV derived from voltage ramps were normalized to the corresponding current amplitudes in the absence of the blocker. Each data point represents the average of three to six cells. Parameters of the fit to equation [1] for Fe$^{2+}$ were IC$_{50}$ 145±10.11 µM, $n_H$=11.8.

4.1.1 Inhibition of ECaC1 by copper

Figure 10C shows the current voltage relationships measured after applying different concentrations of Cu$^{2+}$ and with the nominally free calcium solution as control. Application of 1 µM copper inhibited the inward current at –80 mM by approximately 50%, whereas 100 µM copper nearly reduced the current amplitude to the level that would be observed after substituting extracellular Na$^+$ with NMDG$^+$. Half-maximal inhibition (IC$_{50}$) was obtained at a concentration of 1.09 µM, $n_H$=0.86. The IC$_{50}$ indicates the affinity of the modulator to the epithelial calcium channel. This type of experiments was used in all studies.
4.1.2 Inhibition of ECaC1 by zinc

Figure 10A shows the current behavior when Zn$^{2+}$ was used as inhibitor. Zn$^{2+}$ exerted a similar high sensitivity block of ECaC1 as Cu$^{2+}$. The IC$_{50}$ value of inhibition was 3.8 µM, $n_H$=0.96 The dose response relationship is illustrated in figure 10B.

4.1.3 Inhibition of ECaC1 by cobalt
Application of 10 µM cobalt inhibited the inward current at –80 mV by approximately 50%, whereas 100 µM Co²⁺ reduced the current amplitude to the level observed after substituting extracellular Na⁺ with NMDG⁺. Recovery of the current upon washout of the inhibitor was fast and reversible. Here half-maximal inhibition was obtained at a concentration of 9.5 ± 0.87 µM, nₕ=1.3 ± 0.54 (three concentrations, data not shown).

4.1.4 Inhibition of ECaC1 by Fe²⁺ and Fe³⁺

Compared to all other tested divalent and trivalent cations Fe²⁺ and Fe³⁺ appeared to be less efficient blockers of the epithelial calcium channel. At a concentration of 100 µM, Fe²⁺ blocked the ECaC1 monovalent cation current at –80 mV by 70 ± 13% (n=4) and at a concentration of 500 µM, more than 90% of the monovalent cation current was blocked at –80 mV as it is illustrated in figure 9A and B. From the dose-inhibition curve shown in figure 9C, which was obtained from all voltage step and ramp experiments, we derived an IC₅₀ value of 145 ± 10.22 µM, indicating the relatively low sensitivity of this blocker on ECaC1 currents.

At a concentration of 100 µM, Fe³⁺ blocked the monovalent cation current of ECaC1 at –80 mV by 22 ± 9% (n=4) respectively (data not shown).

4.2 Modulation of ECaC by organic compounds

Besides the inorganic cations some compounds that have been described as weak selective blockers of store-operated Ca²⁺ channels (SOC) (Franzius et al., 1994) have been tested.

4.2.1 Inhibition of ECaC1 by econazol

One of these compounds is the antimycotic drug and imidazole derivative econazol. As shown in figure 11A, econazol is a highly sensitive
blocker of currents through ECaC1. Inhibition had no effect on the reversal potential which is typically around +20 mV for ECaC1 (figure 11B), and was nearly complete at 100 µM. It disappeared slowly after washing out (figure 11C). The concentration for half maximal inhibition measured at –80 mV was 0.43 ± 0.04 µM (figure 11A). It is interesting that econazol exerts its inhibitory effects on ECaC1 in the same concentration range as on a diversity of ion channels investigated by (Franzius et al., 1994). The main conclusion from this result is that econazol is a non-specific blocker of Ca^{2+} entry.
Figure 11: Inhibition of monovalent ECaC1 currents by econazol. (A) Dose-response curve for the effect of econazol on ECaC1. (B) Monovalent cation currents at −80 mV as a function of time. (C) Current voltage relationships from voltage ramps indicated.
4.2.2 Inhibition of ECaC1 by NPPB

