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Abhandlungen der Deutschen Akademie der Naturforscher Leopoldina
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Harald zur Hausen, Vizepräsident der Akademie
Neue Folge, Nummer 336, Band 89

Epithelial Transport of Ions in Health and Disease

Leopoldina Symposium

Deutsche Akademie der Naturforscher Leopoldina in Collaboration with the International Union of Physiological Sciences (IUPS)

Halle/Saale
March 19 to 22, 2003

Organizing Committee:

Irene Schulz, Homburg (Saar)
Member of the Academy

Gerrit Isenberg, Halle (Saale)
Member of the Academy

Deutsche Akademie der Naturforscher Leopoldina, Halle (Saale) 2004
In Kommission bei Wissenschaftliche Verlagsgesellschaft mbH Stuttgart

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With 40 Figures and 4 Tables

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Contents

Epithelial Transporters of Electrolytes and Ion Channels

Preface

Irene SCHULZ (Homburg)

Member of the Academy

Transport of electrolytes, organic substances and products of metabolism in cells are precisely regulated processes. Proteins which transport these molecules through the cell membrane are channels, pores or active transporters, which have been conserved during evolution for more than 700 million years from primitive organisms (e.g. yeast) to human beings. However, mutations in the genes for these proteins lead to hereditary diseases such as cystic fibrosis (Cl^- channel disorders) which manifests in the lungs and pancreas for Bartter's-, Gitelman's- and Liddle's-syndrom (defects of ion transporters) which lead to kidney disease.

The combination of electrophysiological, biochemical and molecular genetic investigations allowed the study of diseases caused by mutations in genes encoding for ion transporters. On invitation of the *Deutsche Akademie der Naturforscher Leopoldina*, in cooperation of the International Union of Physiological Sciences (IUPS) and with support of the *Deutsche Forschungsgemeinschaft* (German Research Foundation) it was possible to bring together leading investigators of ion transporters in health and disease. We welcome participants from all over the world and hope that this symposium will bring new insights into physiological functions and pathophysiological roles of transport-proteins. We will hear about Ca^{2+} signaling and Ca^{2+} transporters in physiological functions as well as about mutations in the gene of the Ca^{2+} transporter SERCA2, which underlies "Darier's disease". The TRP proteins, which belong to a newly discovered family of ion channels, will be discussed. Mutations in magnesium transporting TRP proteins can result in low magnesium blood levels which lead to muscle cramps in infants. We will also hear about "cystic fibrosis" and disorders of Na^+-transporting proteins, which disrupt salt homeostasis or contribute to hypertension.

Hopefully a better understanding of ion-transporter-disorders will help to treat diseases caused by mutations in ion transporting-proteins.

Prof. Dr. Irene SCHULZ
Universität des Saarlandes
II. Physiologisches Institut
Building 58
66421 Homburg (Saar)
Germany
Phone: +49 6841 1626450
Fax: +49 6841 1626655
E-Mail: irene.schulz@uniklinik-saarland.de

Nova Acta Leopoldina NF *89*, Nr. 336, 9–10 (2004)

Welcome Greetings

Volker ter Meulen (Halle/Saale)

President of the Academy

Ladies and gentlemen,

On behalf of the German Academy Leopoldina I would like to welcome you to the Leopoldina symposium "Epithelial transport of ions in health and disease". We are very glad that many of you did not mind to travel so far to come here and to participate at this interesting meeting which is organized in collaboration with the International Union of Physiological Sciences.

The programme looks very interesting, although very specific, and I would like to congratulate both organizers, Dr. Irene Schulz from the University of Homburg and Dr. Gerrit Isenberg, University of Halle, of having succeeded in bringing together so many leading scientists from different countries to Halle to discuss a dysfunction of ion transport in relation to certain important diseases. Without any doubt, through molecular biological studies of ion transport progress has been made in the understanding of the pathogenetic mechanisms of certain genetic diseases. Therefore, I am convinced that many papers presented at this symposion will be of interest not only to molecular biologists, geneticists or physiologists, but also to clinicians, who are interested in the diseases mentioned in your programme.

Since probably some of the foreign participants are not familiar with our academy, I would like to give a brief introduction to the history and structure of the Leopoldina.

Our academy is the oldest one in the German speaking countries, i.e. Germany, Austria, Switzerland, and one of the oldest academies of the world. The academy was founded in 1652 by four physicians of the Free City of Schweinfurt near Würzburg. The goal of the Foundation was to improve knowledge of medical sciences for the individual scholar as well as for the public by exploring nature for the benefit of the human being. These goals have been maintained up to the present time.

Over the period of 351 years, it becomes obvious that the structure of an academy has to be adjusted according to the needs. The last re-organisation of the Leopoldina occurred after the reunification of East and West Germany. At present, the number of elected members is restricted to 1,000, but this number refers to fellows less than 75 years of age. Therefore, the whole "corpus academicum" will be about 25 per cent larger. About 40 fellows can be elected annually. The academy holds 27 scientific sections, grouped in natural sciences such as chemistry, physics, mathematics, biology and technic sciences as well as medicine and empirical humanities. Two thirds of the members come from the German-speaking countries: Germany, Switzerland and Austria, one third from more than 30 other countries throughout the world.

The aims of the academy are directed:

- to the promotion of interdisciplinary and transdisciplinary discussions,
- to the distribution of general scientific knowledge to the public,
- to act as an advisory council for government and public administration in science policy,
- to the promotion of young scientists,
- to maintain relationships with national and international academies of sciences.

The meeting today is an example of how the academy supports scientific exchange and interdisciplinary discussion and also tries to bring such symposia to the attention of the public through our Leopoldina journals.

I wish you a successful meeting with the presentation of new findings as well as interesting discussions to the promotion of your own research. In addition, I hope you will enjoy the stay in the city of Halle.

Prof. Dr. Volker ter Meulen
Deutsche Akademie
der Naturforscher
Leopoldina
Postfach 11 05 43
06019 Halle (Saale)

Keynote Lecture

Nova Acta Leopoldina NF *89*, Nr. 336, 13–23 (2004)

Archaic Transport Systems Alive: Mammalian Proton-dependent Nutrient Transporters

Hannelore Daniel, Member of the Academy, Gabor Kottra, Michael Boll, and Dietmar Weitz (Freising-Weihenstephan)

With 1 Figure and 1 Table

Abstract

Uptake of nutrients into cells is essential to life and occurs in all organisms in expense of energy. In most prokaryotic and simple eukaryotic cells the electrochemical proton gradient across the membrane provides the central driving force for nutrient uptake, whereas in higher developed eukaryots, nutrient uptake is more frequently coupled to the transmembrane sodium gradient established by the activity of the Na^+/K^+ ATPase. With the expression cloning of mammalian nutrient transport proteins it became obvious that "archaic" transport mechanisms utilizing a transmembrane proton-gradient for nutrient influx into the cell are more prominent than anticipated. The present review focuses on selected nutrient transport processes in which proton-coupling in the transport process has been demonstrated.

Zusammenfassung

Die Aufnahme von Nährstoffen in Zellen ist lebensnotwendig und erfolgt in allen Organismen unter Aufwendung von Energie. In den meisten prokaryotischen Zellen und in Zellen niederer Eukaryoten liefert der elektrochemische Protonengradient über der Zellmembran die treibende Kraft zur Aufnahme von Nährstoffen. In höher entwickelten Eukaryoten ist die Nährstoffaufnahme dagegen häufig an einen transmembranären Natriumgradienten gekoppelt, der durch die Aktivität der Na^+/K^+-ATPase aufrechterhalten wird. Erst nach der Expressionsklonierung vieler Nährstofftransporter aus Säugern wurde deutlich, daß die archaisch anmutenden protonengetriebenen Transportmechanismen für die Aufnahme von Nährstoffen weiter verbreitet sind, als ursprünglich vermutet. Nachfolgend werden ausgewählte Nährstofftransportproteine beschrieben, bei denen der Transportprozeß direkt an die Nutzung eines Protonengradienten gekoppelt ist.

1. Introduction

The metabolism of all living cells is protected against the environment by a plasma membrane composed of a lipid bilayer with embedded proteins. Lipid bilayers for themselves are nearly impermeable to ions such as Na^+, K^+, Ca^{2+} and Cl^-, as well as for ionic or polar nutrients such as amino acids, peptides, sugars and nucleotides. Ions and nutrients can permeate into the cell only through the action of specialized membrane transport proteins and even lipophilic nutrients such as cholesterol or fatty acids – so far believed to cross the cell membranes by passive diffusion – appear to require specialized membrane proteins for transport that also control permeation rate and metabolic flow.

Due to thermodynamics, transport through a membrane can be described as passive or active. Passive transport or "facilitated diffusion" allows molecules to flow from the side of high concentration to low concentration depending on their electrochemical gradient. In active

transport processes, ions or metabolites are transported along or against their electrochemical gradient. The endergonic process of an uphill transport must be efficiently coupled with an exergonic process. Two sources of free energy are used in living cells for the uphill transport of molecules: direct ATP hydrolysis (primary active transport) or energy stored in an electrochemical ion gradient across the membrane (secondary active transport).

Whereas the transport pathways that mediate uptake of nutrients into bacteria, yeast and plants are mainly energized by a transmembrane electrochemical proton gradient, the systems mediating nutrient influx into mammalian cells have diverged and are more frequently energized by electrochemical Na^+ gradients provided by the activity of the Na^+/K^+ ATPase. However, in some of the mammalian transport proteins, nutrient uptake is associated with the downhill movement of protons along a shallow proton gradient that in combination with the inside negative membrane potential provides a powerful driving force that allowing nutrients to be accumulated above their extracellular concentrations. The functional expression of these proton-coupled transporters is restricted to specialized cells, mainly epithelial cells and to membranes of intracellular compartments. Representative proton-dependent carrier systems are those for di- and tripeptides, selected free amino acids, some vitamins, sugars and trace elements but also for a number of organic acids from intermediate metabolism.

2. Electrogenic Proton-dependent Peptide Transporters

Mammalian peptide transporters are almost a paradigm for electrogenic proton-dependent nutrient uptake processes. Transmembrane transport of short chain peptides occurs in all living organisms and provides an efficient and energy-saving route for uptake of bulk quantities of amino acids in peptide-bound form. Although accepted as important processes in the nutrition of prokaryotes and simple eukaryotes, the existence of similar transporters in mammals was not anticipated. When the active absorption of short chain peptides in the mammalian gut epithelium was shown in the mid-1970s, it was initially presumed to be energized by the transmembrane Na^+ gradient. During the 1980s evidence accumulated that similar uptake processes for di- and tripeptides are also present in epithelial cells of the kidney tubule and finally, intestinal and renal transport was proven to be energized by an inwardly directed proton gradient across brush-border membranes. The underlying transporter proteins were identified by expression cloning of corresponding cDNA's from rabbit tissues in 1994 and 1996 (Fei et al. 1994, Boll et al. 1996) and the transporters in other mammalian species were identified by homology screening techniques (see reviews Rubio-Aliaga and Daniel 2002, Herrera-Ruiz and Knipp 2003). The intestinal transporter was designated as PEPT1 (SLC15A1) and the renal isoform as PEPT2 (SLC15A2). Functional analysis of the transporters in *Xenopus oocytes* and mammalian cells established PEPT1 to represent the low affinity/high capacity and PEPT2 the high affinity/low capacity variant – nevertheless both proteins essentially transport the same substrates.

The two mammalian di- and tripeptide transporters couple uphill substrate flux to proton movement down an electrochemical proton gradient and accept essentially all possible 400 dipeptides and 8000 tripeptides composed of L-α-amino acids as substrates. In addition ω-amino fatty acids carrying a positively and a negatively charged head group separated by at least four methylene groups are also transported showing that a peptide bond is not a prerequisite for recognition of a substrate. More detailed information on the structural requirements

in determining substrate affinity is provided in recently published reviews (Rubio-Aliaga and Daniel 2002, Terada et al. 2000). Due to this broad substrate specificity it is not surprising that peptide transporters can also recognize a variety of related peptidomimetics. Therapeutic drugs like beta-lactam antibiotics, selected angiotensin-converting enzyme inhibitors and peptidase inhibitors belong to this group of compounds with peptide-like structure, and the peptide transporter have been shown to determine the oral availability and pharmacokinetics of these drugs. Moreover, a new concept for drug delivery involves the design of compounds that by coupling of an amino acid to the drug entity generates a peptidomimetic that than utilizes PEPT1 or PEPT2 for transport. This approach was proven with agents that possess an intrinsic low oral bioavailability such as L-DOPA or the antiviral drug acyclovir which after amino acid derivatization (L-DOPA-Phe and Val-acyclovir) became substrates of PEPT1.

The transport mode of PEPT1 is characterized by the cotransport of one neutral substrate molecule with one hydronium ion. However, the absolute number of transported protons and charges can vary depending on the presence and the number of differently charged amino acid side chains in the peptide and their intramolecular location. The proton to substrate stoichiometric ratios for cotransport of neutral and cationic dipeptides was found to be one, while anionic dipeptides carrying a carboxyl group in the side chain are transported together with two protons (Kottra et al. 2002, Mackenzie et al. 1996, Steel et al. 1997). Differences in transport characteristics of cationic dipeptides depend on the position of the cationic amino acid in the substrate (amino terminal or carboxy terminal location) and suggested that the substrate binding site is asymmetric. Electrophysiological analysis of pre-steady-state currents suggested for hPEPT1 an ordered, simultaneous transport model in which H^+ binds first (Mackenzie et al. 1996). Giant patch clamp experiments provided evidence that PEPT1 is also capable of transporting in the reverse direction, albeit with a considerably lower substrate affinity (Kottra and Daniel 2001). The electrochemical proton gradient for peptide transport in the epithelium of intestine and kidney is mainly generated by the concerted action of Na^+/K^+ ATPase creating a Na^+ gradient across the cell membrane that than is utilized by the apical Na^+/H^+ exchanger isoform NHE-3 that converts the Na^+ gradient into a proton gradient.

The human genes encoding PEPT1 and PEPT2 map to chromosome 13q33 – 34 (Liang et al. 1995) consisting of 23 exons (Urtti et al. 2001) and chromosome 3q13.3 – q21 (Ramamoorthy et al. 1995) consisting of 22 exons (Rubio-Aliago et al. 2000), respectively. The open reading frames consist of 708 (hPEPT1) and 729 amino acids (hPEPT2) with 50 % overall sequence identity and around 70 % similarity. Both transporters show 80 % identity with the corresponding rabbit transporters. The amino acid sequences predict for PEPT1 and PEPT2 twelve transmembrane domains with amino and carboxy termini located intracellular (hPEPT1: Covitz et al. 1998, Liang et al. 1995, hPEPT2: Liu et al. 1995). A large extra-cellular loop of unknown function and lowest homology between the two transporter isoforms is located between transmembrane domain 9 and 10 (Fig. 1). Functional analysis of point mutants and chimeric constructs composed of PEPT1 and PEPT2 have shown that the aminoterminal region including transmembrane domains 1 – 9 plays an important role in determining the substrate affinity and other characteristic features of PEPT1 and PEPT2 (Doring et al. 1996, 2002). Of central importance for the activity of these transporters are amino acid residues in transmembrane domains 2, 3 and 4. Mutation of a single histidine residue in transmembrane domain 2 abolishes transport activity completely (Bolger et al. 1998, Fei et al. 1997, Yeung et al. 1998) implicating that this critical residue might be involved in proton binding.

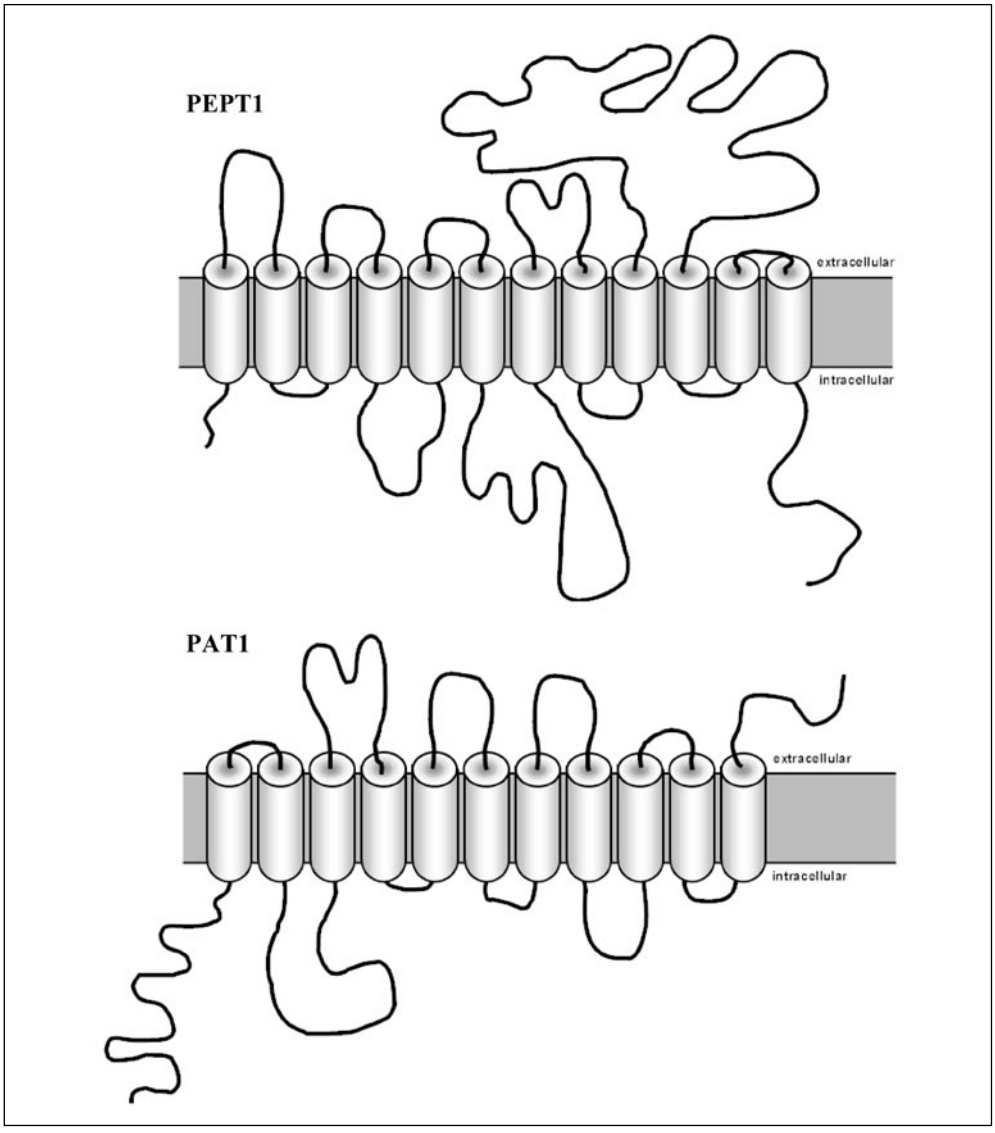

Fig. 1 Proposed membrane topology of a proton-coupled peptide (PEPT1) and amino acid transporter (PAT1)

PEPT1 is mainly expressed in the apical plasma membrane of enterocytes, renal proximal tubular cells and bile duct cells, but a PEPT1-like activity is also present in lysosomal membranes and PEPT1 immunoreactivity was shown in the nuclei of vascular smooth muscle cells (Bockman et al. 1997, Knutter et al. 2002, Lee et al. 1999, Zhou et al. 2000). PEPT2 functions mainly in kidney but is also expressed in a variety of other tissues including glia cells in brain, mammary gland and bronchial epithelium and cells of the choroid plexus (Berger and Hediger 1999, Groneberg et al. 2001, 2002, Shu et al. 2002).

Tab. 1 Distribution of electrogenic transporters for peptides and amino acids in mammalian tissues, cells and subcellular compartments.