The other organic compound used was the well described anion channel blocker NPPB. This modulator is a potent inhibitor of an epithelial Cl-conductance (Wangemann et al., 1986), and completely blocks outwardly-rectifying Cl-channels present in canine tracheal epithelium (Li et al., 1990). On ECaC1 the compound NPPB was nearly ineffective and inhibited currents through ECaC1 by approximately 25% at a concentration of 50 µM as can be seen in the dose-response curve in figure 12C. Application of 10 nM was almost ineffective (figure 12B) whereas the half-maximal inhibition of the inward current at -80 mV was obtained at a concentration of 550.65 µM, \( n_H = 3.05 \) (figure 12C). Blocking in the micromolar range is not considered to be specific. The registration of monovalent cation currents at –80 mV over this long period of time (almost 20 minutes) hints at the slow action of the inhibitor on the calcium channel and the delayed but reversible recovery of monovalent cation current upon washout (figure 12A). During voltage steps ECaC1 currents did not show a time-dependent decay indicating that the block was voltage independent (figure 12C). Here again NMDG\(^+\) was applied to detect leak currents.
Figure 12 A and B: Block of monovalent ECaC1 currents by NPPB.

(A) Same screening protocol as in figure 9A for inhibition of ECaC1 by NPPB. Note the slow recovery of the current after removal of 50µM NPPB. Perfusion with solution containing NMDG⁺ is indicated. (B) Current voltage relationships from voltage ramps measured under control conditions (cont) and at three different concentrations of NPPB.
Figure 12C: Inhibition of ECaC1 monovalent currents by NPPB.

Dose response relationship for currents normalized to the control current before application of NPPB were fitted to equation 1. Data points are from three to six cells. Parameters of the fit are IC\textsubscript{50} 550.65 and n\textsubscript{H} = 3.05.
Figure 12D: Inhibition of ECaC1 currents by NPPB. Voltage steps of 60 ms from +100 to –140 mV (decrement 40 mV, holding potential is +20 mV) under control conditions (cont) and at 4 different concentrations of NPPB. No time dependent current decay could be observed.
The inhibiting effects of all the compounds which were tested are summarized in figure 12 and table 2.

![Figure 13: Synopsis of the various inhibitors on ECaC1 currents. Fraction of the blocked current (inhibition) at -80 mV by various substances (concentrations are in µM).](image)

Table 2: Summary of the pharmacological data.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Fraction blocked</th>
<th>IC$_{50}$ (µM)</th>
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</thead>
<tbody>
<tr>
<td>10 Cu$^{2+}$</td>
<td>0.1</td>
<td>1.09</td>
</tr>
<tr>
<td>10 Zn$^{2+}$</td>
<td>0.29</td>
<td>3.8</td>
</tr>
<tr>
<td>100 Fe$^{2+}$</td>
<td>0.3</td>
<td>145</td>
</tr>
<tr>
<td>10 Co$^{2+}$</td>
<td>0.59</td>
<td>9.5</td>
</tr>
<tr>
<td>100 Fe$^{3+}$</td>
<td>0.74</td>
<td>N.D.</td>
</tr>
<tr>
<td>10 econazol</td>
<td>0.1</td>
<td>0.43</td>
</tr>
<tr>
<td>100 NPPB</td>
<td>0.6</td>
<td>550</td>
</tr>
</tbody>
</table>
5. Discussion and Perspectives

5.1 Introduction

The objective of this work was a pharmacological study of the novel epithelial calcium channel ECaC1 in human embryonic kidney cells. Therefore cloning of epithelial calcium channels (ECaC1 and ECaC2) has provided a molecular basis to analyze the process of calcium entry in a variety of non-excitable and excitable cells by using the patch-clamp technique.

A complete understanding of the functional features of the channels of the ECaC family will significantly depend on the availability of selective modulators. Therefore, we have searched for compounds that might affect the current through one member of this family, the epithelial calcium channel ECaC1. Considering the proposed role of ECaC1 in transepithelial calcium transport in the distal tubule of the kidney, ECaC1 has a substantial calcium conductance when the membrane potential is typically around –70 mV in the distal nephron (Friedmann, 2000), permitting basal calcium influx in the epithelial cell. This influx can be enhanced when the driving force is increased by decreasing \([\text{Ca}^{2+}]_{\text{cyt}}\) or by hyperpolarizing the membrane potential (Friedman and Gesek, 1993). The screening was restricted to the modulation of monovalent cation currents through ECaC1, since these are very stable currents and in this way provide an excellent probe for studying the modulating effects of various compounds.

5.2 Preparation of the cells

In order to test the different pharmacological modulators on ECaC1, HEK 293 cells had to be transfected and prepared by trypsinization. To obtain isolated single cells with a non-rigid cell membrane firmly attached to the coverslips measurements were optimally done two to maximally four days after transfection. In optimal conditions a good seal between pipette and cell with a negligible leak could be obtained and whole cell currents were measured at a low series resistance.
Incubation of the HEK 293 cells more than two days after transfection lead to formation of cell clusters which made registration of single cell currents impossible.

5.3 Electrophysiological characteristics of ECaC1

Currents through ECaC1 could be evoked by hyperpolarizing the cell membrane without any other activating maneuver. This observation indicates that under physiological conditions used in all experiments ECaC1 is constitutively open.

A study of the biophysical properties of ECaC1 in the presence of divalent cations is extremely difficult because of the fast and pronounced calcium-induced current decay under these conditions. Even at physiological magnesium and calcium concentrations currents through ECaC1 still are relatively small. Because of this reason solutions free from calcium and magnesium were used to obtain large currents. The calcium selectivity of ECaC1 is significantly higher than observed for homologous ion channels, including TRPV1 and TRPV4 and more distantly related members of the TRP vanilloid subfamily of proteins (see introduction). Furthermore the current-voltage relationships of TRPV1 and TRPV4 show dominant outward rectification of the corresponding currents, whereas ECaC1 constitutes inwardly rectifying currents and will mediate a large monovalent cation influx at negative membrane potentials.

5.4 Application of inhibitors

5.4.1 Inorganic compounds

Previously, it has been described that extracellular divalent cations effectively inhibit currents through ECaC1. The IC$_{50}$ value for block by Mg$^{2+}$ at $-80$ mV was 62 µM in the absence of extracellular calcium and 328 µM at 100 µM [Ca$^{2+}$]$_{ext}$, respectively. Barium currents were blocked by lanthanum and gadolinium, which are well-known potent blockers of calcium channels. The IC$_{50}$ values at $-80$ mV were 4.6 and 1.1 µM
respectively. Currents through ECaC1 in the presence of 100 µM $[\text{Ca}^{2+}]_{\text{ext}}$ were inhibited by Cd$^{2+}$ with an IC$_{50}$ of 2.5 µM (Vennekens et al., 2000b). Here novel cations with IC$_{50}$ values ranging between 1.09 µM and 145 µM have been tested in their efficiency to block the ECaC1 channel. After adding the novel inorganic inhibitors the overall sequence of block is now Cu$^{2+}$=Gd$^{3+}$>Cd$^{2+}$>Zn$^{2+}$>La$^{3+}$>Co$^{2+}$>Fe$^{2+}$>>Fe$^{3+}$.

Though all inorganic compounds were soluble in water attention was necessary when preparing the appropriate measuring solution from the stock solution and the final concentrations of one compound, especially with Fe$^{3+}$ which has a relatively low solubility product which made that it easily precipitated. Optimizing the hydrosolubility of Fe$^{3+}$ by acidifying with HCl would have had a to important impact on osmolarity and pH of the solution and would have modified the membrane current since lowering extracellular pH inhibits both monovalent and divalent currents through ECaC1 (Vennekens et al., 2000a).

### 5.4.2 Organic compounds

In comparison to the inorganic compounds the organic inhibitors econazol and NPPB had to be dissolved in DMSO. The concentration of DMSO had to be less than 1:1000 to forestall cytotoxic effects.

As described previously, the 5-nitro-2-(3-phenylpropylamino)-benzoic acid derivative NPPB has been frequently used as a blocker of volume-activated, calcium–activated and cAMP-activated chloride channels (Li, et al., 1990; Wangemann, et al., 1986). It has also been described that NPPB blocks basolateral epithelial potassium channels and calcium-impermeable non-selective cationic channels in brain and capillary endothelial cells and in the basolateral membrane of epithelial cells from exocrine pancreas, although, much less effectively by chloride channels. Based on the fact that this frequently used anion channel blocker significantly inhibited capacitative calcium entry in endothelial cells (Gericke et al., 1994) we used NPPB in our measurements. Unfortunately in this experiment working with NPPB was extremely difficult as very high concentrations (around 500 µM) had to be applied to obtain an inhibitory
effect on ECaC1 implying that NPPB is not an ideal inhibitor for this channel. Also, no voltage-dependent effect of NPPB could be observed.