Transporter	Tissue	Localization
PEPT1 (SLC15A1)	Small intestine	Brush border membrane of enterocytes
	Kidney	Brush border membrane of epithelial cells of the proximal tubule S1 segment
	Bile duct	Apical membrane
	Pancreas	Lysosomes of acinar cells
	Smooth muscle	Nuclei of vascular smooth muscle cells
PEPT2 (SLC15A2)	Kidney	Brush border membrane of epithelial cells of the proximal tubule S2 and S3 segment
	CNS	Epithelial cells of choriod plexus, ependymal cells and astrocytes
	PNS	Glial cells
	Lung	Apical membrane of bronchial and tracheal epithelial cells, pneumocytes
	Mammary gland	Epithelial cells of glands and ducts
	Colon, pancreas	
PAT1 / LYAAT1 (SLC36A1)	Brain	Neurons
	Intestine	Epithelial cells, brush border membrane
	Colon, Kidney, Lung, Liver	
	Spleen	
PAT2 (SLC36A2)	Lung, heart, kidney, muscle, testis, spleen, adrenal gland, thymus, sciatic nerve	

3. Novel Electrogenic Proton-dependent Amino Acid Transporters

Amino acid/auxin permease transport proteins are grouped in the AAAP transporter family and are found virtually in all eukaryotic organisms, like yeast, plants, invertebrates, and vertebrates (Young et al. 1999, Wipf et al. 2002). In mammals three different subfamilies of the AAAP transporter class have been identified, including the vesicular GABA transporter VGAT (McIntire et al. 1997), the various system A/N transporters (Chaudhry et al. 1999, Reimer et al. 2000, Sugawara et al. 2000, Nakanishi et al. 2001) and the LYAAT/PAT subfamily (Sagne et al. 2001, Boll et al. 2002). Although amino acid sequence identity and homology is rather low among the members of the family, a common characteristic signature within the proteins is identified (Young et al. 1999). Membrane topology predictions based on hydropathy analysis (McIntire et al. 1997, Chaudhry et al. 1999, Reimer et al. 2000) reveal similar secondary structures and most of the mammalian AAAP family members are comprised of 11 putative transmembrane domains (Fig. 1). All so far functionally characterized mammalian AAAP transporters possess at least the two common features: firstly, they all recognize amino acids or closely related compounds as substrates (McIntire et al. 1997, Reimer et al. 2000, Boll et al. 2002) and secondly, their activity is dependent on changes in the intra- or extracellular proton concentration. Protons are either cotransported with the substrate as in the PAT transporters (Sagne et al. 2001, Boll et al. 2002) or are transported in opposite direction in an antiport mode

as in VGAT and system N transporters (McIntire et al. 1997, Chaudhry et al. 1999) or serve as negative regulators of transport activity as in the system A transporters (Reimer et al. 2000).

The mammalian members of the AAAP family are represented by the rat LYAAT1 and the mouse and human PAT1 and PAT2 transporters and operate as electrogenic proton-dependent amino acid cotransporters. LYAAT1 and PAT1 are orthologous, identified independently by Sagne et al. (2001) and Boll et al. (2002) by homology based cloning. Recently, Chen et al. (2003) and Boll et al. (2003a) reported the cloning of a human PAT1- and PAT2-cDNA. Beside these two functionally characterized members, the PAT family comprises in addition two orphan transporters (Boll et al. 2003a,c). PAT1 and PAT2 are proton-dependent transporters for small amino acids, such as glycine, alanine, and proline. Uptake of these amino acids is electrogenic and leads to an intracellular acidification suggesting a proton/amino acid symport mechanism (Boll et al. 2002). Beside the small L-α-amino acids, GABA and betaine have also been shown to interact with the transporters binding site (Boll et al. 2003b). Whereas PAT1 shows K_m values for substrates from 2.5 – 15 mM, PAT2 represents the high affinity subtype with K_m values for the same substrates below 0.6 mM. Northern blot analysis revealed different expression patterns for both transporters in mouse tissues. Whereas PAT1-mRNA has been detected in virtually all tissues examined, with particularly high expression levels in brain, small intestine, colon and kidney, expression of PAT2-mRNA is more restricted with high expression levels observed in lung and heart and lower levels in kidney, testis, muscle, and spleen (Boll et al. 2002). In epithelial cells of small intestine, the PAT1 protein is exclusively found in the brush-border membrane where it contributes to intestinal amino acid absorption (Chen et al. 2003). Additionally the PAT1/LYAAT1 protein, but not PAT2, has been detected in the plasma membrane and in lysosomes of neurons in rat brain (Sagne et al. 2001, Wreden et al. 2003, Agulhon et al. 2003). For PAT2, no immunohistochemical data are yet available. However, when over-expressed in Hela cells the protein was detectable in the plasma membrane (Boll et al. 2002) suggesting that it also may play a role in cellular uptake of small amino acids. From the present point of view, it seems that PAT1 can play a dual role in the mammalian organism. While in epithelial cells the transporter is located in the apical membrane mediating the uptake of small amino acids into the cell, in other cells like in brain the protein is also localized in lysosomes mediating proton-coupled efflux of small amino acids from lysosomes into the cytosol. Further experiments will elucidate how different targeting of one and the same protein in different cell types is regulated.

4. Proton-dependent Monocarboxylate Transporters

The exchange of monocarboxylates between tissues and organs is of central importance for a variety of metabolic processes including the release of lactate from muscle tissue and other cells followed by its re-uptake into liver for gluconeogenesis (Cori cycle) or the release and uptake of ketone bodies in states of starvation and ketoacidosis. Pyruvate, butyrate, propionate and acetate or the keto-derivatives of the branched chain amino acids also show significant interorgan fluxes. Transporters identified for the various monocarboxylates have been grouped in the MCT family, and transmembrane monocarboxylate fluxes occur in most cases electroneutral by symport of the organic anion with a proton. Currently the monocarboxylate transporters MCT-1 to MCT-9 are known as human genes (HUGO: SLC16A1 to SLC16A9) with a large number of related genes in other pro- and eukaryotic organisms (Halestrap and Price 1999).

MCT-1 is ubiquitously expressed in mammals. Very strong expression is found in heart and red muscle, where MCT-1 is up-regulated in response to increased work, suggesting its special role in lactic acid delivery for oxidation (Bonen et al. 1997). It is interesting to note that MCT-1 in muscle is also found in mitochondrial membranes based on immunostaining, which suggests that it may be targeted to plasma as well as to mitochondrial membranes (Brooks et al. 1999). The transporter MCT-4 is most prominent in white muscle and other cells with a high rate of glycolysis including tumor cells and white blood cells with a particular high demand for efficient lactic acid efflux (Bonen 2000). MCT-2 is a high affinity type transporter expressed in renal proximal tubules, sperm tails and neurons. MCT-3 is uniquely expressed in the retinal pigment epithelium (Halestrap and Price 1999).

Functional analysis of heterologously expressed MCT-4 as the major transporter isoform present in white skeletal muscle shows low affinity for L-lactate and L-pyruvate [28 and 153 mM] whereas those of the isoform MCT-1 range from 4.4 mM for L-lactate and 2.1 mM for L-pyruvate (Juel and Halestrap 1999). The kinetics of pyruvate and lactate transport into red blood cells have been thoroughly characterized and represent the characteristics of MCT-1 as the only MCT present in the red-blood-cell plasma membrane (Poole and Halestrap 1993). Transport involves proton binding to the transporter as the initial step, followed by binding of lactate and the translocation of the complex with the sequential release of the solutes from the transporter on the other side of the membrane. The transporter can operate in both directions depending on the concentration of substrate and cotransported protons on both sides of the membrane. The equilibrium is reached when $[\text{lactate}]_{in}/[\text{lactate}]_{out} = [H+]_{out}/[H+]_{in}$ (Halestrap and Price 1999).

In colon epithelium transport of weak organic acids like butyrate, lactate, propionate and acetate is also mediated by MCT-family members. Monocarboxylates are generated in the colon in huge quantities by microorganisms metabolizing soluble fibers (i.e. inulin, oligofructoses) and non-digested starch. Luminal concentrations of short chain fatty acids may reach 100 to 150 mM and uptake across the brush border membrane provides the fuel for colonocytes (butyrate) and delivers others to circulation (propionate, lactate, acetate). In addition to its role as an energy substrate, butyrate promotes cell growth in normal colon but inhibits proliferation in established colon adenomas and cancers. Kinetic properties of butyrate transport into colonocytes are consistent with the properties of MCT-1 and therefore this transporter appears to be very important in tissue homeostasis and control of intestinal adaptation (Stein et al. 2000). However, there are conflicting results whether MCT-1 is localized in the basolateral or the apical membrane of colonocytes (Orsenigo et al. 1999, Ritzhaupt et al. 1998). The importance of specialized transporters for short chain fatty acids was discussed controversially in view of the high permeability of cell membranes for the non-charged species of these low molecular weight compounds. pH-dependent partition between the two sides of the membrane with unrestricted diffusion of the non-polar species was considered to be the sole mechanisms for transport. Of course, this process represents a background route for permeation of monocarboxylates as it is determined by the physicochemical characteristics of the weak acids. However, the transport proteins such as MCT-1 and the modulation of the expression level of these carriers provides a level of control for the flux of the various organic weak acids in and out of a cell that is mandatory for metabolic homeostasis.

5. Proton-dependent Vitamin Transporters

Proton-dependent transporters that mediate transport of folates and thiamine and derivatives have been cloned and characterized recently (Matherly 2001). The first identified mammalian membrane folate carrier was the RFC-1 (SLC19A1). It mediates a sodium-independent but strongly pH-dependent folic acid uptake process that was identified functionally in intact tissues prior to cloning. Cellular uptake of reduced folic acid, methyl–tetrahydrofolate and methotrexate increases with a decrease in extracellular pH suggesting a H^+/folate symport or an OH^-/folate anion exchange process with folate anions exchanged for intracellular hydroxyl ions. A comparison of the amino acid sequence of human RFC-1 with other protein sequences helped to identify human ThTr1, the thiamine transporter (SLC19A2), as the closest relative with a sequence identity of 40 % and similarity of 55 % at the amino acid level (Dutta et al. 1999). Very recently a third member of this family of proteins was cloned from human and mouse (SLC19A3) and identified as a second thiamine transporter (Rajgopal et al. 2001). The unique feature of thiamine as a water soluble vitamin is its character as a cation. Depending on pH, it may carry one or two positive charges. ThTr1, cloned from human placenta, induces specific thiamine influx in transfected cells that shows the characteristics of a thiamin-proton exchange process with a pronounced pH dependence and reduced flux rates at low extracellular pH values.

6. Summary

A variety of proton-dependent membrane transport proteins for nutrients are found in mammals that show phenotypic characteristics similar to transporters in lower eukaryotes and prokaryotes. Most of these mammalian carrier proteins are proton-dependent symporters that utilize a transmembrane proton gradient for influx or efflux of solutes. In the mammalian system, transmembrane proton gradients are shallow and appear to compartimentalize with pH microdomains in close vicinity of the cell membrane. Such a membrane microenvironment (microclimate) in which the pH is in disequilibrium with the extracellular or intracellular bulk solution is a characteristic phenomenon at membrane interfaces. Proton gradients can be generated by Na^+/H^+ antiporters that allow protons to accumulate on the outer membrane surface or that are a consequence of a high metabolic production of weak acids that are not entirely buffered by endogenous buffer systems. Representative transporters using such proton gradients for bi-directional transport of organic solutes are the monocarboxylate transporters which operate as electroneutral symporters for metabolically important intermediates such as lactate, pyruvate, acetate, propionate or butyrate. Proton-coupled nutrient carriers that are in contrast electrogenic in nature are the peptide transporters and selected amino acid transporters. As the majority of peptides and amino acids are non-charged at physiologically pH, proton/substrate symport by these systems is strongly dependent on the membrane potential as the classical electromotive force. Why these "archaic" systems survived evolution in contrast to most of the other nutrient transporters in mammals remains to be determined.

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Prof. Dr. Hannelore Daniel
Wissenschaftszentrum Weihenstephan
Lehrstuhl für Ernährungsphysiologie
Institut für Lebensmittel und Ernährung
Hochfeldweg 2
85350 Freising-Weihenstephan
Germany
Phone: +49 81 61713400
Fax: +49 81 61713999
E-Mail: daniel@weihenstephan.de

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Structure and Function of IP_3 Receptor

Katsuhiko Mikoshiba, Kozo Hamada, Hideaki Ando,
Takayuki Michikawa, Songbai Zhang, Akinori Kuruma,
and Takafumi Inoue (Tokyo)

With 1 Figure

Abstract

IP_3 receptor is a IP_3-gated Ca^{2+} release channel and works as an intracellular Ca^{2+} oscillator. IP_3 receptor has many unique biochemical properties different from the Ca^{2+} channel on the plasma membrane. In addition, IP_3 receptor interacts with many of the molecules which may modify the function of IP_3-induced Ca^{2+} releasing activity. These properties may be important for IP_3 receptor to work as a metabolic center playing an important role in the various tissues during development as well as in adult stage.

Zusammenfassung

Der IP_3-Rezeptor ist ein IP_3-gesteuerter Ca^{2+}-Freisetzungskanal und wirkt als ein intrazellulärer Ca^{2+}-Oszillator. Der IP_3-Rezeptor hat viele einzigartige biochemische Eigenschaften, die ihn vom Ca^{2+}-Kanal in der Plasmamembran unterscheiden. Außerdem wirkt der IP_3-Rezeptor mit vielen jener Moleküle zusammen, die die IP_3-induzierte Ca^{2+}-Freisetzungsaktivität modifizieren können. Diese Eigenschaften könnten für den IP_3-Rezeptor wichtig sein, um als Stoffwechselzentrum zu wirken, das sowohl während der Entwicklung als auch im Adultstadium eine wichtige Rolle in verschiedenen Gewebe spielt.

1. IP_3 Receptor as a Developmentally Regulated P400 Enriched in Cerebellar Purkinje Neuron

We have been long working on the P400 protein which increases during development but is greatly decreased in the cerebellar mutant mice where Purkinje cells are deficient or spines of Purkinje cells are absent. We discovered that this developmentally regulated P400 protein is IP_3 receptor (Furuichi et al. 1989). We found that IP_3R is essential for Ca^{2+} oscillation, since specific function-blocking antibody against IP_3R1 blocks Ca^{2+} oscillation during fertilization (Miyazaki et al. 1992).

2. Unique Biochemical Properties of IP_3 Receptor

IP_3 receptor (IP_3R) is an IP_3-gated Ca^{2+} channel (Miyawaki et al. 1990, Maeda et al. 1991). We found by intensive molecular and biochemical studies that IP_3R has unique biochemical properties as following:

(*i*) IP_3R allosterically and dynamically changes its form (windmill form in the presense of Ca^{2+} and square form in the absence of Ca^{2+}) (Hamada et al. 2003).
(*ii*) IP_3R is functional (both IP_3-binding activity and Ca^{2+}-releasing activity) even though it is fragmented by mild trypsin digestion into several pieces.

3. Proteins which Associate with IP_3 Receptor

We found that IP_3R interacts with Trp channel on the plasma membrane (Boulay et al. 1999). We found 4.1N cytoskeletal protein associates with C-terminal tail of IP_3R1 (Zhang et al. 2003). Overexpression of 4.1N results in a translocation of IP_3R1 to the plasma membrane region (Zhang et al. 2003). We found CARP (carbonic anhydrase related protein) which binds to the modulatory domain of IP_3R1 lowers the affinity to IP_3 (Hirota et al. 2003). Since CARP is exclusively expressed in Purkinje cells, the fact that IP_3 sensitively for Ca^{2+} release in Purkinje cell is low could be due to co-expression of CARRP with IP_3R in Purkinje cells and inhibitory effects on IP_3 binding. We purified and identified a new protein, IRBIT (IP_3R-binding protein released with inositol 1,4,5-trisphosphate) which interacts with IP_3-binding site of IP_3R1. IRBIT is released upon IP_3 raising the possibility that IRBIT acts as a signaling molecule downstream from IP_3R (Ando et al. 2003).

4. A New Five-domain Structure Model of IP_3 Receptor for IP_3-gated Ca^{2+} Release

Using intrinsically IP_3R-deficient cells as the host cells for the expression of mutant form of IP_3 receptor, we obtained results to indicate that two regions of IP_3R1, namely amino acid residues 1 – 223 and 651 – 1130, are critical for the IP_3-induced gating. We therefore named 1 – 223 region as N-terminal coupling domain, and we named the region down to channel region as internal coupling domain which includes amino acid residues 651 – 1130. We also identified a highly conserved cystein residue at position 2513, which is located within the C-terminal tail, as being essential for the channel opening. We therefore propose a novel five-domain structure model in which both N-terminal and internal coupling domains transduce ligand-binding signals to the C-terminal tail, which acts as a gatekeeper that triggers opening of the activation gate of IP_3R1 following IP_3 binding (Uchida et al. 2003).

5. Development of a Novel Recombinant Hyper-affinity IP_3 Absorbent (IP_3 Sponge) that Results in Specific Inhibition of IP_3-mediated Ca^{2+} Signaling

We have developed a novel recombinant hyper-affinity inositol 1,4,5-trisphosphate (IP_3) absorbent, called the IP_3 sponge, which we construct on the basis of the ligand-binding site of the mouse type 1 IP_3 receptor (IP_3R1) (Uchiyama et al. 2002). It exhibits approximately 1000-fold higher affinity for IP_3 than the parental IP_3R1 and specifically competes with the endogenous IP_3R for binding to IP_3. Trapping IP_3 with the IP_3 sponge inhibits IP_3-induced Ca^{2+} release (IICR) from cerebellar microsomes in a dose-dependent manner. We succeeded in obtaining 3-dimensional structure at 2.3 Å (Bosanac et al. 2002). It shows a unique structure. IP_3 sponge injected into oocyte blocks fertilization, and suppresses CREB (cAMP responsible element binding protein) phosphorylation suggesting that IP_3-induced Ca^{2+} release is involved in the phosphorylation of CREB (Uchiyama et al. 2002). We utilized a novel IP_3R antagonist, the "IP_3 sponge", to investigate the role of IP_3 during fertilization of the starfish oocyte. The IP_3 sponge strongly, specifically and dose-dependently competes with endogenous IP_3R for binding to IP_3. By injecting IP_3 sponge into starfish oocyte, the increase in intracellular Ca^{2+} and formation of the fertilization envelope are both dramatically blocked, although oocyte

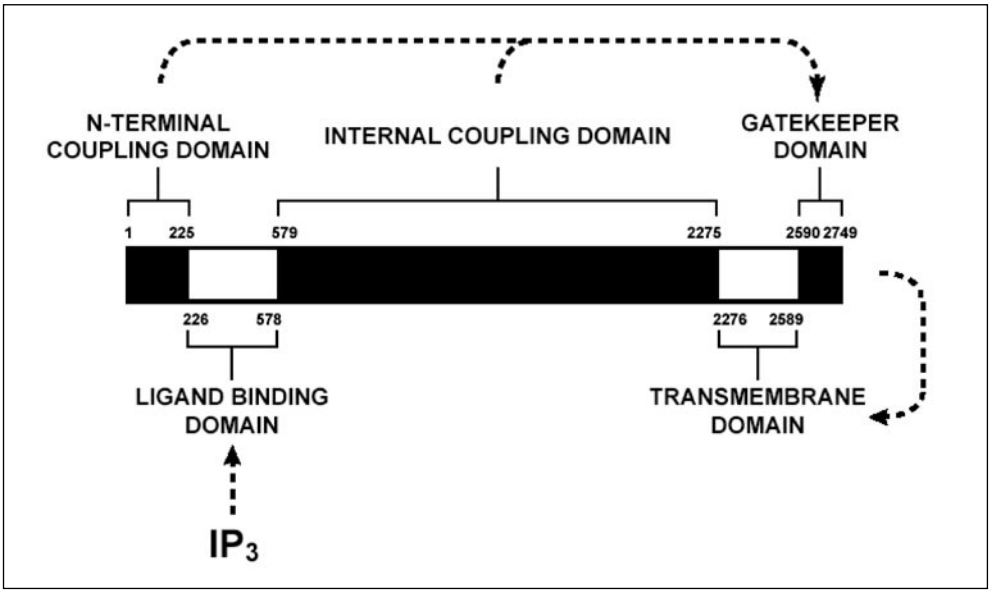

Fig. 1 A five-domain structure model of the IP_3R1. In this model, the structure of the IP_3R1 is divided into five functional domains, namely, as N-terminal coupling domain (amino acid residues 1–225), ligand-binding domain (226–578), internal coupling domain (579–2275), transmembrane domain (2276–2589), and gatekeeper domain (2590–2749). The signal of IP_3 binding is transferred through both the N-terminal and internal coupling domains to the gatekeeper domain which triggers conformational change of the activation gate.

maturation is not blocked (Iwasaki et al. 2002). Since we found that IP_3 sponge works even *in vivo*, we are planning to apply it to study the effect on the development and neural plasticity of the nervous system.

6. Developmental Role of IP_3 Receptor

Dorso-ventral axis formation is an essential step for the body plan. Early in embryonic growth, the signaling pathway specifies the development pattern. Evidence has been obtained that blockage of PI turnover in the ventral part of the embryo by injecting lithium chloride leads to the conversion of the ventral mesoderm to the dorsal mesoderm, thereby generating ectopic dorsal axes. Injection of the function blocking antibody against IP_3 receptor converted ventral mesoderm to dorsal mesoderm. This finding suggests a role for the IP_3/Ca^{2+}-signaling pathway as a ventralizing signal in addition to the already known pathway through glycogen synthase kinase 3β (Kume et al. 1997). We recently found that downstream target of IP_3 receptor is NFAT. Dominant negative form of NFAT converts ventral mesoderm to dorsal mesoderm (Saneyoshi et al. 2002).

7. Type1 IP_3R Is Involved in Neuronal Plasticity (LTD in the Cerebellum and LTP in the Hippocampus)

Type 1 IP_3 receptor (IP_3R1) is the major neuronal member of the IP_3R family in the central nervous system. We found that most IP_3R1-deficient mice generated by gene targeting die *in utero*, and born animals have severe ataxia and tonic or tonic-clonic seizures and die by the weaning period. Electroencephalograms have shown that they suffer from epilepsy, indicating that IP_3R1 is essential for proper brain function (Matsumoto et al. 1996). We found that the IP_3-receptor-deficient mice could be used as valuable model animals in studying the role of IP_3 receptor in neuronal plasticity.