Since the half maximal inhibition of econazol measured at –80 mV was 0.43 ± 0.04 µM a high-affinity ligand could be found in econazol, which might be used as a probe for the biochemical isolation of the channel protein ECaC1. One obvious aim to search for efficient ECaC1 blockers is to evaluate the function of these channels under more physiological conditions in different native systems. In this context it would be of high interest to repeat these measurements with the endogenously expressed ECaC1 channel.

5.5 Perspectives

ECaC channels are expressed in a wide range of tissues as described in the introduction (see table 1) and not confined to epithelial cells as their name could misleadingly propose. ECaC1 is expressed in brain tissue whereas both ECaC1 and 2 can be detected in pancreas. Until now the role of ECaC channels in these tissues remains to be explored, which would be a challenging field of research.

From a pathophysiological point of view it would be interesting to study the role of ECaC channels in diseases affecting the mentioned tissues in which a defect in calcium influx mechanism plays an important role, such as Alzheimer’s disease affecting the nervous tissue (Yoo et al., 2000), pancreatitis (Raraty et al., 2000) and Scott syndrome causing primary hemostatic disorders (Kunzelmann-Marche et al., 2001).

Since expression of ECaC1 and the highly similar channel ECaC2 (CaT-1) have been correlated with tumor-grade and malignancy of prostate cancer (Peng et al., 2001) it would also be tempting to analyze in how far these channels play a functional role in prostate malignancies and whether they are merely markers for the tumor-grade or follow up as an alternative or addition to the relatively unspecific marker prostate specific antigen (PSA).

Furthermore, the central role of ECaC channels in active calcium (re)absorption makes it a prime target for pharmacological manipulation
and several disorders related to calcium homeostasis, including idiopathic hypercalciuria and osteoporosis could benefit from such developments. Therefore, a better understanding of the ECaC permeation and regulation of its gating will be crucial to understand the pathophysiology.

All these open questions point at the necessity to find a specific high affinity blocker for ECaC channels that will improve our knowledge of these channels in disorders and might lead us to the development of new and more specific therapeutical options in the treatment of these diseases. In this work several blockers of ECaC1 have been tested but the search for more specific high affinity blockers of ECaC channels by a more thorough pharmacological profile of these channels will be one of the most important goals in the future in this field of research. To improve our knowledge of the physiological role of these channels, measurement of ECaC in native cell types will be inevitable. Exploring the regulation in physiological conditions will be the basis for understanding dysfunctions and possible diseases caused by ECaC channels.
6. Summary

The aim of my stay at the research lab was (1) to obtain more insight in the pharmacological modulation of monovalent cation currents through the novel epithelial calcium channel ECaC1 and (2) to become more familiar with general techniques used in a physiology laboratory (patch clamp techniques, cell culture and transfection,...).

At the moment I joined the laboratory, preliminary results suggested that ECaC1 belonging to the transient receptor potential (TRP) gene family is a central component of transcellular calcium transport in 1,25-dihydroxyvitamin D3-responsive epithelia. Cloning and characterization of the two homologous calcium channels ECaC1 and ECaC2 suggested those to be highly calcium selective and show inward rectification and calcium dependent feedback inhibition.

The aim of my project was to investigate a number of pharmacological tools that inhibit ECaC1 currents carried by monovalent cations. To measure these currents the whole cell patch clamp technique was applied to transfected human embryonic kidney (HEK 293) cells that were visually identified by exciting green fluorescent protein (GFP) in the patch clamp set up. To prevent the fast calcium dependent inactivation of ECaC1 cells were dialyzed with 10 mM BAPTA or 10 mM EGTA.

Here several reasonably sensitive blockers of ECaC1 have been tested. Finding a specific high affinity blocker will improve our knowledge of ECaC channels in diseases caused by defective calcium reabsorption. This could possibly open up new therapeutical options in the treatment of these disorders.

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8. Acknowledgements

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In the first place I would like to thank my promoter, Professor Dr. L. Pott, who supported me in my wish to do research work in a foreign country. I am very grateful for the confidence and enthusiasm you had in sending me to another research laboratory. I would like to thank my copromoter Professor Dr. Bernd Nilius as well, who accepted me in his research group and in this way gave me the great opportunity to work in a foreign laboratory. I am very grateful for the work possibilities, comments and knowledge you transmitted to me in the fascinating world of channels and electrophysiology.

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Dit werk draag ik op aan mijn familie.
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