Since the IP_3 receptor is highly enriched in Purkinje neurons in the cerebellum, a test was conducted as to whether long-term depression (LTD), involving the cerebellar memory system is altered or not in the IP_3-receptor-deficient mice. Purkinje neuron from the IP_3-receptor- deficient mice and that injected with the IP_3 receptor antibody shows a blockage of LTD, suggesting the importance of the IP_3 receptor in cerebellar plasticity (Inoue et al. 1998). Then, we studied LTP in hippocampal slice of IP_3R1-deficient mice. Long term potentiation (LTP) of CA1 hippocampus of the IP_3R1-deficient mice shows no significant difference from wild type by standard tetanus. LTP is induced by short tetanus only in the mutant. LTD at CA1 hippocampus is induced by short low frequency stimuli (LFS) in both wild type and mutant. Depotentiation of LTP after standard tetanus is observed in the wild type, but the depotentiation is abolished in the mutant (Fujii et al. 2000). LFS induces LTD but the LTD suppresses subsequent LTP induced by standard tetanus (LTP suppression). The LTP suppression is abolished in the mutant (Fujii et al. 2000). Further more, we found that IP_3R1 is involved in the determination of polarity and input specificity of synapse (Nishiyama et al. 2000). These data clearly show that IP_3 receptor is involved in development and neuronal plasticity.

8. Dynamics of Ca^{2+} and Na^+ in the Dendrites of Mouse Cerebellar Purkinje Cells Evoked by Parallel Fiber Stimulation

We studied dynamics of Ca^{2+} and Na^+ transients by simultaneous monitoring in Purkinje cell dendrites in mouse cerebellar slices. High frequency parallel fiber stimulation depolarizes Purkinje cells, and Ca^{2+} transients are observed at the anatomically expected sites. The magnitude of the Ca^{2+} transients increases linearly with increasing numbers of parallel fiber inputs. With 50 stimuli, Ca^{2+} transients last for seconds, and the peak [Ca^{2+}] reaches ~100 μM which is much higher than that reported previously, although it is still confined to a part of the dendrite. In contrast, Na^+ transients are sustained for tens of seconds and diffused away from the stimulated site. Pharmacological interventions revealed that Na^+ influx through AMPA receptors and Ca^{2+} influx through P-type Ca channels are essential players, that AMPA receptors does not operate as Ca^{2+} influx pathway.

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Prof. Dr. Katsuhiko Mikoshiba
University of Tokyo
Institute of Medical Science
Department of Molecular Neurobiology
Minato-ku
Tokyo 108-8639
Japan
Phone: +81 3 54495316
Fax: +81 3 54455420
E-Mail: mikosiba@ims.u-tokyo.ac.jp

Festliche Übergabe des Präsidentenamtes von Benno Parthier an Volker ter Meulen

am 13. Februar 2003 im Freylinghausen-Saal der Franckeschen Stiftungen zu Halle (Saale)

Nova Acta Leopoldina N. F., Bd. *89*, Nr. 335
Herausgegeben vom Präsidium der Akademie
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Die Übergabe des Präsidentenamtes in der Deutschen Akademie der Naturforscher Leopoldina lieferte die Gelegenheit für eine erneute Standortbestimmung der Akademie, nachdem die Leopoldina erst 2002 ihr 350. Gründungsjubiläum begangen hatte. Nach der Begrüßung durch Leopoldina-Vizepräsident Ernst-Ludwig Winnacker (Bonn/München) beschäftigte sich der scheidende Präsident Benno Parthier (Halle/Saale) mit den erforderlichen Weichenstellungen und Entwicklungen der Wirkungsfelder der Akademie in den zurückliegenden Jahren seiner Präsidentschaft. In seiner Antrittsrede umriß der neue Präsident Volker ter Meulen (Würzburg) die weiteren Aufgaben der Leopoldina und ging dabei auch auf die heißdiskutierte Frage einer nationalen Akademie für Deutschland ein. In seinem Festvortrag sprach Wolfgang Frühwald (München) zum Thema »Eine liebenswerte Wissenschaft und ein glücklicher Sisyphos. Zum Leben und zur Arbeit einer nationalen Akademie«.

In Kommission bei Wissenschaftliche Verlagsgesellschaft mbH Stuttgart

Nova Acta Leopoldina NF *89*, Nr. 336, 33–36 (2004)

The Endoplasmic Reticulum in Polarized Epithelial Cells: Differential Sensitivity to Ca^{2+} Releasing Messengers Including Ca^{2+} itself

Ole H. Petersen (Liverpool)

Abstract

The pancreatic acinar unit is composed of classically polarized acinar cells. Even in isolation, these cells retain their polarity and this has made them particularly useful for Ca^{2+} signaling studies. In 1990, we discovered that this cell has the capability to produce cytosolic Ca^{2+} signals, both locally and globally. The mechanisms underlying this signal generation have now been established. Furthermore it has become clear that the local signals are sufficient for the control of both fluid and enzyme secretion, whereas prolonged global signals are dangerous and give rise to a disease process in which the pancreas digests itself, namely acute pancreatitis.

Zusammenfassung

Ein Pankreasazinus ist aus mehreren polarisierten Azinuszellen zusammengesetzt. Auch nach Isolation dieser Zellen *in vitro* behalten sie ihre Polarisierung, und dies machte sie für Ca^{2+}-Signal-Untersuchungen besonders geeignet. Wir entdeckten 1990, daß diese Zelle die Fähigkeit hat, lokale und globale zytosolische Ca^{2+}-Signale zu produzieren. Der Mechanismus, der dieser Signalentstehung zugrunde liegt, ist nun beschrieben worden. Weiterhin ist geklärt worden, daß die lokalen Signale für die Kontrolle von Flüssigkeits- und Enzymsekretion ausreichen, während langanhaltende globale Signale gefährlich sind und einen Krankheitsprozeß initiieren können, in dem das Pankreas sich selbst verdaut, was zur Pankreatitis führt.

1. Introduction

More than 30 years ago, it was demonstrated that neurotransmitters eliciting fluid and enzyme secretion from exocrine glands, activate the acinar cells by liberating Ca^{2+} into the cytosol from a store in the endoplasmic reticulum (ER) (Nielsen and Petersen 1972). Twenty years ago, work on pancreatic acinar cells directed by Irene Schulz and Michael Berridge (Streb et al. 1983) provided the original evidence for the Ca^{2+} releasing action of inositol 1,4,5-trisphosphate (IP_3). Further work on the pancreatic acinar cells presented some problems for the general view that IP_3 acts on the ER, since the primary intracellular Ca^{2+} release site turned out to be in the apical pole, which is dominated by the secretory (zymogen) granules, but contains little ER (Thorn et al. 1993). It is now evident that all cytosolic Ca^{2+} signals elicited by neurotransmitter or hormone stimulation are initiated in the apical secretory granule area and in many cases are confined to this region (Petersen et al. 1994). These local apical Ca^{2+} signals control not only exocytosis (Park et al. 2004), but also acinar fluid secretion *via* regulation of Ca^{2+}-activated Cl^- channels specifically located in the apical plasma membrane (Park et al. 2001). This very brief review explains how the acinar cells manage to generate these local apical Ca^{2+} signals, which are crucial for their physiological function.

2. Long-range Intracellular Communication

It is now well established that G protein coupled receptors in very many different cell types activate phospholipase C causing breakdown of membrane-bound phosphatidyl inositol bisphosphate (PIP_2) resulting in the formation of IP_3 and diacyl glycerol. It is possible to visualize the breakdown of PIP_2 and formation of IP_3 in real time in single cells by fluorescence measurements of the translocation of the GFP-conjugated PH domain of PLC. In such studies one can clearly observe the breakdown of PIP_2 in the basolateral membrane and the appearance of IP_3 in the cytosol in response to ACh stimulation (Ashby et al. 2002).

Long distance communication between muscarinic receptors and intracellular Ca^{2+} release channels was revealed by carbachol uncaging inside a patch pipette attached to the basal membrane and monitoring of the cytosolic Ca^{2+} concentration. These studies showed that local stimulation of muscarinic receptors at the base gave rise to cytosolic Ca^{2+} signals at the other side of the cells in the apical (granular) pole. With strong stimulation, such Ca^{2+} signals would spread as a wave from the apex to the base (Ashby et al. 2003).

3. Local and Global Ca^{2+} Spikes

Osipchuk et al. (1990) discovered that acinar cells can produce local or global cytosolic Ca^{2+} signals, depending on the agonist concentration used. Flooding the cell interior with IP_3 causes local Ca^{2+} spiking specifically in the apical pole (Thorn et al. 1993). The same result is obtained when using the more novel Ca^{2+} releasing messengers cyclic ADP-ribose (cADPR) or nicotinic acid adenine dinucleotide phosphate (NAADP) (Cancela et al. 2002). It is now clear that the specific localization of mitochondria on the border between the granular pole and the basolateral part of the cell, surrounding the nucleus, is crucial for confining Ca^{2+} signals initiated in the apical pole to that region (Tinel et al. 1999). When all three intracellular messengers are put together, there is marked potentiation resulting in global Ca^{2+} signals progressing as waves from the apical pole to the base of the cells (Cancela et al. 2002).

4. The Endoplasmic Reticulum

We have recently mapped the Ca^{2+}-sensitive Ca^{2+} release sites from the endoplasmic reticulum (ER) in pancreatic acinar cells, using local uncaging of caged Ca^{2+}, and shown that Ca^{2+}-induced Ca^{2+} release (which does not involve IP_3 formation) can only be triggered in the apical pole and is dependent on both functional IP_3 and ryanodine receptors (Ashby et al. 2002). Ryanodine itself can also trigger Ca^{2+} waves, which always start in the apical pole.

A detailed investigation of the distribution of ER in living acinar cells, using a number of different ER-specific fluorescent probes in conjunction with confocal and two-photon microscopy, shows that although the bulk of the ER is clearly located in the basolateral part of the cells, there is significant invasion of ER into the granular pole and each secretory granule is surrounded by strands of ER (Gerasimenko et al. 2002). These data provide the framework for a coherent and internally consistent theory for cytosolic Ca^{2+} signal generation in the secretory pole, where the primary Ca^{2+} release occurs from ER terminals in the apical pole supplied with Ca^{2+} from the main store at the base of the cell *via* the tunnel function of the ER (Petersen et al. 2001).

5. Mechanisms of Ca^{2+} Release

Recent work has defined the mechanism of action of NAADP on isolated nuclei from pancreatic acinar cells. Ca^{2+} release from the envelope of these nuclei (plus adhering ER strands) could be activated by NAADP as well as by IP_3 and cADPR. Each of these agents reduced the Ca^{2+} concentration inside the nuclear envelope and this was associated with a transient rise in the nucleoplasmic Ca^{2+} concentration. NAADP released Ca^{2+} from the same thapsigargin-sensitive pool as IP_3 and cADPR. The NAADP action was specific since, for example, NADP was ineffective. The Ca^{2+} release was unaffected by procedures interfering with acidic organelles (bafilomycin, brefeldin, nigericin). Ryanodine could block the Ca^{2+}-releasing effects of NAADP, cADPR and caffeine, but not IP_3. Ruthenium red also blocked the Ca^{2+} release elicited by NAADP but not by IP_3. IP_3 receptor blockade did not inhibit the Ca^{2+} release elicited by NAADP or cADPR. We conclude that the nuclear envelope/ER contains both functional ryanodine and IP_3 receptors, which can be activated separately and independently: the ryanodine receptors by either NAADP or cADPR and the IP_3 receptors by IP_3. These data are most easily understood by assuming that the NAADP receptor is not itself an ion channel. The characteristics of NAADP- and cADPR-elicited Ca^{2+} release from the nuclear envelope are essentially identical except that cADPR can still act to release Ca^{2+} normally when the NAADP receptors have been inactivated by a high NAADP concentration. The simplest explanation is that occupied cADPR and NAADP receptors can both interact with ryanodine receptors to increase their open state probability (Gerasimenko et al. 2003).

6. Pathophysiology

Excessive stimulation (hyperstimulation) of acinar cells evokes sustained global Ca^{2+} signals, which are associated with trypsin activation in the secretory granules and transformation of the normally electron dense granules into vacuoles (Raraty et al. 2000). Later, it has been shown that bile acids, implicated in the generation of pancreatitis, also have the ability to generate prolonged global Ca^{2+} signals (Voronina et al. 2002).

A recent study, dealing with the actions of alcohol and alcohol metabolites on Ca^{2+} transports in pancreatic acinar cells shows that whereas ethanol, even in very high concentrations, has little effect on the cytosolic Ca^{2+} concentration, several non-oxidative alcohol metabolites generate substantial and sustained global elevations of the cytosolic Ca^{2+} concentration and depolarize completely the inner mitochondrial membrane. The toxic effects of excessive alcohol intake are therefore not caused by ethanol directly, but rather by metabolites such as fatty acid ethyl esters. These act by emptying the intracellular Ca^{2+} stores and, because the mitochondrial function is also impaired, the cell is unable to dispose of the Ca^{2+} released into the cytosol (Criddle et al. 2004).

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Prof. Ole H. Petersen FRS
MRC Secretory Control Research Group
The Physiological Laboratory
University of Liverpool
Crown Street
Liverpool L69 3BX
UK
Phone: +44 151 7945342
Fax: +44 151 7945323
E-Mail: o.h.petersen@liv.ac.uk

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SERCA2b Mutations Associated with Darier's Disease

Dong Min SHIN[1], Wooin AHN[2], and Shmuel MUALLEM[3]

With 3 Figures

Abstract

Darier's Disease (DD) is a high penetrance, autosomal dominant mutation in the *ATP2A2* gene, which encodes the SERCA2 Ca^{2+} pump. Although more than 100 mutations in the *ATP2A2* were identified, no apparent relation between genotype/phenotype emerged. To begin to understand the basis of the disease and the relationship between phenotype/genotype we analyzed Ca^{2+} signaling in SERCA2$^{-/-}$ mice and the effect of DD-associated mutations on activity of WT SERCA. Deletion of one copy of the SERCA2 gene resulted in adaptation of the Ca^{2+} signaling to reduced Ca^{2+} oscillations frequency. Nonetheless, agonist-stimulated Ca^{2+}-dependent functions such as exocytosis was identical in cells from wild type and SERCA2$^{+/-}$ mice due to adaptation in the levels of the Ca^{2+} sensors for exocytosis. The adaptations can explain the normal function of most physiological systems in DD patients.

In a second approach we analyzed twelve DD-associated mutations from all regions of SERCA2b to study the underlying pathologic mechanism of DD and to elucidate the role of dimerization in SERCA2b activity. Most mutations markedly affected protein expression, partially due to enhanced proteasome-mediated degradation. All mutants showed lower activity than the WT pump. Notably, several mutants that cause relatively severe disease phenotype inhibited the activity of the endogenous and the co-expressed WT SERCA2b. Co-immunoprecipitation experiments showed that SERCA2b monomers interact to influence the activity of each other. These findings reveal multiple molecular mechanisms to account for the plethora of pathologic states observed in DD and provide evidence for the importance of SERCA2b dimerization in pump function *in vivo*.

Zusammenfassung

Darier-Krankheit (DK) ist eine autosomal dominante Mutation im ATP2A2 Gen, das für die SERCA2-Ca^{2+}-Pumpe kodiert. Obwohl mehr als 100 Mutationen im ATP2A2 identifiziert wurden, war keine Beziehung zwischen Genotyp/Phänotyp ersichtlich. Um die Grundlagen der Krankheit und die Beziehung zwischen Phänotyp/Genotyp zu verstehen, haben wir Ca^{2+}-Signale in SERCA2$^{-/-}$-Mäusen und den Effekt von DK-assoziierten Mutationen auf die Aktivität des Wild-Typs (WT) der SERCA analysiert.

Deletion einer Kopie des SERCA2-Gens resultierte in Anpassung der Ca^{2+}-Signale zu reduzierten Ca^{2+}-Oszillations-Frequenzen. Trotzdem waren Agonisten-stimulierte, Ca^{2+}-abhängige Funktionen wie „Exozytose" in Zellen von Wild-Typ- und SERCA$^{-/-}$-Mäusen infolge der Adaption der Ca^{2+}-Sensor-Schwelle für die Exozytose identisch. Die Adaption kann die normale Funktion der meisten physiologischen Systeme in DK-Patienten erklären.

In einem zweiten Schritt haben wir 12 DK-assoziierte Mutationen von allen SERCA2b-Bereichen analysiert, um die zugrunde liegenden pathologischen Mechanismen der DK zu untersuchen und die Rolle der Dimer-Bildung für die SERCA2b Aktivität herauszufinden. Die meisten Mutationen hatten Effekte auf die Protein Expression, teilweise bedingt durch erhöhten Proteosom-abhängigen Abbau. Alle Mutanten zeigten niedrigere Aktivität der Ca^{2+}-Pumpe als der Wild-Typ. Bemerkenswerterweise hemmten mehrere Mutanten, die einen relativ schweren Krankheitsphänotyp verursachten, die Aktivität der endogenen und der ko-exprimierten WT SERCA2b. Ko-Immunopräzipitations-Experimente zeigten, daß SERCA2b-Monomere interagieren und die gegenseitige Aktivität beeinflussen.

Diese Untersuchungen zeigen mehrfache molekulare Mechanismen auf, die der Vielfältigkeit der pathologischen Zustände, die bei der DK beobachtet wurden, zugerechnet werden können und Hinweise geben auf die Wichtigkeit der SERCA2b-Dimerisierung für die Ca^{2+}-Pump-Funktion *in vivo*.

1 Department of Oral Biology, Yonsei University, Seoul, Korea.
2 Department of Pharmacology, Yonsei University, Seoul, Korea.
3 Department of Physiology, University of Texas Southwestern Medical Center, Dallas, TX 75390.

Darier's disease is an autosomal dominant disorder characterized by skin lesions due to loss of adhesion of epidermal cells and aberrant keratinization (Burge and Wilkinson 1992). DD is also associated with an increased prevalence of neuropsychiatric disorders, including bipolar disorders, schizophrenia, epilepsy, and mental retardation (Burge and Wilkinson 1992). The disease was recently mapped to mutations in the *ATP2A2* gene encoding the sarco/endoplasmic reticulum Ca^{2+}-ATPase pump type 2 isoform, which codes for the cardiac SERCA2a and the house keeping SERCA2b isoforms (Sakuntabhai et al. 1999). The ubiquitous SERCA2b has many critical functions in Ca^{2+} signaling. For example, SERCA2b buffers $[Ca^{2+}]_i$ in resting cells, SERCA2b controls I_{crac} activity in stimulated cells by preventing accumulation of Ca^{2+} at the site of Ca^{2+} influx, it mediates Ca^{2+} uptake into the ER, including the agonist-mobilizable Ca^{2+} pool, at the end of cell stimulation and during Ca^{2+} oscillations and it regulates the speed of Ca^{2+} waves in polarized cells (Kiselyov et al. 2003, Liu et al. 1998, Zhao et al. 2001, Carafoli 2002).

Considering the diverse roles of SERCA2b in Ca^{2+} signaling and the involvement of $[Ca^{2+}]_i$ in virtually all cellular functions (Carafoli 2002), the question arise is how cells adapt to partial deletion of such a vital molecule. Previous work showed that alteration in SERCA2 pump function by over-expression leads to adaptation of the activity of other Ca^{2+} transporting proteins, such as the plasma membrane Ca^{2+} ATPase (PMCA) pump (Brini et al. 2000). Whether adaptation of the Ca^{2+} signaling machinery takes place *in vivo* and the extent of adaptation is not known. This question of adaptation of Ca^{2+} signaling is particularly important in the context of DD. There is a lack of consistent defect in DD patients platelet and heart function, in which SERCA2a is the major pump in the ER/SR (Tavadia et al. 2001). By contrast, all DD-associated mutations cause moderate to severe skin disorders and several mutations cause neuropsychiatric disorders, suggesting particular susceptibility of these tissues to reduction in SERCA2 activity. These observations raise the questions of how mutations associated with DD affect SERCA2 pump activity; is there a phenotype-genotype relationship in SERCA2 mutations and disease state. To address these questions we characterized Ca^{2+} signaling in cells from the $SERCA2^{+/-}$ mice and characterized the effect of DD mutations on SERCA pump activity. Here, the highlights of the findings from three recent publications will be summarized. A more detailed account can be found in the references (Zhao et al. 2001, Ahn et al. 2003, Dode et al. 2003)

Measurements of Ca^{2+} signaling in pancreatic acini prepared from the mouse model of DD, the $SERCA2^{+/-}$ mouse, revealed marked adaptation of Ca^{2+} signaling and Ca^{2+}-dependent cell function to a deletion of one copy of the SERCA2 gene. Measurement of the global Ca^{2+} signals showed that the maximal $[Ca^{2+}]_i$ increase in Ca^{2+}-containing and Ca^{2+}-free media was the same in WT and $SERCA2^{+/-}$ cells. However, $SERCA2^{+/-}$ cells reduced $[Ca^{2+}]_i$ towards basal level much faster that WT cells. Analysis of SERCA pump activity confirmed the reduction in pumping rate. However, Ca^{2+} content of stores was not markedly affected, probably due to reduced Ca^{2+} leak from the ER. Equally, Ca^{2+} influx was not altered by partial deletion of SERCA2. Finally, IP_3-mediated Ca^{2+} release was the same in WT and $SERCA2^{+/-}$ cells. The only major adaptation was in the expression and activity of PMCA (Fig. 1). Figure 1*A–C* shows that PMCA activity and Figure 1*E–F* show that PMCA protein is approximately two-fold higher in $SERCA2^{+/-}$ cells. In fact Figure 1*G* shows that specifically PMCA isoform 4b (Fig. 1*G*), that is expressed at the apical pole of these polarized cells, was up-regulated (Fig. 1*H*, *I*) in the $SERCA2^{+/-}$ cells.

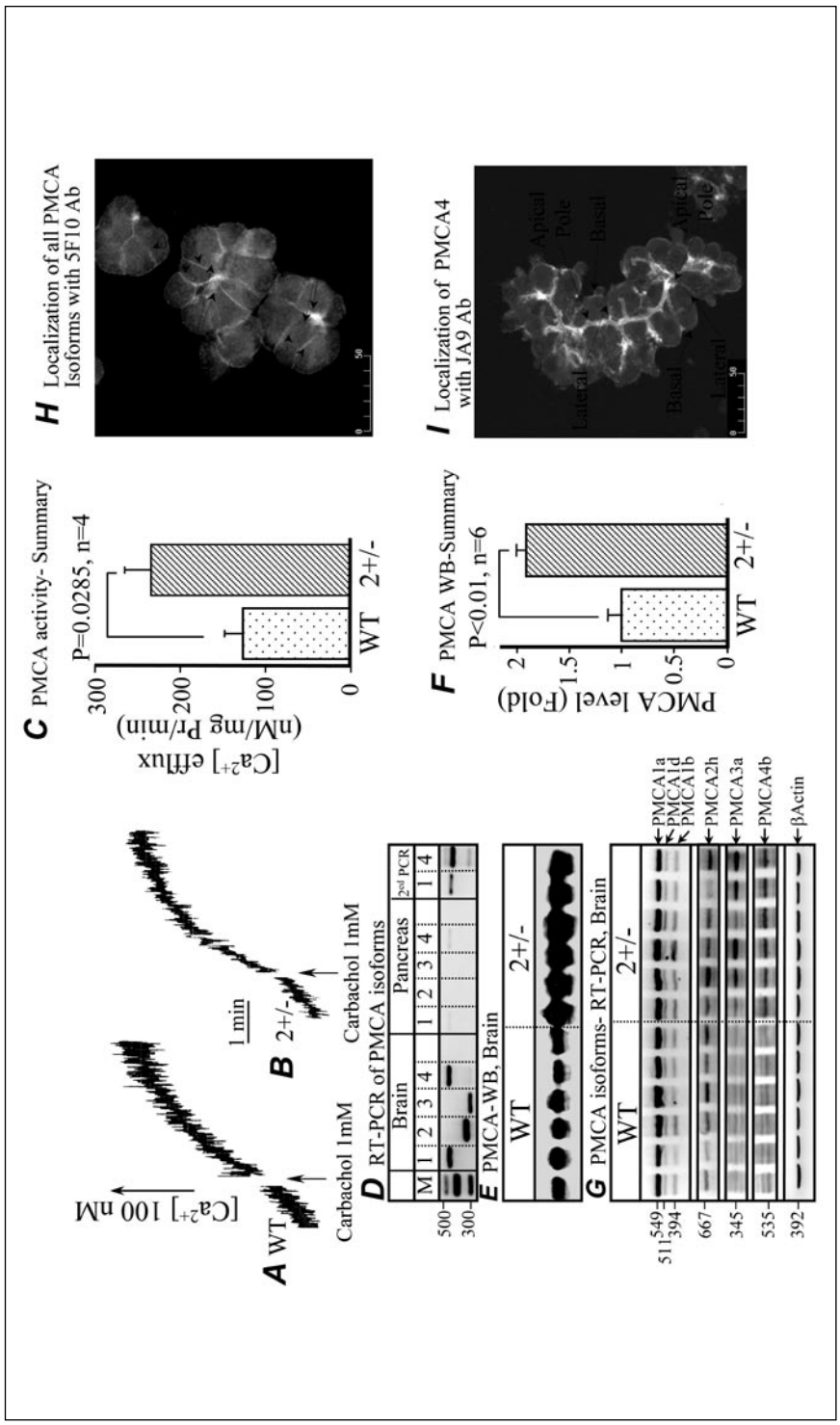

Fig. 1 Up-regulation of PMCA mRNA, protein and activity in SERCA2$^{+/-}$ cells. Panels (*A–C*) show PMCA activity in WT and SERCA2$^{+/-}$ cells. Panel (*D*) shows RT-PCR analysis of PMCA isoforms in brain and pancreatic acini and panel (*E*) shows up-regulation of PMCA protein in SERCA2$^{+/-}$ cells. RT-PCR analysis in panel (*G*) shows the mRNA levels for the different PMCA isoforms in WT and SERCA2$^{+/-}$ cells. Panels (*H*) and (*I*) show confocal images of immunolocalization of PMCA in pancreatic acini. The Figure was reproduced from Zhao et al. 2001.

The change in SERCA2b and PMCA4b activity resulted in modified cellular Ca^{2+} handling in SERCA2$^{+/-}$ cells. Figure 2*A–E* shows that the change in Ca^{2+} handling caused about 50 % reduction in $[Ca^{2+}]_i$ oscillation frequency stimulated by GPCRs expressed in these cells. Ca^{2+} oscillation frequency codes many cellular functions and therefore reduction in Ca^{2+} oscillations frequency was expected to alter many cellular activities in SERCA2$^{+/-}$ cells. Yet, the SERCA2$^{+/-}$ mouse has very mild phenotype (Periasamy et al. 1999). In an attempt to understand the mild phenotype of the SERCA2$^{+/-}$ mouse we followed the properties of a classical Ca^{2+}-regulated activity in pancreatic acini, that of agonist-stimulated exocytosis. Remarkably, in spite of the reduction in Ca^{2+} oscillations frequency, the agonist stimulated exocytosis was identical in WT and SERCA2$^{+/-}$ cells (Fig. 2*F*). This paradox was resolved when the Ca^{2+} dependence of exocytosis was measured. Figure 2*F* shows an adaptation in this process such that exocytosis was stimulated at lower Ca^{2+} concentrations in SERCA2$^{+/-}$ cells. Further studies showed that the adaptation of the exocytotic machinery was achieved by adaptation in the levels of the Ca^{2+} sensors for exocytosis synaptotagmins I and III in cells from SERCA2$^{+/-}$ mice. These findings reveal a remarkable plasticity and adaptability of Ca^{2+} signaling and Ca^{2+}-dependent cellular functions *in vivo* and could explain the normal function of most physiological systems in DD patients.

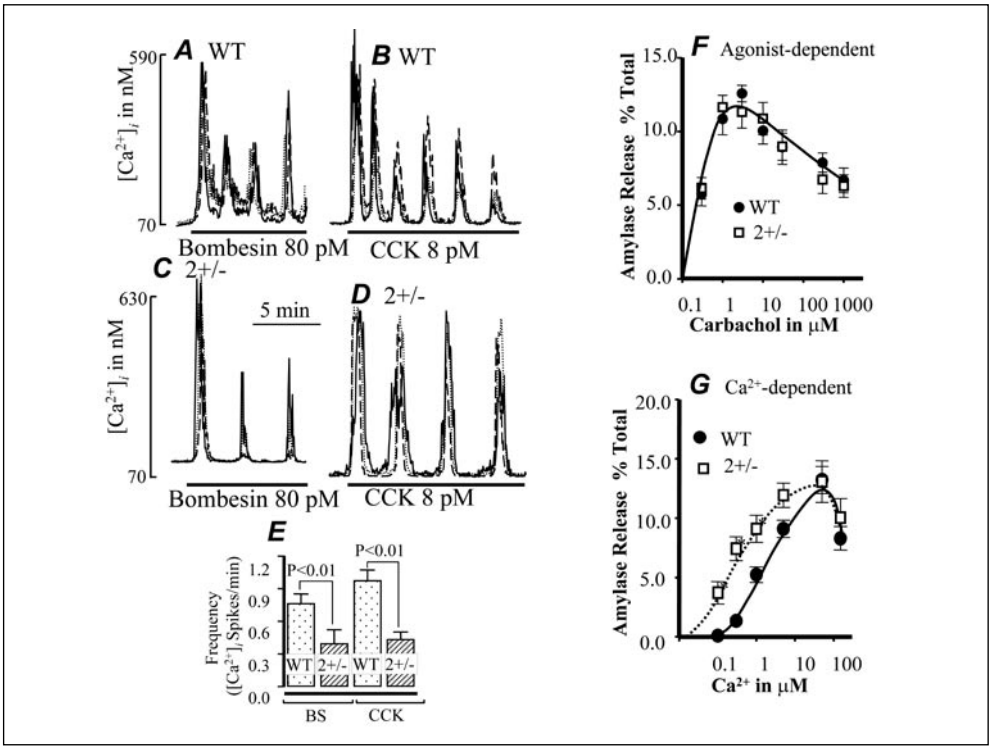

Fig. 2 $[Ca^{2+}]_i$ oscillations and exocytosis in pancreatic acinar cells from WT and SERCA2$^{+/-}$ mice. Pancreatic acini from WT (*A*, *B*) or SERCA2+/– mice (*C*, *D*) were stimulated with the indicated agonists. The frequency of the oscillations is summarizes in panel (*E*). Panel (*F*) shows agonist-stimulated exocytosis intact pancreatic acini and panel (*G*) shows Ca^{2+}-stimulated exocytosis in permeabilized pancreatic acini from WT in SERCA2+/– mice. The Figure was reproduced from Zhao et al. 2001.

To better understand the variable phenotype of DD it was necessary to characterize the effect of DD mutations on pump activity and on the activity of WT pump resulting from the normal allele. Three hypotheses were examined. Since haploinsufficiency cannot explain the variable phenotype of DD patients, one hypothesis is that the severity of the disease correlates with protein expression. This hypothesis was refuted in two studies that examined expression and stability of the protein and found no correlation between protein expression and the reported phenotype (Ahn et al. 2003, Dode et al. 2003).

The second hypothesis was correlation between pump activity of the mutant and disease phenotype. This hypothesis was examined by measuring the activity of heterologously expressed mutants. The results obtained with 12 mutants are shown in Figure 3. Several conclusions emerged from this work. First, all SERCA2b mutants reduced Ca^{2+} uptake activity. Subsequent work showed that the reduced activity could result from inhibition of various steps in the turnover cycle of the pump (Dode et al. 2003). Importantly, several mutants inhibited Ca^{2+} pumping of the native SERCA2b (Fig. 3) or WT SERCA2b co-expressed with the mutant protein (Ahn et al. 2003). Inhibition of WT-SERCA2b by the mutants required that SERCA2b functions as a dimmer and interaction between monomers. Indeed, such interaction could be demonstrated for most mutants by co-immunoprecipitation of WT and mutant pumps (Ahn et al. 2003).

The significance of interaction of the WT and mutant SERCA2b monomers *in vivo* is that it provides a mechanism to explain reduced Ca^{2+} pumping below the 50 % expected from the recessive nature of the disease. This provides a likely mechanism to account for the variable clinical features of DD. That is, the essential role of SERCA2b in Ca^{2+} signaling makes it vital

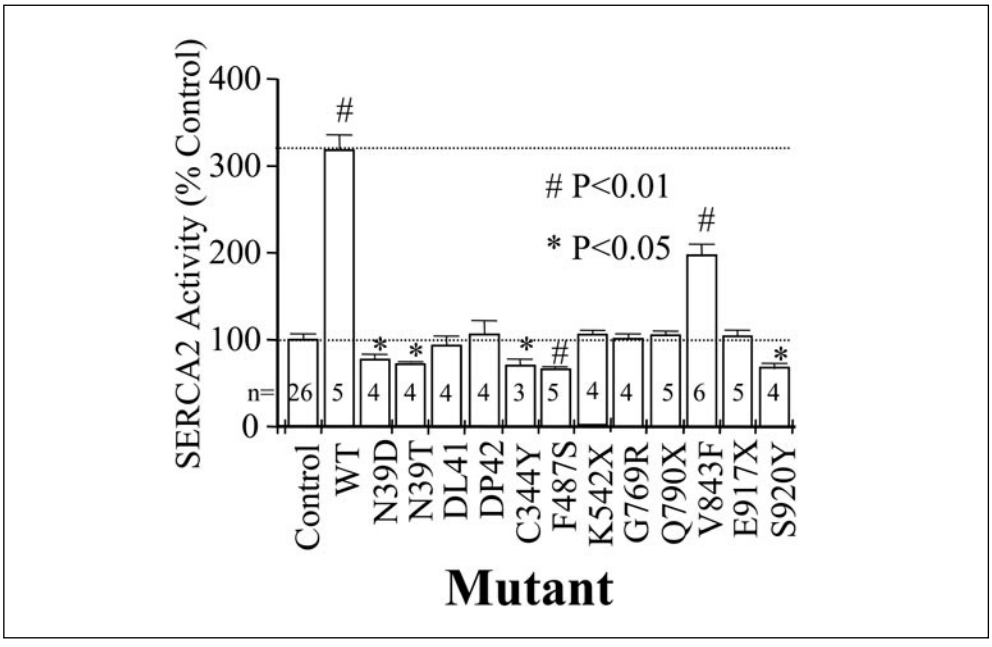

Fig. 3 Ca^{2+} transport by WT and mutant SERCA2b pumps. Cell transfected with the indicated mutant and permeabilized with SLO were used to evaluate pump activity of all mutants. The Figure was reproduced from Ahn et al. 2003.

for life and even 50 % reduction in pump activity is not tolerated, leading to detachment of skin keratinocytes. Other cellular activities, such as Ca^{2+}-dependent exocytosis (Fig. 2) and myocytes contractility (Periasamy et al. 1999), undergo adaptation to the modified Ca^{2+} response. However, interaction of WT and the DD causing mutants that leads to further reduction of SERCA2b pump activity is likely to affect additional cells and organs, such as the nervous system. In summary, although the poor clinical definition of DD phenotype makes it difficult to draw a precise correlation between the mutants that inhibited WT-SERCA2 activity and the disease phenotype, the tendency found is that mutants that cause a relatively severe phenotype inhibit noticeably more the endogenous and expressed WT-SERCA2b activity. The finding of reduced WT pump activity by the mutants provides the first molecular mechanism that can account for the variable DD phenotypes.

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Prof. Dr. Shmuel Muallem
University of Texas
Department of Physiology
Southwestern Medical Center
5323 Harry Hines Vivd
USA-Dallas, TX 75390-9040
Phone: +1 214 6482593
Fax: +1 214 6488879
E-Mail: Shmuel.muallem@utsouthwestern.edu

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Extracellular Calcium Signaling

Aldebaran M. Hofer[1] and Annunziata De Luisi[2]

Abstract

Activation of plasma membrane pathways for Ca^{2+} influx and efflux can produce measurable alterations in $[Ca^{2+}]$ in the local microenvironment at the external face of cells during intracellular Ca^{2+}-signaling events. The extracellular Ca^{2+}-sensing receptor (CaR) is a widely expressed G-protein-coupled receptor capable of translating information about $[Ca^{2+}]$ in the extracellular milieu to the interior of the cell. The activation of this receptor is coupled to intracellular calcium signaling cascades in many cell types. Previously we provided evidence that CaR-expressing cells are able to detect Ca^{2+}-signaling activity in adjacent cells as a consequence of local extracellular $[Ca^{2+}]$ changes that occur outside the stimulated cell. In the present study we explored the possibility that Ca^{2+} extrusion *via* the plasma membrane Ca^{2+}ATPase (PMCA) can amplify Ca^{2+} signals through a positive feedback loop involving CaRs on the same cell. Using fura-2 imaging of cytoplasmic $[Ca^{2+}]$, we observed that stimulation of CaR in HEK293 cells stably expressing the receptor was frequently manifested as an oscillatory Ca^{2+} response. Ca^{2+} oscillations were also triggered in Ca^{2+}-free solutions by the CaR agonist spermine (1 mM). Exogenous Ca^{2+} buffers based on BAPTA free acid or citrate attenuated or eliminated Ca^{2+} oscillations, even though the free $[Ca^{2+}]_{out}$ was maintained at a constant level. A variety of PMCA inhibitors ($HgCl_2$, orthovanadate, and Caloxin 2A1) also attenuated or eliminated Ca^{2+} oscillations. Measurement of extracellular $[Ca^{2+}]$ using the near membrane probe fura-C_{18} revealed that external $[Ca^{2+}]$ rose following receptor activation, sometimes displaying an oscillatory pattern. Our data suggest that PMCA-mediated cycling of Ca^{2+} across the plasma membrane leads to localized increases in $[Ca^{2+}]_{out}$ that increase the excitability of CaR, which is then translated into an oscillatory response inside the cell.

Zusammenfassung

Die Aktivierung von Plasmamembran-Bahnen für den Ca^{2+}-Ein- und -Ausstrom kann meßbare Veränderungen in der $[Ca^{2+}]$ in der lokalen Mikroumgebung an der äußeren Oberfläche von Zellen während intrazellulärer Ca^{2+}-Signalisierungsereignisse hervorrufen. Der extrazelluläre Ca^{2+}-sensitive Rezeptor (CaR) ist ein weit exprimierter G-Protein-gekoppelter Rezeptor, der in der Lage ist, Informationen über die $[Ca^{2+}]$ in der extrazellularen Umgebung in das Innere der Zelle zu übertragen. Die Aktivierung dieses Rezeptors ist in vielen Zelltypen mit intrazellulären Calcium-Kaskaden gekoppelt. Zuvor wurden Hinweise gefunden, daß CaR-exprimierende Zellen fähig sind, Ca^{2+}-Signalaktivität in angrenzenden Zellen als Folge lokaler extrazellulärer $[Ca^{2+}]$-Veränderungen, die außerhalb der stimulierten Zelle auftreten, zu detektieren. In der vorliegenden Studie untersuchten wir die Möglichkeit, daß die Ca^{2+}-Extrusion durch die Plasmamembran-Ca^{2+}-ATPase (PMCA) Ca^{2+}-Signale über eine positive Feedback-Schleife unter Einschluß der CaRs auf der selben Zelle vergrößern kann. Unter Verwendung des Fura-2-Imagings der zytoplasmatischen $[Ca^{2+}]$ wurde beobachtet, daß die Stimulierung der CaR in HEK293-Zellen, die den Rezeptor stabil exprimieren, sich oft

1 West Roxbury VAMC and the Department of Surgery, Harvard Medical School, Boston MA, USA.

2 Dipartimento di Fisiologia Generale ed Ambientale, Universitá di Bari, Via Amendola 165/A, I-70126 Bari, Italy.

als eine oszillatorische Ca^{2+}-Antwort manifestiert. Ca^{2+}-Oszillationen wurden in Ca^{2+}-freien Lösungen auch durch den CaR-Agonisten Spermin (1 mM) ausgelöst. Exogene Ca^{2+}-Ionen buffern, basierend auf BAPTA-freier Säure oder Zitrat, abgeschwächte oder eliminierte Ca^{2+}-Oszillationen ab, gerade auch dann, wenn die freie $[Ca^{2+}]_{out}$ auf einem konstanten Level gehalten wurde. Eine Reihe von PMCA-Inhibitoren ($HgCl_2$, Orthovanadat und Caloxin 2A1) schwächen oder eliminieren Ca^{2+}-Oszillationen. Die Messung der extrazellulären $[Ca^{2+}]$ unter Verwendung von Fura-C_{18} ergab, daß die externale $[Ca^{2+}]$ in Folge der Rezeptor-Aktivierung anwuchs und manchmal ein oszillatorisches Muster zeigte. Unsere Versuchsergebnisse deuten darauf hin, daß der PMCA-vermittelte Transport von Ca^{2+} über die Plasmamembran zu einem lokalen Anwachsen der $[Ca^{2+}]_{out}$ führt, die die Erregbarkeit der CaR erhöht, die wiederum in eine oszillatorische Reaktion innerhalb der Zelle umgesetzt wird.

1. Introduction

In multicellular organisms, cells are crowded together in organized communities, surrounded by an interstitial fluid of extremely limited volume. Local communication between adjacent cells is known to occur through gap junctions in cells that are physically connected, or through the release of paracrine signaling molecules (e.g. ATP, glutamate, nitric oxide) that diffuse to their target receptors through the extracellular microenvironment. We recently reported a novel form of intercellular communication mediated by external Ca^{2+} and the extracellular calcium-sensing receptor (CaR) (Hofer et al. 2000, Thomas 2000).

2. The Extracellular Ca^{2+}-sensing Receptor

The CaR is a widely-expressed seven-transmembrane-spanning cell surface receptor for divalent cations that was originally identified in the parathyroid gland (Brown et al. 1993, see Brown and MacLeod 2001 for review). This receptor couples, *via* heterotrimeric G-proteins, to several different signaling cascades, including the phosholipase C (PLC)/phosphoinositide pathway. This typically results in the production of inositol 1,4,5 trisphosphate ($InsP_3$), and the release of $InsP_3$-sensitive internal Ca^{2+} stores. CaR activation can therefore be conveniently assayed in many cell types by measuring the cytoplasmic Ca^{2+} response using fluorescent Ca^{2+} indicators. It is important to note that the CaR is also activated by Mg^{2+} and certain polycations (including some endogenous polyamines such as spermine and spermidine). Brown and colleagues recently showed that the CaR is also activated by physiological concentrations of amino acids, and that its response can be influenced by shifts in extracellular pH (Conigrave et al. 2000). It is therefore possible that multiple agonists may act in concert to stimulate the receptor physiologically.

In our previous studies (Hofer et al. 2000) we used a co-culture model system in order to show that Ca^{2+} extrusion from stimulated cells can activate CaR on neighboring cells. We also considered the possibility that Ca^{2+} extrusion might influence CaR on the same cell, thus forming the basis for a special type of autocrine signaling.

3. Evidence that PMCA Activity Reinforces Intracellular Ca^{2+} Oscillations

We, along with others (Breitwieser and Gama 2001), have noted that cell types expressing CaR frequently display intracellular Ca^{2+} oscillations. "Classical" mechanistic explanations

for Ca^{2+} oscillations include complex positive-negative feedback loops by Ca^{2+} on the $InsP_3$ receptor, and other types of feedback inhibition involving PKC and PLC (Thomas et al. 1996). Our data suggest an additional dynamic in cells expressing CaR that derives from a feedback loop involving Ca^{2+} extrusion by the PMCA and subsequent sensing of extruded Ca^{2+} ions by CaR (De Luisi and Hofer 2003). In contrast to oscillations induced by carbachol in wild-type HEK cells, oscillations in CaR-expressing HEK cells were sensitive to PMCA inhibitors ($HgCl_2$, orthovanadate, and Caloxin 2A1). Moreover, exogenous Ca^{2+} buffers based on BAPTA-free acid or citrate-attenuated or eliminated Ca^{2+} oscillations, even though the free $[Ca^{2+}]_{out}$ was maintained at a constant level (see De Luisi and Hofer 2003, for details). Our immunolocalization data for CaR and PMCA showed these plasma membrane proteins to be highly co-localized. The physical geometry of HEK 293 cells might also contribute to interactions between PMCAs and CaR. Using confocal optical sectioning through clusters of HEK cells bathed with an impermeant high-molecular weight fluorescent marker (fluorescein dextran, 10,000 M. W.), we observed that the fluorescent marker was able to penetrate into minute clefts in between cells, revealing an elaborate, diffusionally limited extracellular space in the immediate vicinity between the cells.

We hypothesized that Ca^{2+} spiking in HEK CaR cells may result in part from a unique positive feedback loop in which the Ca^{2+} signal, once initiated, results in Ca^{2+} extrusion *via* the PMCA, activating CaRs on the same cell through local increases in $[Ca^{2+}]_{out}$. This would result in the initiation of another spike in intracellular Ca^{2+}, Ca^{2+} export, activation of the CaR, etc., thereby reinforcing oscillatory behavior. If this model were correct, we would predict that local *extracellular* $[Ca^{2+}]$ should oscillate. *Alternatively*, repetitive spiking may simply result from local accumulation of external Ca^{2+} in microdomains outside of the cell due to PMCA activity. This might induce a generalized increase in CaR activation and consequently higher basal $[InsP_3]$. The elevated levels of $InsP_3$ might induce oscillations *via* the $InsP_3$ receptor on internal stores through well-described positive/negative feedback effects. The key to distinguishing between these two possibilities was to measure *extracellular* $[Ca^{2+}]$ directly, to determine whether it also oscillates.

Our experiments using the near-membrane fluorescent Ca^{2+} indicator $furaC_{18}$ indicated that extracellular $[Ca^{2+}]$ at the external membrane was substantially, but transiently elevated following CaR activation. In ~50 % of the cells, small oscillations in extracellular $[Ca^{2+}]$ lasting several minutes were also observed. However, the transient nature of this response suggests that the extracellular $[Ca^{2+}]$ increase cannot account entirely for maintenance of intracellular oscillations, which are sustained for at least ten minutes in the absence of extracellular Ca^{2+}, and for up to an hour in the presence of Ca^{2+} in the bathing solution. We conclude that PMCA activity serves to increase the 'excitability' of CaR by causing local extracellular increases in $[Ca^{2+}]$, and that this is manifested as an oscillatory intracellular Ca^{2+} response.

4. Significance

CaR desensitizes slowly, yet the receptor is chronically exposed physiologically to a wide variety of CaR activators. Ca^{2+} spiking is a mode of signaling that allows continuous encoding of information without subjecting the cell to the potentially deleterious effects of persistent elevations in cytoplasmic $[Ca^{2+}]$. Our data suggest that appropriately timed cycling of Ca^{2+} across the plasma membrane by the PMCA helps to initiate the oscillatory spike by ampli-

fying the actions of other CaR agonists (including extracellular Ca^{2+}). It is possible that in CaR expressing cells, several mechanisms may coexist that converge to reinforce oscillatory behavior. These seemingly redundant mechanisms may be crucial to ensure that extracellular signals are translated into Ca^{2+} oscillations, in spite of prolonged exposure to maximal concentrations of CaR activators.

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Prof. Dr. Aldebaran M. Hofer
Harvard Medical School
West Roxbury VAMC and Brigham and Women's Hospital
1400 VFW Parkway; Room 2B111
West Roxbury MA 02132
USA
Phone: +1 617 3237700 ext 5902
Fax: +1 617 3635592
E-Mail: ahofer@rics.bwh.harvard.edu

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Physiological Role of Agonist-induced Extracellular [Ca^{2+}] Fluctuations in Intact Polarized Epithelia

Rosa Caroppo[1], Gregorio Fistetto[1], Andrea Gerbino[1,2], Matilde Colella[1], Lucantonio Debellis[1], Aldebaran M. Hofer[2], and Silvana Curci[1,2]

Abstract

Ca^{2+} may act extracellularly, in a first messenger capacity, to communicate signals that can be "sensed" by cells harboring the extracellular Ca^{2+}-sensing receptor (CaR) (Hofer et al. 2000). Using Ca^{2+}-selective microelectrodes in an intact polarized epithelium, the amphibian gastric mucosa, we recently demonstrated that stimulation with the intracellular Ca^{2+}-mobilizing agonist, carbachol, resulted in substantial local *increases* (as large as 0.5 mM) in extracellular [Ca^{2+}] at the apical face of the acid-secreting cells. A comparable *depletion* of external [Ca^{2+}] occurred at the basolateral aspect of the cell (Caroppo et al. 2001). These local fluctuations in $[Ca^{2+}]_{ext}$ would be sufficient to modulate CaR, which partially co-localized with the plasma membrane Ca^{2+} ATPase and the gastric H^+/K^+ ATPase in the apical membrane of the acid-secreting cells. In this study we investigated whether these changes in extracellular [Ca^{2+}] might represent a "signal" that can be transduced into functional responses. Electrophysiological studies performed with pH-sensitive double-barreled microelectrodes in the gland lumen of the amphibian stomach indicated that mimicking the $[Ca^{2+}]_{extr}$ changes elicited by carbachol (i. e. simultaneously decreasing basolateral [Ca^{2+}] while elevating luminal [Ca^{2+}]) resulted in an alkalinization of the gastric gland lumen that was very similar to the alkalinization induced by carbachol itself. Simultaneous, asymmetrical changes of $[Ca^{2+}]_{extr}$ also transiently increased pepsinogen secretion, another response of the stomach known to be stimulated by carbachol. Additional studies are in progress to assess the role of CaR in these responses. The alkalinization induced by carbachol may play a cytoprotective role in preventing the activation of pepsinogen to pepsin in gastric gland lumen.

Zusammenfassung

Ca^{2+} kann extrazellulär, mit *First-Messenger*-Kapazität, wirken, um Signale zu übertragen, die von solchen Zellen wahrgenommen werden, die den extrazellulären Ca^{2+}-empfindlichen Rezeptor (CaR) tragen (Hofer et al. 2000). Unter Verwendung von Ca^{2+}-selektiven Mikroelektroden in einem intakten polarisierten Epithel, der amphibischen Magenschleimhaut, konnten wir unlängst zeigen, daß die Stimulation mit dem intrazellulären Ca^{2+}-mobilisierenden Agonisten Carbocha zu deutlichen lokalen Anstiegen (von 0,5 mM) in der extrazellulären Calciumkonzentration an der apikalen Oberfläche der Säure-sekretierenden Zellen führt. Eine vergleichbare Verringerung der [Ca^{2+}] kommt im basolateralen Bereich der Zellen vor (Caroppo et al. 2001). Diese lokalen Fluktuationen in der externen [Ca^{2+}] würden ausreichen, um die CaR zu modulieren, die teilweise mit der Plasmamembran-Ca^{2+}-ATPase und der gastrischen H^+/K^+-ATPase in der Apikalmembran der Säure-sekretierenden Zellen kolokalisiert sind. In dieser Studie untersuchten wir, ob diese Veränderungen in der extrazellulären [Ca^{2+}] ein „Signal" darstellen könnten, das in funktionelle Reaktionen umgewandelt werden kann. Elektrophysiologische Untersuchungen, die mit pH-sensitiven Rundmikroelektroden im Drüsenlumen des Amphibienmagens durchgeführt wurden, zeigten, daß die Nachahmung der $[Ca^{2+}]_{extr}$-Veränderungen,

1 Dipartimento di Fisiologia Generale ed Ambientale, Universita di Bari, Bari, Italy.

2 Department of Surgery, Harvard Medical School, Brigham and Women's Hospital and VA Boston Healthcare System, Boston and West Roxbury, MA, USA.

die durch Carbachol hervorgerufen werden (z. B. die gleichzeitige Verringerung der basolateralen [Ca^{2+}] während sich die luminale [Ca^{2+}] erhöht) zu einer Alkalisierung des gastrischen Drüsenlumens führt, die der Alkalisierung, die durch Carbachol ausgelöst wird, ähnlich ist. Simultane asymmetrische Veränderungen der $[Ca^{2+}]_{extr}$ erhöhen auch vorübergehend die Pepsinogen-Sekretion, eine weitere Reaktion des Magens, von der bekannt ist, daß sie durch Carbachol stimuliert wird. Weitere Untersuchungen sind im Gange, um die Rolle der Ca^{2+}-empfindlichen Rezeptoren in diesen Antworten zu bestimmen. Die Alkalisierung, die durch Carbachol bewirkt wird, könnte eine zellschützende Rolle durch die Verhinderung der Aktivierung von Pepsinogen zu Pepsin spielen.

1. Extracellular Ca^{2+} as a "Signal"

We recently showed that cells are able to communicate with each other through extracellular [Ca^{2+}] increases that result from the extrusion of cellular Ca^{2+} across the plasma membrane during Ca^{2+}-signaling events (Hofer et al. 2000, Thomas 2000). These studies employed a co-culture model system to show that activation of one cell type to mobilize its intracellular Ca^{2+} stores resulted in local increases of *extracellular* [Ca^{2+}], likely as a result of active extrusion of Ca^{2+} from the stimulated cell by the plasma membrane Ca^{2+} ATPase (PMCA). We further showed that these increases in external [Ca^{2+}] were of sufficient magnitude to be detected by neighboring cells expressing the extracellular Ca^{2+}-sensing receptor (CaR), a widely expressed sensor for extracellular Ca^{2+} first identified by Brown, Hebert, and colleagues in the parathyroid gland (Brown et al. 1993). An important implication of this finding is that every time an *intracellular* Ca^{2+} signal occurs, it may potentially be translated into an *extracellular* Ca^{2+} signal detected by CaR located either on neighboring cells or on the same cell. Since the CaR is found in a wide variety of cell types, we proposed that this interaction might constitute a universal means by which cells talk to each other in the living organism (Hofer et al. 2000).

In the present study we have asked whether this sort of "paracrine" communication takes place in "real" living tissues. However, in intact tissues, there are additional levels of complexity that may be envisaged, particularly in epithelial tissues, where there may be a polarized distribution of CaR, Ca^{2+} extrusion mechanisms, and Ca^{2+} entry pathway (Petersen et al. 1999, Belan et al. 1996, Camello et al. 1996, Carafoli and Penniston 1985, Caroppo et al. 2001, Lee et al. 1997). Collectively, these factors will determine whether extracellular [Ca^{2+}] fluctuations can serve as signals acting through CaR to potentially alter the function of the tissue.

2. Determinants of Extracellular Ca^{2+} "Signals" Generated by Intracellular Signaling Events

2.1 The PMCA and Other Modes of Ca^{2+} Export

Plasma membrane Ca^{2+} ATPases (PMCAs) are present in all mammalian cells (Guerini et al. 1998). It is well known that Ca^{2+} released from internal stores following Ca^{2+}-signaling events is largely extruded from the cell through the actions of this ubiquitous pump in many cell systems (Hofer et al. 1998, Muallem et al. 1988a,b, Zhang et al. 1992). Four genes encoding the PMCAs have been identified, and alternative splicing gives rise to numerous isoforms. It is important to note that these isoforms are expressed in a cell- and tissue-specific manner, and moreover, they possess dramatically different kinetic properties, some being "fast" pumps, and others "slow" (Penniston et al. 1997). Our own data and those of others have shown that in

polarized epithelial cell types, the PMCA is confined to discrete apical or basolateral domains (see PETERSEN et al. 1999 for review). Thus the ability of the PMCA to produce *extracellular* Ca^{2+} "signals" will depend on the kinetic properties of the specific isoforms expressed, and their localization within a tissue (i.e. apical *versus* basolateral). In addition to the PMCA, Na^{2+}/Ca^{2+} exchangers (found mainly in excitable cells) can rapidly export Ca^{2+} following intracellular Ca^{2+} elevations (MUALLEM et al. 1988a,b). Secretory granules contain considerable amounts of total Ca^{2+}, which have been shown by direct measurement to be lost to the cell exterior following exocytosis (BELAN et al. 1998).

2.2 Ca^{2+} Entry via SOCs

Depletion of Ca^{2+} from intracellular stores (as occurs during intracellular Ca^{2+} signals) results in the opening of "store operated channels" or SOCs in the plasma membrane. During signaling events, activation of SOCs and extrusion mechanisms may be temporally and spatially distinct, thus when considering the net impact of these pathways on extracellular [Ca^{2+}], entry and extrusion do not necessarily cancel one another out. Again our own data and those of others have shown a discrete localization of Ca^{2+} entry pathways in polarized epithelial cells (see PETERSEN et al. 1999 for review). Our data have further shown that localized Ca^{2+} depletions occur outside of cells following activation of these channels (CAROPPO et al. 2001).

2.3 Other Factors

Both the gradient for extrusion and the cytosolic Ca^{2+}-buffering capacity will influence rates of Ca^{2+} export from the cell. In polarized epithelia, the transepithelial potential can cause passive redistribution of Ca^{2+} across the tissue. Fluid secretion may dilute or concentrate Ca^{2+} in the interstitial spaces, and secreted Ca^{2+} buffers (HCO_3^-, oxalate, PO^{4-} etc.) may potentially influence the free extracellular Ca^{2+} concentration as well.

3. Direct Measurement of [Ca^{2+}] in the Local Extracellular Microenvironment of an Intact Polarized Epithelium

Using Ca^{2+}-selective microelectrodes in the intact amphibian gastric mucosa we found that stimulation with an intracellular Ca^{2+}-mobilizing agonist, carbachol, resulted in substantial local increases (as large as 0.5 mM) in extracellular [Ca^{2+}] at the apical face of oxyntic cells (CAROPPO et al. 2001). A comparable depletion of external [Ca^{2+}] occurred at the basolateral aspect of the cell. These shifts in extracellular [Ca^{2+}] would be large enough to modulate CaR. Based on our findings using inhibitors of various Ca^{2+} transport pathways, we concluded that Ca^{2+} released from internal stores is rapidly extruded from the cell by the PMCA into the apical domain, causing an increase in the local extracellular [Ca^{2+}]. Additionally, depletion of stores leads to opening of SOCs (store-operated channels) localized predominantly at the basal cell side, resulting in localized diminution of extracellular [Ca^{2+}]. Interestingly, immunostaining of the acid- and enzyme-secreting oxyntic cells displayed a curious distribution of CaR and the PMCA. Both of these proteins partially co-localized with the gastric H^+/K^+ ATPase, which resides in an elaborate apical tubulovesicular network (CAROPPO et al. 2001). Our data highlight an important point: Whether cells send or receive signals mediated by extracellular Ca^{2+} will

depend on the specific localization of Ca^{2+} entry and exit pathways in the plasma membrane, as well as the location of the CaR and/or other extracellular Ca^{2+} sensors that may potentially detect the extracellular signal.

4. Do These Extracellular [Ca^{2+}] Changes in the Gastric Mucosa Have Any Functional Consequences?

In our new unpublished studies (Caroppo, R., Gerbino, A., Fistetto, G., Debellis, L., Hofer, A. M., and Curci, S.: Physiological changes in extracellular [Ca^{2+}] regulate the secretory activity of gastric cells. Manuscript in preparation) H^+-selective microelectrodes were advanced into the gastric gland lumen, allowing real-time measurement of pH changes in individual intact gastric glands within the mucosa (see Debellis et al. 1998 for details of the methods). Remarkably, a stepwise elevation in luminal [Ca^{2+}] and concurrent decrease in basolateral [Ca^{2+}] (as our direct measurements indicated would occur following carbachol stimulation) caused the lumen of the gastric gland to alkalinize significantly (Caroppo et al., unpublished). We have shown previously using this same technique that stimulation with carbachol also actually causes a similar alkalinization of the gastric gland lumen (Debellis et al. 1998). It is therefore possible that cholinergic stimulation, which is associated with the secretion of pepsinogen in the amphibian, causes this alkalinization indirectly as a consequence of extracellular [Ca^{2+}] changes. Additional studies are in progress to determine whether CaR is involved in any aspect of this response. In parallel experiments pepsinogen secretion was assayed. Remarkably, a modest increase in external luminal [Ca^{2+}] concurrent with a decrease in basolateral [Ca^{2+}] also caused an enhancement of pepsinogen secretion (Caroppo et al., unpublished).

5. Possible Physiological Significance

In the actively secreting tissue there must be coordination of acid and enzyme secretion. Since pepsinogen is cleaved to its active form (pepsin) by low pH, it is possible that the alkalinization induced by carbachol (and/or alterations in extracellular [Ca^{2+}]) may prevent the premature activation of the proenzyme while it is still in the gland lumen.

6. Conclusions and Future Prospects

The transmission of information between cells is crucial for the proper function of tissues and organs. We have identified a novel, and potentially universal form of intercellular crosstalk that relies on extracellular Ca^{2+} messages. It is possible that this mechanism serves to amplify or propagate signals from stimuli delivered focally (Ca^{2+}-mobilizing hormones or neurotransmitters) on one cell to its neighbors. Importantly, our data demonstrate that *physiological* extracellular [Ca^{2+}] changes can alter the functional status of the tissue. Our studies have thus revealed that there is a second dimension to intracellular Ca^{2+}-signaling events; namely the generation of an extracellular Ca^{2+} message that may have specific functional consequences.

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Prof. Dr. Aldebaran M. Hofer
Harvard Medical School
West Roxbury VAMC and Brigham and Women's Hospital
1400 VFW Parkway; Room 2B111
West Roxbury MA 02132
USA
Phone: +1 617 3237700 ext 5902
Fax: +1 617 3635592
E-Mail: ahofer@rics.bwh.harvard.edu

350 Jahre Leopoldina – Anspruch und Wirklichkeit

Festschrift der Deutschen Akademie der Naturforscher Leopoldina 1652–2002

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Die älteste deutschsprachige Akademie prüft »Anspruch und Wirklichkeit« ihrer Vergangenheit und läßt 350 Jahre wechselvoller Geschichte in ihren naturwissenschaftlichen und medizinischen Rahmenbedingungen Revue passieren. Die Festschrift wendet sich an eine interessierte Öffentlichkeit, die allmählich diese besondere Akademie in der deutschen und internationalen Akademienlandschaft mit ihrer spezifischen wissenschaftlich-kulturellen Bedeutung wahrnimmt, nachdem die Wirkungen von 40 Jahren defizitärer Existenz »hinter dem eisernen Vorhang« überwunden werden konnten. (Klappentext)

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TRPM Cation Channels, an Unlikely Family

Andrea Fleig (Honolulu)

With 4 Figures

Abstract

The transient receptor potential (TRP) cation channel family originally described in *Drosophila* photoreceptors has seen intensive research efforts in order to identify new members and to elucidate their physiological functions. At least 20 new mammalian TRP-related genes have been cloned and the resulting channels characterized. The focus here will be on two members of the TRPM channel subfamily, TRPM5 and TRPM7. While TRPM5 is a calcium-activated cation ion channel gated by fast changes in intracellular calcium concentration, TRPM7 is a constitutively active divalent ion channel pathway regulated by intracellular Mg-ATP and free Mg^{2+} concentrations. Although the gating mechanisms of these two channels are quite different, they both play an important role in the regulation of cellular Ca^{2+} homeostasis.

Zusammenfassung

Die ursprünglich in den Photorezeptoren von *Drosophila* beschriebene Kationenkanal-Familie mit transistorischem Rezeptorpotential (TRP) ist intensiv untersucht worden, um neue Mitglieder zu identifizieren und deren physiologische Funktionen aufzuklären. Mindestens 20 neue TRP-verwandte Säugetier-Gene sind geklont und die von ihnen ausgebildeten Kanäle charakterisiert worden. Der Schwerpunkt dieses Beitrages liegt auf zwei Mitgliedern der TRPM-Kanal-Unterfamilie, TRPM5 und TRPM7. Während TRPM5 ein Calcium-aktivierter Kationenkanal ist, der durch schnelle Veränderungen in der intrazellulären Calciumkonzentration reguliert wird, ist TRPM7 ein konstitutiv aktiver divalenter Ionenkanalweg, der durch intrazelluläres Mg-ATP und freie Mg^{2+}-Konzentrationen reguliert wird. Obwohl der Schleusenmechanismus dieser zwei Kanäle recht verschieden ist, spielen beide eine wichtige Rolle bei der Regulation der zellulären Ca^{2+}-Homöostase.

1. Introduction

The TRP-channel family has been divided into three main subfamilies of channel proteins, TRPC for 'canonical', TRPM for 'melastatin-like' and TRPV for 'vanilloid receptor-like' (Fig. 1, Montell et al. 2002). New subfamilies are likely to be found in the future, with the newest member, the cold-activated ANKTM channel (Story et al. 2003), now designated $TRPA_1$. TRP channels can be found in a variety of tissues, and they have been implicated in a breadth of physiological functions, ranging from thermo-sensation to magnesium homeostasis and apoptosis (Hara et al. 2002, Jordt et al. 2003, Schmitz et al. 2003). The TRPM subfamily is unusual in that three of its member proteins are "chanzymes" or "channelases", that is, they are ion channels that have enzymatic domains embedded in their carboxy-terminal. One of these chanzymes is TRPM2 and its enzymatic domain has pyrophosphatase activity specific

for ADP ribose (Perraud et al. 2001, Sano et al. 2001). The other two proteins are TRPM6 and TRPM7, which both contain an atypical kinase domain related to the recently identified family of alpha kinases (Nadler et al. 2001, Riazanova et al. 2001, Runnels et al. 2001, Ryazanov et al. 1997, Schlingmann et al. 2002, Walder et al. 2002, Yamaguchi et al. 2001). Both TRPM6 and TRPM7 are constitutively active channels regulated by intracellular Mg-ATP and free Mg^{2+} (Nadler et al. 2001, Voets et al. 2003). The other TRPM family members lack enzymatic domains. Two of these TRPM channels have been shown to respond to changes in intracellular free Ca^{2+} concentrations (TRPM4 and TRPM5) (Hofmann et al. 2003, Launay et al. 2002, Liu and Liman 2003, Prawitt et al. 2003), whereas TRPM8 is sensitive to cold temperatures (McKemy et al. 2002, Peier et al. 2002). The gating mechanisms for TRPM1 and TRPM3 are less clear (Grimm et al. 2003, Lee et al. 2003). We will focus in this report on two TRPM family members. First, we will review TRPM5, a TRPM channel family member whose gating mechanisms has been elucidated only recently (Fig. 2). This will be followed by an update on the magnesium ion channel TRPM7 (Fig. 2).

2. TRPM5

Functional analysis of the chromosomal region (11p15.5) associated with loss of heterozygosity in a variety of childhood and adult tumors and the Beckwith-Wiedemann Syndrome revealed a central role of the TRPM5 gene (Prawitt et al. 2000). A variety of fetal and adult human and

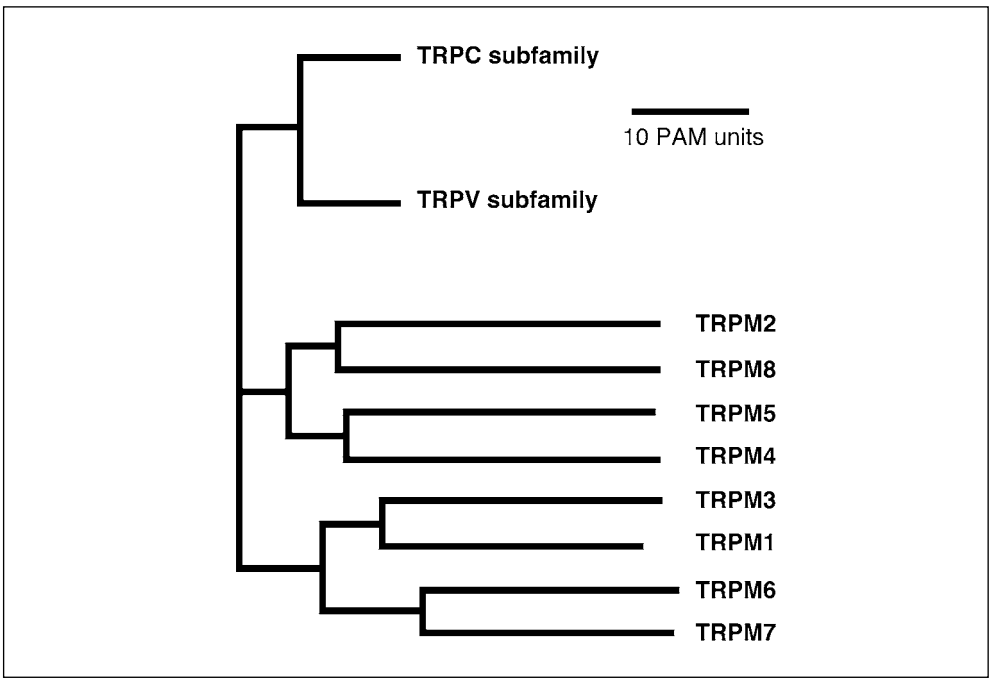

Fig. 1 Phylogenetic tree concentrating on the TRPM subfamily. The tree was adapted from Montell et al. (2002).

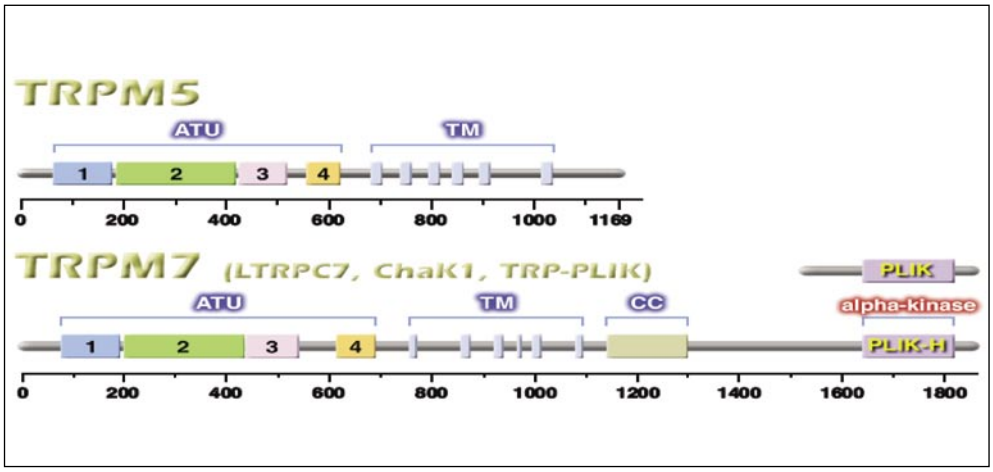

Fig. 2 Proposed molecular structure of TRPM5 and TRPM7. TRPM5 and TRPM7 proteins are composed of a unique amino terminal region (ATU), six putative transmembrane domains (TM) and a slightly hydrophobic pore-forming region, while both N- and C-terminal domains are intracytoplasmic. TRPM7 is notable in that it contains an alpha protein kinase domain (ChaK1, TRP-PLIK-H, LTRPC7) within its C-terminal sequence. This alpha kinase can also be found independently in the cell (alpha kinase, PLIK).

murine tissues express TRPM5. TRPM5 is structurally related to TRPM4. Initial studies of TRPM5 suggested that it constitutes a store-operated and a Ca^{2+}-permeable ion channel functioning as a sensor for bitter taste in sensory neurons (Perez et al. 2002). Zuker and colleagues proposed a receptor-mediated mechanism that depends on PLC activation independent of Ca^{2+} changes (Zhang et al. 2003). However, recent evidence suggests that TRPM5 may rather be a calcium-activated cation channel selective for monovalent ions only (Hofmann et al. 2003, Liu and Liman 2003, Prawitt et al. 2003). Our own studies find no evidence for activation of TRPM5 by Ca^{2+} store depletion nor do we see channel gating in the absence of Ca^{2+}. Instead, we find that the protein is directly activated by elevated $[Ca^{2+}]i$ in both whole-cell recordings and in excised membrane patches (Fig. 3). TRPM5 has a single channel conductance of 25 pS, which is similar to TRPM4. TRPM5 is a monovalent-specific ion channel that is activated by fast increases in $[Ca^{2+}]i$, whereby receptor agonists producing inositol 1,4,5-trisphosophate (IP3) and subsequent Ca^{2+} release can gate the channel (Prawitt et al. 2003). TRPM5 is therefore the second calcium-activated non-selective Ca^{2+} (CAN) ion channel whose molecular identity has been identified after TRPM4 (Launay et al. 2002). However, while TRPM4 requires Ca^{2+} influx for maximal activation and does not respond well to Ca^{2+} release alone, TRPM5 is strongly activated by receptor-mediated Ca^{2+} release. Unlike the sustained activation of TRPM4, TRPM5 exhibits only transient activation. Furthermore, TRPM5 does not simply mirror changes in Ca^{2+}, but requires fast changes in Ca^{2+} to activate. Thus, TRPM5 is uniquely situated to allow TRPM5-expressing cells to translate a receptor-mediated elevation in $[Ca^{2+}]i$ into an electrical response that ultimately results in transmitter release. This is a compelling concept, especially in view of our findings that TRPM5 expression is not limited to taste receptor cells, as we have identified TRPM5-like currents in pancreatic β cells. This would argue for a more generalized role of the channel in coupling agonist-induced intracellular Ca^{2+} release to electrical activity and subsequent cellular responses such as neurotransmitter or insulin release.

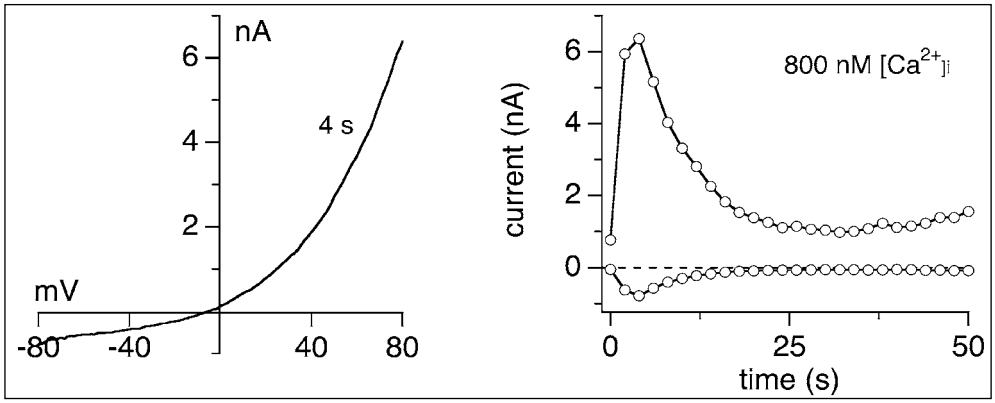

Fig. 3 Current-voltage relationship (I/V) and whole-cell currents of TRPM5. The right panel illustrates the typical activation time course of TRPM5 whole-cell currents in HEK-293 cells stably expressing TRPM5 recorded at +80 and – 80 mV, respectively. Currents were activated by perfusing cells with intracellular solutions containing 800 nM [Ca^{2+}]i. The left panel shows a typical I/V relationship of TRPM5 evoked by a voltage ramp of 50 ms duration at the peak of the current (4 s in this case). Data are not leak subtracted.

3. TRPM7

Virtually all mammalian cells express the TRPM7 ion channel (Nadler et al. 2001, Runnels et al. 2001). TRPM7 seems to be essential in cellular survival, as knocking the protein out in DT-40 cells results in cell death (Nadler et al. 2001). However, recent data show that these TRPM7-deficient cells can be rescued and remain viable by supplementation of extracellular Mg^{2+}, indicating that a primary cell biological function of TRPM7 relates to Mg^{2+} transport (Schmitz et al. 2003). TRPM7 is unique in its protein structure as it contains a protein kinase domain within its C-terminal sequence (Fig. 2). TRPM7 shares this feature with the closely related TRPM6 ion channel. The latter has been implicated in hypomagnesemia (Schlingmann et al. 2002, Walder et al. 2002). However, in contrast to TRPM2, where a C-terminal nudix hydrolase domain is responsible for channel gating, TRPM7's kinase domain seems to have a regulatory function (Schmitz et al. 2003) rather than being involved in gating of the channel (Runnels et al. 2001). TRPM7 has been shown to be constitutively active with both Mg^{2+}-nucleotide complexes and free [Mg^{2+}]i regulating channel availability (Fig. 4, Nadler et al. 2001). Additional regulators of TRPM7 include G protein activation (Hermosura et al. 2002) and phosphatidylinositol 4,5-bisphosphate (PIP2) hydrolysis (Runnels et al. 2002). Conflicting data have been presented regarding TRPM7 channel permeation characteristics, suggesting both nonselective conduction of Na^{+} and Ca^{2+} (Runnels et al. 2001) and complex permeation with selectivity towards divalent cations (Nadler et al. 2001). Our own work shows that TRPM7 is highly selective for divalent cations and is regulated by both intracellular Mg-ATP and cytoplasmic levels of free [Mg^{2+}]i. Based on these characteristics, we have named native TRPM7-like currents MagNuM for Magnesium-Nucleotide-regulated Metal ion currents (Hermosura et al. 2002, Nadler et al. 2001). In resting cells, physiological levels of these molecules strongly suppress the activity of TRPM7 channels, and only small constitutive activity remains, sufficient to maintain basal divalent cation fluxes. Intracellular solutions that lack Mg-ATP or are reduced in free Mg^{2+} recruit TRPM7-mediated currents that exhibit a

highly non-linear current-voltage relationship with pronounced outward rectification (Fig. 3). While outward currents at positive potentials are carried by monovalent ions (e.g. Cs^+ or K^+), the small inward currents at more physiological, negative potentials are exclusively carried by divalent ions such as Ca^{2+} and Mg^{2+}. The channel also permeates a variety of other divalent ions such as Zn^{2+}, Ni^{2+}, Co^{2+}, Ba^{2+}, Sr^{2+} and Cd^{2+} (Monteilh-Zoller et al. 2003). Native MagNuM currents share some features with the store-operated calcium influx pathway I_{CRAC}, in that they will conduct large monovalent currents in the absence of divalent ions such as Ca^{2+} and Mg^{2+}. Furthermore, MagNuM is activated under experimental conditions that have traditionally been used to study I_{CRAC} at the single channel level. It is now generally accepted that the 40 pS channels observed under these conditions are not due to CRAC channels but in fact represent TRPM7 channel activity (Hermosura et al. 2002, Kozak et al. 2002, Prakriya and Lewis 2002). In recent work, we analyzed human TRPM7 proteins that had either the kinase domain removed or that included point mutations in this region that abolish phosphotransferase activity. These data show that while the protein's C-terminal kinase domain is not essential for channel activation, it determines the sensitivity of the channel to intracellular levels of Mg^{2+} and Mg-ATP (Schmitz et al. 2003). By virtue of its sensitivity to physiological Mg-ATP levels, it is tempting to hypothesize that TRPM7 is involved in fundamental processes that adjusts plasma membrane divalent cation fluxes according to the metabolic state of the cell. TRPM7 may thus play an important role in pathophysiological circumstances such as ischaemia or hypoglycemia.

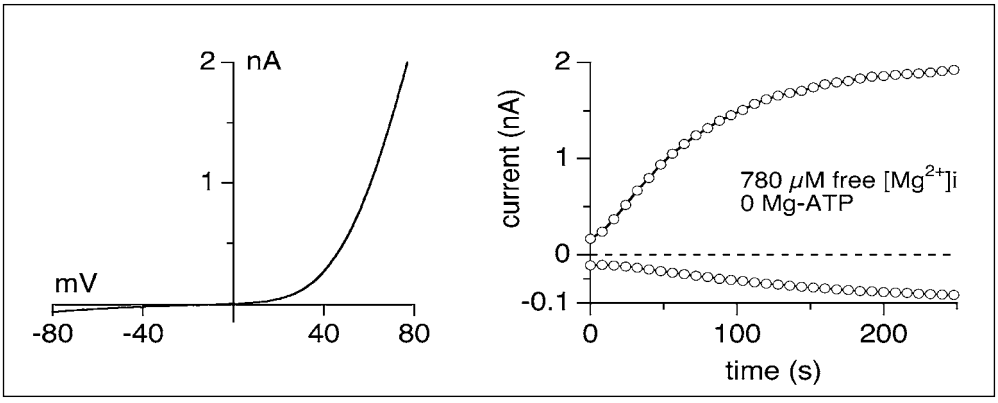

Fig. 4 Current-voltage relationship (I/V) and whole-cell currents of TRPM7. The right panel illustrates the typical activation time course of TRPM7 whole-cell currents in HEK-293 cells over-expressing TRPM7 recorded at +80 and −80 mV, respectively (note the different scale of the y-axis for inward and outward currents). Currents were induced using intracellular solutions that lacked ATP and in which $[Mg^{2+}]i$ was buffered to 780 μM. The left panel shows the typical I/V relationship of TRPM7 evoked by a voltage ramp of 50 ms duration at the peak of the current (200 s into the experiment). The I/V shows significant outward rectification at positive voltages due to permeation block of divalent ions at negative membrane potentials.

4. Summary

The TRPM subfamily is clearly a quite heterogeneous group of ion channels, although channels that seem to form evolutionary "sister" channels seem to share activation mechanisms (Fig. 1, Montell et al. 2002). For example, both TRPM4 and TRPM5 are gated by $[Ca^{2+}]i$, although their activation kinetics and the preferred source of calcium seem to differ. TRPM6 and TRPM7, however, are virtually indistinguishable and appear to be regulated in exactly the same way involving Mg nucleotides and free $[Mg^{2+}]i$. On the other hand, TRPM2 and TRPM8 clearly have different modes of activation. A common denominator of all TRPM channels characterized so far is that they serve as second messenger-operated channels. Similarly, the selectivities of individual channels vary widely with some members being strictly monovalent with no Ca^{2+} permeation (TRPM4 and TRPM5), others being non-selective and Ca^{2+}-permeable (TRPM2 and TRPM8) and two members being exquisitely divalent-specific (TRPM6 and TRPM7). Nevertheless, all of these channels have significant impact on Ca^{2+} signaling, either directly by permeating the ion or indirectly by controlling the membrane potential and thereby setting the driving force for Ca^{2+} influx. For example, the properties of TRPM5 are ideally suited to produce a transient depolarizing stimulus and may thus contribute to the electrical activity in electrically excitable cells. In regards to non-excitable cells, the channel may serve as a negative feedback mechanism for calcium influx and thus sharpening calcium oscillations. TRPM7 (and possibly its closest relative TRPM6) appear to control the influx of Ca^{2+}, Mg^{2+} and presumably other divalent ions, thereby directly influencing divalent ion homeostasis in mammalian cells.

Acknowledgements

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Andrea FLEIG, Ph. D.
Center for Biomedical Research
The Queen's Medical Center
1301 Punchbowl Street – UHT 8
Honolulu, HI 96813
USA
Phone: +1 808 5377931
Fax: +1 808 5377939
E-Mail: afleig@hawaii.edu

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Multiple Interactions between TRPCs and IP3Rs: TRPC3-interacting Domains of IP3R Serve to Identify TRPCs as Components of CCE Channels; yet, IP3R Activation Does Not Appear to Activate TRPC3 Channels

Lutz Birnbaumer[1], Alexander Dietrich[2], Brian Kawasaki[1], and Michael Xi Zhu[3]

With 5 Figures

Abstract

The biochemical evidence accumulated in our laboratories on the physical interactions of the TRPC3 channel protein with various regions of the type-3 IP3 receptor is summarized in this note. The data are discussed in the context of the hypothesis that these interactions play a key role in agonist and store depletion activated calcium entry and take into consideration results from published functional tests that both, are and are not, in support of the hypothesis.

Zusammenfassung

In diesem kurzen Bericht fassen wir unsere biochemischen Befunde zur physikalischen Interaktion des TRPC3-Kanals mit verschiedenen Regionen des IP3-Rezeptors Typ 3 zusammen. Die Daten werden im Zusammenhang mit der Hypothese diskutiert, daß diese Interaktion eine zentrale Rolle im zellulären Calcium-Einstrom spielt, der durch Agonisten und/oder Speicherentleerung aktiviert wird. Es werden sowohl Ergebnisse der Funktionsstudien berücksichtigt, die diese Hypothese unterstützen, als auch Daten, die ihr widersprechen.

Two questions have been addressed:

(*i*) Are TRPCs part of CCE channels? And:
(*ii*) Is IP3R activation essential for TRPC3 activation?

Using the interaction of IP3R3 and TRPC3 as primary model molecules, we have identified three regions of the IP3R in GST pull-down experiments, termed F2q, F2g, and F5c, that have significant affinity for the C-termini of TRPCs. Of these, two, F2q and F2g, interact each with two regions of TRPC3, labeled C7 and C3 (and a subfragment, C5). F5c interacts only with C3 and its derivative, C5. C3 comprises 65 amino acids immediately after the last transmembrane segment of the channel proper and includes at its N-terminus the EWKFAR TRPC motif. C7 comprises the next 50 amino acids. F2q and F2g lie within a 120 amino acid stretch ca. 100 amino acids C-terminal to the receptor's IP3-binding site, F2g being C-terminal to F2q. F5c

1 National Institute of Environmental Health Sciences (NIH, DHHS), Research Triangle Park, North Carolina 27709, USA.
2 Institut für Pharmakologie und Toxikologie, Universität Marburg, Marburg, Germany.
3 Neurobiotechnology Center and Department of Neuroscience, Ohio State University, Columbus, Ohio 43210, USA.

comprises the C-terminal cytosolic sequence of the IP3R, immediately after its channel domain. These findings are summarized in Figure 1 through 4.

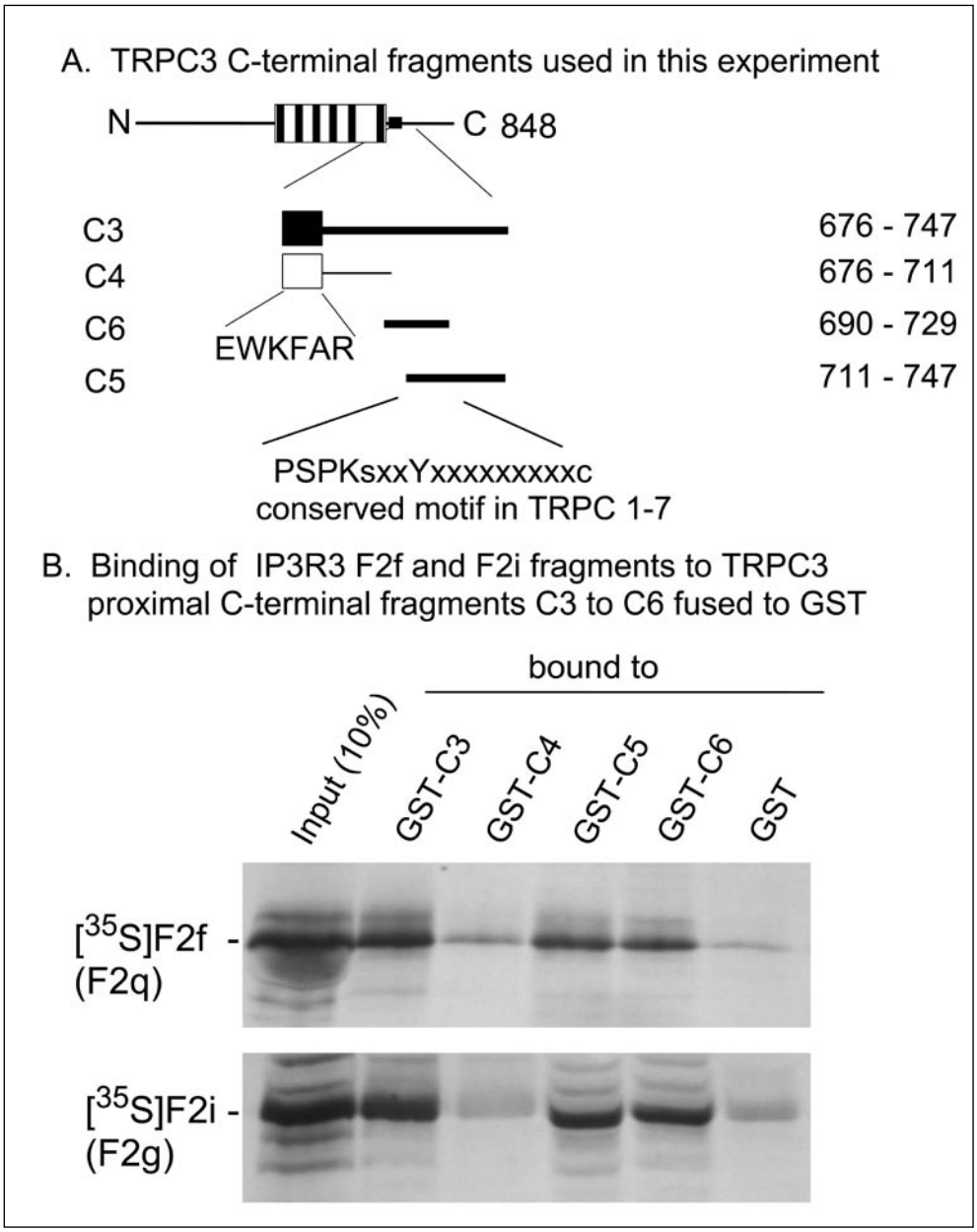

Fig. 1 Binding of *in-vitro*-translated IP3R fragments to TRPC3 fragments fused to GST. In this and the subsequent figures, heavy lines denote positive results in GST pull-down experiments. For methodological details see Boulay et al. (1999).

C7 not only interacts with F2q and F2g, but also with Ca calmodulin (CaCaM). Restricting the size of C7 to give C8 (last 21 aa of C7) retains F2q and CaCaM binding but almost totally looses F2g binding. We believe that this rather complex pattern of interactions plays a role in activation of TRPC channels for which the details are further subjects of current investigations in our laboratories. Sequence comparisons among TRPCs and among IP3Rs, and limited binding tests, suggest that these interactions are of a general nature, i.e., all TRPCs and all IP3Rs have these interaction sites, leading to the model shown in Figure 5. See also Boulay et al. (1999), Zhang et al. (2001) and Tang et al. (2001).

Expression of a TRPC-binding IP3R fragment containing F2q modulates thapsigargin-induced CCE negatively. Expression of a TRPC-binding fragment containing F2g has a positive effect (Boulay et al. 1999). F5c, had so far given equivocal results. Taken together the data argue for TRPCs being intrinsic components of CCE channels.

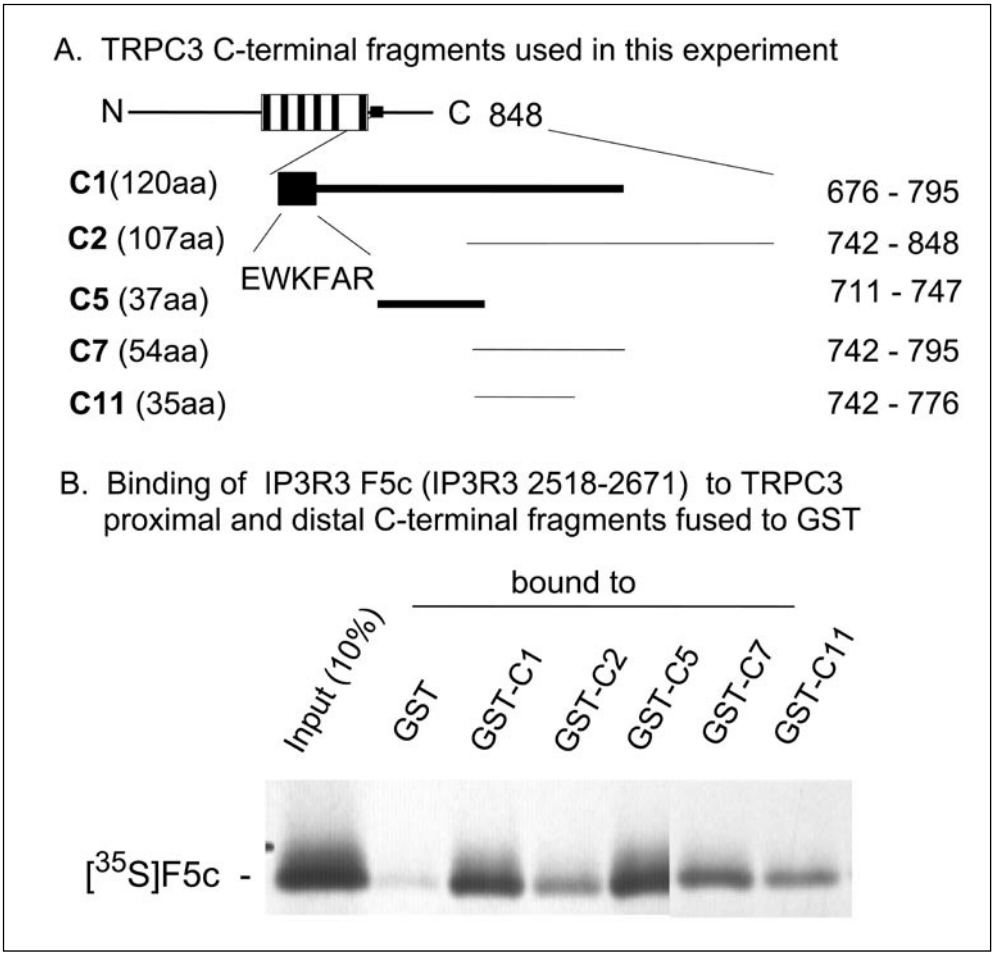

Fig. 2 Binding of *in-vitro*-translated F5c fragment of IP3R-3 to C-terminal fragments of TRPC3 fused to GST. For methodological details see Boulay et al. (1999).

Interestingly, Jim PUTNEY'S group recently described that activation of IP3R with IP3 and ensuing store depletion does not activate TRPC3 channels found in cells over-expressing TRPC3. In these cells TRPC3 channel activation depends on activation of the Gq-PLC pathway and

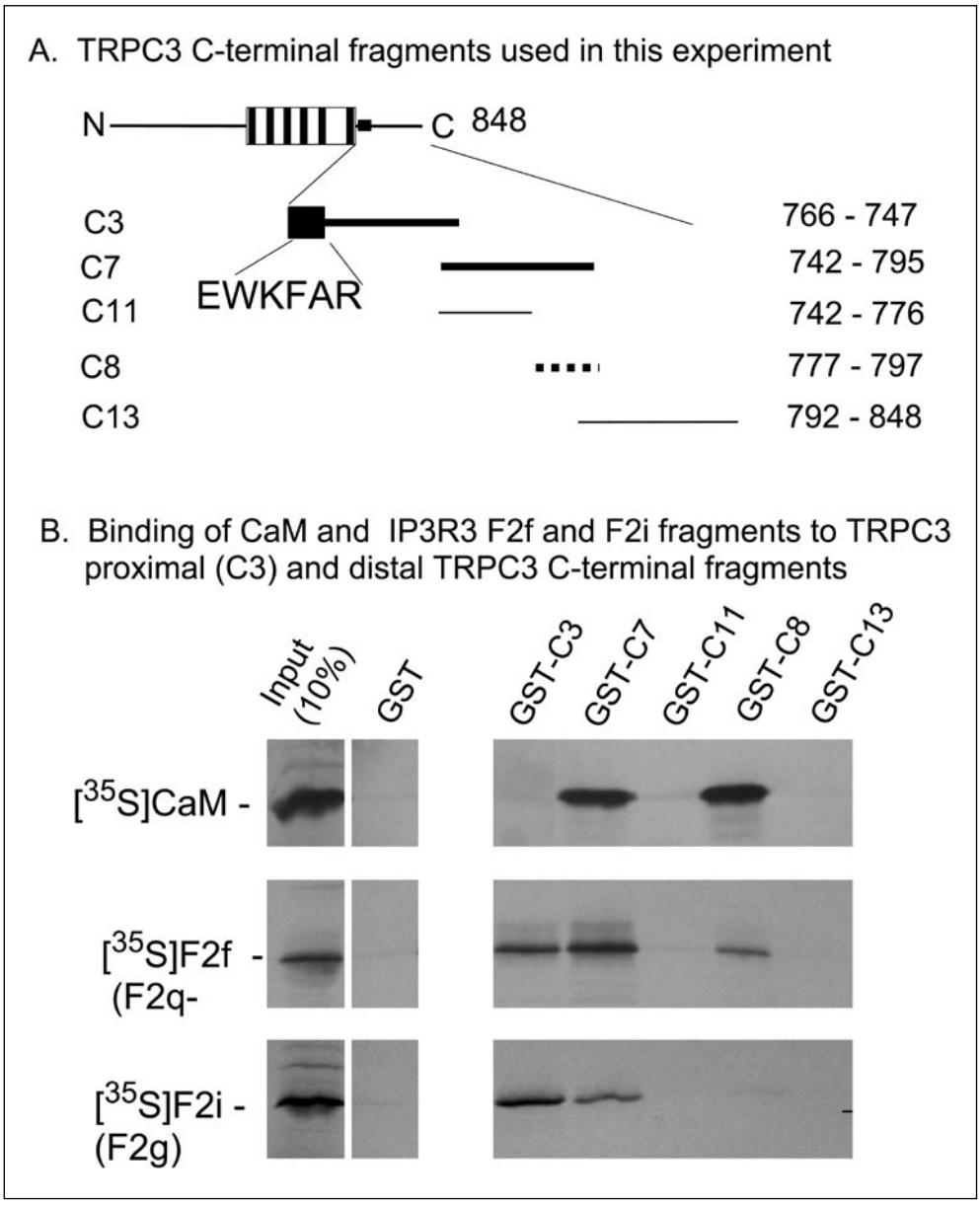

Fig. 3 Binding of *in-vitro*-translated calmodulin (CaM) and F2g and F2q-derived IP3R-3 fragments to C-terminal fragments of TRPC3 fused to GST. Dotted line: positive for CaM binding, weak for binding of F2q-containing fragments, and negative for binding of F2g-derived fragments. For methodological details see BOULAY et al. (1999).

cannot be obtained by microinjection of IP3-attendant store depletion, or by thapsigargin-mediated store depletion. Both these maneuvers activate CCE channels. Moreover, while IP3-induced store depletion and activation of CCE can be blocked by heparin, TRPC3 activation by a stimulation of a Gq-coupled receptor is not (Trebak et al. 2003). TRPC3 activation may be due to diacylglycerol formation, which bypasses IP3 and store depletion altogether (Hofmann et al. 1999).

The analysis of both TRPC3 activation and modulation of CCE (thapsigargin-activated calcium entry) commented on here, was done in HEK cells. Calcium entry mediated by TRPC3 was "filtered" from entry by endogenous channels and other mechanisms by working in the presence of gadolinium. Working in the absence of gadolinium and analyzing channel activity by electrophysiological means, instead of changes in Fura2 fluorescence, reveals that HEK cells stably expressing TRPC3 show channel activities that are never seen in control HEK cells. While this is reassuring, in terms of what is being measured in TRPC3-expressing cells,

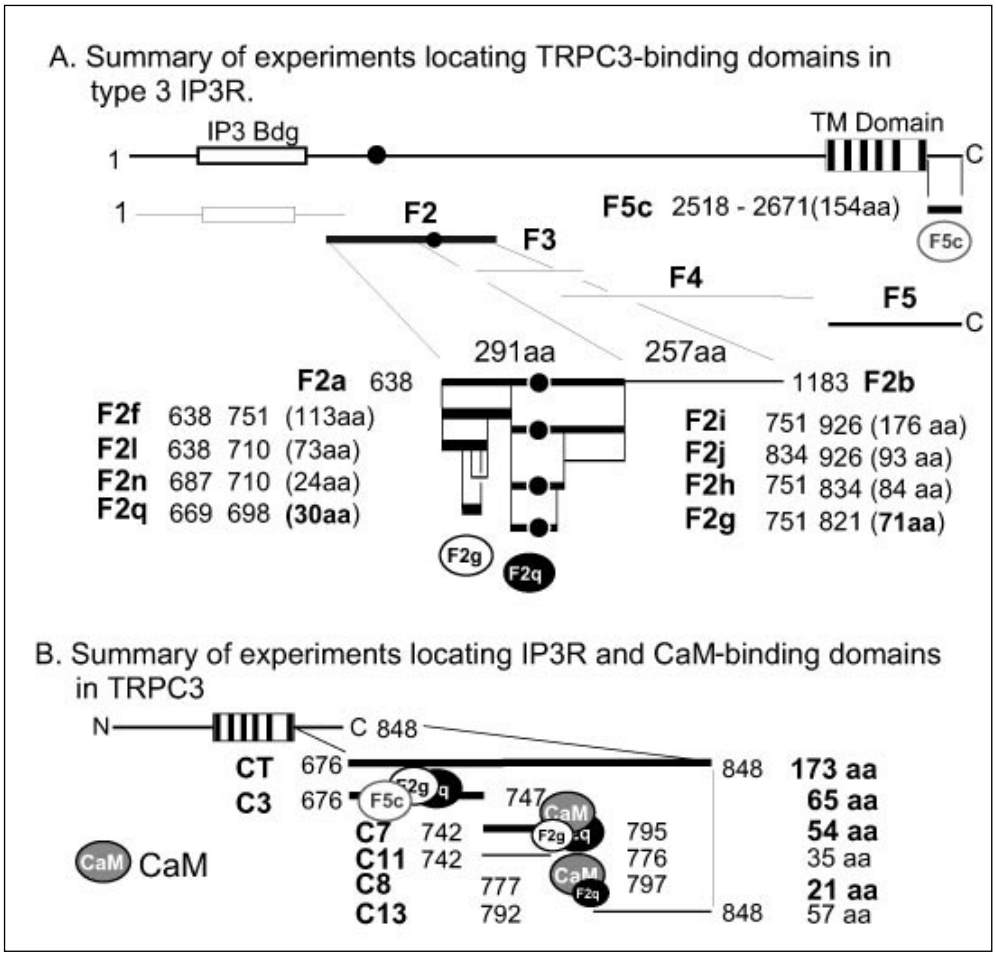

Fig. 4 Interactions studied in the experiments of Fig. 1 through 3, and summary of results.

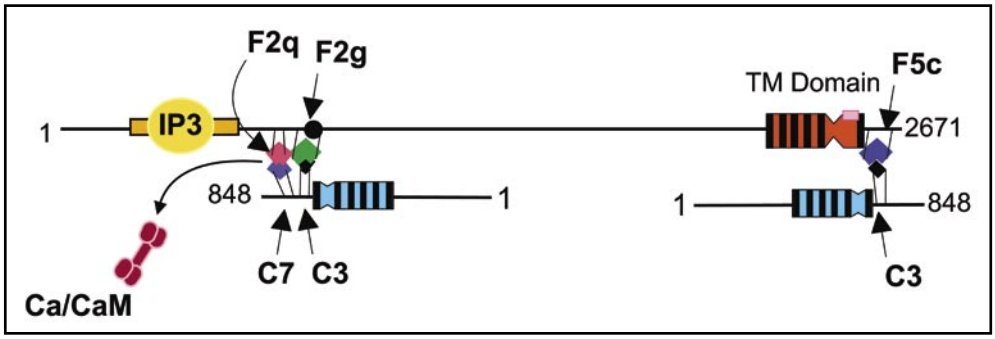

Fig. 5 Model of IP3R-TRPC interactions.

it raises concerns about why similar channels or gadolinium-resistant calcium entry is not seen in control cells. The concern is raised by the fact that HEK cells strongly express mRNA encoding TRPC3. Why then are 'TRPC3' channels and gadolinium-resistant calcium entry only seen upon transfection with exogenous mRNA? A reasonable explanation is probably that over-expression of TRPC3 mRNA drives synthesis of homomultimeric TRPC3 channels, while normally TRPC3 forms part of channels that incorporate other TRPCs. TRPC5 and TRPC6 are expressed about as abundantly as TRPC3 mRNA and coexist with lower quantities of TRPC1 and TRPC4 mRNAs. It follows that homomultimeric TRPC3 channels are not likely to exist and are unlikely to provide definite answers about the regulation of native TRPC3 containing channels. On the other hand, since TRPC-binding fragments of IP3R modulate CCE, in otherwise naive cells with an unaltered complement of TRPC mRNAs, it may be safe to conclude that TRPCs are intrinsic members of CCE channels.

As to the second question posed at the beginning of this article, it would seem that further studies are needed to determine if IP3R indeed activates TRPC channels. There is no question however, that receptor mediated activation of Gq leads to activation of gadolinium resistant TRPC3 channels. It is likely that the same is true for endogenous channels that incorporate TRPC3.

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Prof. Dr. Lutz BIRNBAUMER
National Institute of Environmental Health Sciences
(NIEHS)
Research Triangle Park
111 TW Alexander Dr.
North Carolina 27709
USA
Phone: +1 919 5413205
Fax: +1 919 5415002
E-Mail: lutzb@niehs.nih.gov

Academia 350
Die Leopoldina-Feiern in Schweinfurt und Halle 2002

Vorträge der Festveranstaltungen aus Anlaß des 350jährigen
Gründungsjubiläums der Deutschen Akademie der Naturforscher Leopoldina am
17. und 18. Januar 2002 in Schweinfurt und vom 18. bis 20. Juni 2002 in Halle (Saale)

Nova Acta Leopoldina N. F. Bd. *87*, Nr. 325
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Die Deutsche Akademie der Naturforscher Leopoldina feierte im Jahre 2002 ihre Gründung vor 350 Jahren mit zwei Festveranstaltungen. Im Januar in der Gründungsstadt Schweinfurt standen die historischen Wurzeln der Akademiegründung im 17. Jahrhundert im Mittelpunkt. Nach der Begrüßung durch Akademiepräsident Benno Parthier untersuchte Lorraine Daston (Berlin) das Thema „Die Akademien und die Neuerfindung der Erfahrung im 17. Jahrhundert". Richard Toellner (Rottenburg) nannte seine Ausführungen zu den Quellen der Akademiegründung „Im Hain des Akademos auf die Natur wißbegierig sein: Vier Ärzte der Freien Reichsstadt Schweinfurt begründen vor 350 Jahren eine Naturforscherunternehmung". Der Schweinfurter Festakt brachte nach der Begrüßung durch Frau Oberbürgermeisterin Gudrun Grieser und einer Folge von Grußadressen (u. a. vom bayerischen Ministerpräsidenten Edmund Stoiber) abschließend eine Standortbestimmung der ältesten deutschen Akademie in der Gegenwart von Präsident Parthier („Die Leopoldina heute"). Außerdem wird die im Rahmen der Feierlichkeiten erfolgte diesjährige Verleihung des Carus-Preises der Stadt Schweinfurt dokumentiert. Die Juniveranstaltung in Halle (Saale) widmete sich nach Grußworten des Bundespräsidenten Johannes Rau und des sachsen-anhaltinischen Ministerpräsidenten Wolfgang Böhmer den internationalen Aspekten des Leopoldina-Wirkens mit einem Symposium „Science und Society", das gemeinsam von Vertretern der ältesten europäischen Akademien, der Royal Society (Lord Robert May, London), der Acadèmie des Sciences (Jean-Pierre Kahane, Paris), der Accademia Nazionale dei Lincei (Sergio Carrà, Rom) und der Leopoldina (Hans Mohr, Freiburg i. Br.), getragen wurde. Die Beiträge behandeln das gesamte Spannungsfeld von Wissenschaft, Politik, Gesellschaft und Demokratie. Darüber hinaus dokumentiert der Band den von der Jungen Akademie, einer Tochterinstitution von Leopoldina und Berlin-Brandenburgischer Akademie, im Rahmen der Leopoldina-Jubelfeier veranstalteten Workshop „Science und Society: Science Goes Pop?", der versuchte, der Popularisierung von Wissenschaft und dem Bild des Wissenschaftlers in der Öffentlichkeit nachzugehen.

In Kommission bei Wissenschaftliche Verlagsgesellschaft mbH Stuttgart

Nova Acta Leopoldina NF *89*, Nr. 336, 69–74 (2004)

TRP, TRPV6 and Cancer

Ulrich Wissenbach[1], Helmut Bonkhoff[2], and Veit Flockerzi[1],
Member of the Academy (Homburg)

With 1 Figure and 1 Table

Abstract

Calcium (Ca^{2+}) is an ubiquitous intracellular signal that is responsible for a plethora of cellular processes such as fertilization, secretion, contraction, neuronal signaling and learning. In addition, changes in intracellular Ca^{2+} have been known to influence cell proliferation and differentiation for more than three decades. Recent studies have indicated that members of the transient receptor potential (TRP) family of ion channels may be a primary mode of Ca^{2+} entry into cells and may have roles in growth control. Moreover, changes in the expression of these channels may contribute to certain cancers. In the following, recent results concerning the expression of the TRP related protein TRPV6 in prostate cancer are summarized.

Zusammenfassung

Calzium-Ionen sind ubiquitär vorkommende intrazelluläre Botenstoffe, die verschiedene Prozesse wie Fertilisation, Sekretion, Kontraktion, neuronale Abläufe und das Lernverhalten beeinflussen. Außerdem ist seit mehreren Dekaden bekannt, daß Veränderungen der intrazellulären Calziumkonzentration Zellteilung und Differenzierung von Zellen beeinflussen. Aktuelle Untersuchungen zeigen, daß Mitglieder der „transient receptor potential" (TRP)-Familie von Ionenkanälen wichtige, an der Calziumregulation beteiligte Komponenten darstellen und somit an der Regulation der Wachstumskontrolle beteiligt sein könnten. Weiterhin werden in einigen Geweben Änderungen der Expressionslevel von TRP-Kanälen mit der Entwicklung von Krebs in Zusammenhang gebracht.

Im folgenden sind die Ergebnisse der Expression von TRPV6, einem TRP-Protein, in Prostatakarzinomen zusammenfassend dargestellt.

1. Introduction

In resting cells, intracellular free Ca^{2+} is maintained at a concentration of approximately 100 nM, but on stimulation this level can rise globally to in excess of 1 μM. This increase can be generated from sources both within and outside the cell. Internal Ca^{2+} stores are composed of the extensive endoplasmic reticulum network (the sarcoplasmic reticulum in muscle), where

1 Experimentelle und Klinische Pharmakologie und Toxikologie, Medizinische Fakultät, Universität des Saarlandes, 66421 Homburg.

2 Institut für Allgemeine und Spezielle Pathologie, Medizinische Fakultät, Universität des Saarlandes, 66421 Homburg.

release is controlled by various channels, of which the inositol 1,4,5-trisphosphate (InsP3) receptor and ryanodine receptor families have been the most extensively studied. The entry of external Ca^{2+} into cells is controlled by several mechanisms, including voltage-operated channels and receptor-operated channels that open in response to binding of a ligand to a receptor which is an integral part of a plasma membrane ion channel. A third class of plasma membrane ion channels include the members of the transient receptor potential (TRP) family of ion channels (Montell et al. 2002), which can function as Ca^{2+} influx channels both in excitable and non-excitable cells. This latter group include ion channels activated by binding of extracellular and intracellular messengers, temperature, mechanical stress, and, apparently, depletion of intracellular calcium stores. Based on structural similarities the TRP family is subdivided in three main subfamilies, the TRPC (canonical or classical) group, the TRPV (vanilloid) group and the TRPM (melastation) group (Montell et al. 2002). At least two other subfamilies of TRP-related gene products exist, TRPP and TRPML, which include PKD2 and mucolipin, respectively. Mutations in PKD2 or polycystin-2 lead to autosomal dominant polycystic kidney disease (Grantham 2001) whereas mutations within the mucolipin gene (Bargall et al. 2000) cause mucolipidosis type IV, a developmental neurodegenerative disorder. Mutations in mucolipin 3, another member of the TRPML subfamily is associated with deafness and pigmentation defects in varitint-waddler mice (Di Palma et al. 2002).

2. TRPV6 Transcript Expression Correlates with the Malignancy of Prostate Cancer

TRPV6 is the sixth member of the TRPV subfamily of TRP-related proteins and was originally identified as Ca^{2+} transport protein or CaT in the rat intestine (Peng et al. 1999). Subsequently the corresponding gene products were cloned from human and mouse tissues and called TRP8 (Wissenbach et al. 2000), CaT-like (Wissenbach et al. 2001) and ECaC2 (Hoenderoop et al. 2003). TRPV6 is a glycosylated (Hoenderoop et al. 2003, Hirnet et al. 2003) Ca^{2+} calmodulin-binding protein (Niemeyer et al. 2000, Hirnet et al. 2003). Unlike rat TRPV6 (Bödding et al. 2002) the human TRPV6 appears not to be expressed in duodenum but is present in the trophoblasts and syncytiotrophoblasts of the placenta, in pancreatic acinar cells but not in pancreatic ductal epithelial cells, and in myoepithelial cells of the salivary glands (Wissenbach et al. 2001). So far, little is known of the Ca^{2+} entry pathways in these tissues, but the Ca^{2+} permeation properties of the TRPV6 channel renders TRPV6 a good candidate for Ca^{2+}secretion coupling and transcellular Ca^{2+} transport in these cells.

TRPV6 transcripts are also expressed in locally advanced prostate cancer, metastatic and androgen-insensitive prostatic lesions, but are undetectable in healthy prostate tissue, benign prostate hyperplasia and high grade intraepithelial neoplasia (HGPIN), a premalignant prostatic lesion (Wissenbach et al. 2001). Extensive studies using prostate tissue specimens from more than 140 prostate cancer patients revealed that the expression of TRPV6 transcripts is tightly correlated with the malignancy of prostate cancer. In very small tumors and tumors of the stage pT1 which are confined to the prostate no TRPV6 expression occurs. A minor fraction of tumors of the stage pT2 which are still confined to the prostate does express significant levels of TRPV6 transcripts. The fraction of TRPV6 expressing cases rises considerably at tumors of the stage pT3a (advanced prostate cancer with extraprostatic extension) and almost all tissue specimens of patients with tumor stage pT3b (prostate carcinoma with infiltration of the seminal vesicles) do express TRPV6 transcripts (Fixemer et al. 2003). In summary, these results indicate that

TRPV6 expression in clinically organ-confined tumors correlates significantly with the Gleason score, which defines the histopathological grading, the pathological stage and extraprostatic extension. These features make TRPV6 a promising molecular marker in the preoperative staging for prediction of clinically significant disease and extraprostatic extension.

As described, TRPV6 transcript expression is detected in advanced stages of the disease but, interestingly, only in subsets of tumor cells. The latter finding suggests a specific function of these TRPV6-expressing tumor cells on the process of tumor progression and, maybe, hormone therapy failure. Prostate cancer is the most commonly diagnosed malignancy in men and is the second leading cause of cancer-related death in Western countries (Denmeade and Isaacs 2002). When organ-confined at the time of diagnosis, prostate cancer can be cured by radical prostatectomy. Unfortunately, more than 50 % of cancers that are considered clinically confined prior to surgery show extracapsular extension upon pathological analysis and thus represent a high risk of progression. In fact, locally advanced cancer is still a fatal disease for which presently no curative treatment is available (Denmeade and Isaacs 2002). There is great need for new molecular markers predicting tumor progression and the clinical outcome. The observation that TRPV6 transcripts are undetectable in most of normal human tissues including the prostate but present at high levels in locally advanced, metastatic, and recurrent prostatic leasons suggest, that TRPV6 is a promising marker for detection of prostate cancer and its molecular staging.

Interestingly, up-regulation of TRPV6 transcript expression has not been observed in other malignancies such as pancreatic carcinoma and melanoma, arguing against TRPV6 being a general marker of cell proliferation. However, recent results indicate that TRPV6 transcripts are also expressed in endometrial carcinoma but not in healthy endometrium (Fig. 1).

3. TRP-related Gene Products other than TRPV6 and Cancer

TRPV6 is not the only member of the family of TRP-related proteins associated with growth control and cancer. Recently it has been demonstrated that TRPC3 (Li et al. 1999) and TRPV2 (Kanzaki et al. 1999) are activated by brain derived neurotrophic factor (BDNF) and insulin-like growth factor I (IGF-I), respectively, apparently by translocation to the plasma membrane. The expression of melastatin or TRPM1, the founding member of the TRPM subfamily, was shown to correlate inversely with thickness of human primary cutaneous melanomas (Duncan et al. 1998, 2001). Subsequently it was concluded, that down-regulation of TRPM1 mRNA in the primary cutaneous tumor is a prognostic marker for metastasis in patients with localized malignant melanoma (Tab. 1).

Human TRPM5, previously called MTR1, has been located in the chromosomal region 11p15.5 (Prawitt et al. 2000). Alterations in this region are associated with the Beckwith-Wiedemann syndrome and predisposition to a variety of neoplasias, including Wilms' tumors, rhabdoid tumors and rhabdomyosarcomas. TRPM5 transcripts are expressed in a wide variety of tissues and cells including taste receptor cells (Perez et al. 2002), where TRPM5 appears to be involved in signal pathways coding sweet, bitter and umami tastes (Zhang et al. 2003). TRPM8, another member of the TRPM subfamily, has recently been called Trp-p8 (Tsavaler et al. 2001). Trp-p8 was originally identified as a prostate-specific gene, expressed in normal prostate epithelial cells, which is up-regulated in prostate cancer. In addition Trp-p8 expression appears to be up-regulated in other malignancies as well, such as primary

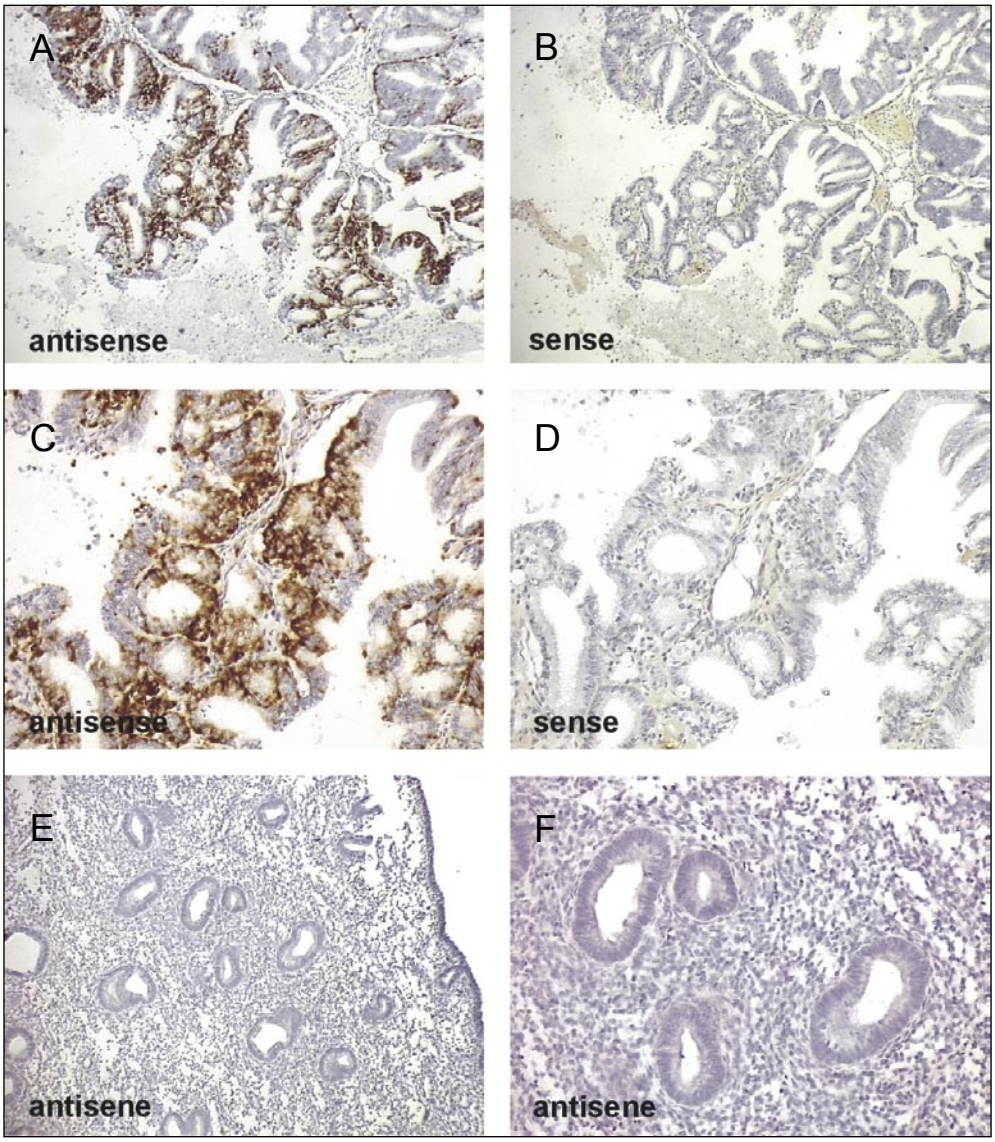

Fig. 1 TRPV6 transcripts are expressed in endometrial cancer. (*A*)–(*D*), *in-situ* hybridization with tissue specimens from a bone metastasis of an endometrial cancer, (*E*), (*F*), healthy endometrium, (*A*), (*B*), (*E*), magnification × 25; (*C*), (*D*), (*F*), magnification × 40

tumors of breast, colon, lung, and skin origin, whereas its transcripts were hardly detected in the corresponding normal human tissues (Tsavaler et al. 2001). TRPM8 is also expressed in dorsal root ganglia and trigeminal ganglia, and cells over-expressing TRPM8 can be activated by cold temperatures and by cooling agents such as menthol (Peier et al. 2002, McKemy et al. 2002).

Tab.1 TRP-related proteins associated with cell proliferation/growth control

TRP	Synonyme	
TRPV6	(CaT-L, TRP8, EcaC2, CaT1)	Up-regulated in prostate and endometrial cancer (Wissenbach et al. 2001)
TRPM1	(Melastatin)	Down-regulation in metastasis of localized malignant melanoma (Duncan et al. 1998)
TRPM5	(MTR1, Taste channel)	Associated with Beckwith-Wiedemann syndrome (predisposition to childhood-tumors) (Prawitt et al. 2000)
TRPM8	(TRP10, TRP-P8, Cold Receptor)	Up-regulated in prostate cancer, melanoma and colorectal adenocarcinoma (Tsavaler et al. 2001)

In summary, there is recent and increasing evidence, that a number of TRP-related proteins may have roles in growth control, and changes in the expression of these channels may contribute to certain cancers. Up-regulation of TRPV6 mRNA and, maybe, TRPM8, in prostate cancer, like down-regulation of TRPM1 mRNA in cutaneous malignant melanoma might be a prognostic marker to classify these cancers on a molecular level. In addition, as plasma membrane ion channels these TRP-related proteins might well represent promising targets for drugs used to treat these cancers.

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Prof. Dr. Veit Flockerzi
Experimentelle und Klinische
Pharmakologie und Toxikologie
Universität des Saarlandes
66421 Homburg
Germany
Phone: +49 6841 1626400
Fax: +49 6841 1626402
E-Mail: veit.flockerzi@uniklinik-saarland.de

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Calcium Transport in T Lymphocytes

Désirée Griesemer[1], Ariel Quintana[1], Laurence Méry[2], Eva Schwarz[1], and Markus Hoth[1]

With 1 Figure

Abstract

Ca^{2+} transport across membranes is a key event during T lymphocyte activation. Following stimulation through the T cell receptor, several signaling cascades are activated ultimately leading to a T lymphocyte dependent immune response. One of these cascades activates Ca^{2+} release from the endoplasmic reticulum and subsequently also Ca^{2+} influx from the extracellular space into the cytosol through Ca^{2+} selective ion channels. The resulting complexity of the cytosolic Ca^{2+} signals such as Ca^{2+} oscillations leads to differential gene expression which regulates the immune response. The present paper focuses on some recent developments regarding Ca^{2+} transport in T lymphocytes.

Zusammenfassung

Ca^{2+}-Transport über die Plasmamembran ist ein Schlüsselereignis während der T-Zellaktivierung. Nach Stimulation des T-Zell-Rezeptors wird eine Reihe von Signalkaskaden aktiviert, die zur T-Zell-abhängigen Immunantwort führen. Eine dieser Kaskaden aktiviert die Ca^{2+}-Freisetzung aus dem Endoplasmatischen Retikulum und führt daran anschließend zum Ca^{2+}-Einstrom aus dem Extrazellulärraum in das Zytosol durch Ca^{2+}-selektive Ionenkanäle. Die sich daraus ergebende Komplexität der Ca^{2+}-Signale, wie z. B. Ca^{2+}-Oszillationen, ermöglicht eine differentielle Genexpression, die die Immunantwort reguliert. Der folgende Beitrag präsentiert einige wichtige neue Erkenntnisse auf dem Gebiet des Ca^{2+}-Transports in T-Lymphozyten.

1. Introduction

T lymphocytes are usually quiescent. Following stimulation with an agonist antigen and co-stimulatory signals, they become activated to proliferate and secrete IL-2 and other cytokines, resulting in the initiation of an immune response (Lewis 2001). This response must not be activated inappropriately, because this could lead to autoimmunity, nor must the response be inefficient, which could result in the immune system not killing an infecting agent quickly enough to prevent damage of the whole organism. T cell activation is thus carefully balanced between dangerous alternatives.

Stimulation of the T cell receptor requires agonist peptides presented through MHC proteins, for instance by dendritic cells, macrophages or B lymphocytes (Fig. 1). One of the signaling

1 Department of Physiology, University of the Saarland, Homburg, Germany.
2 INSERM 442, Université Paris-Sud, Batiment 443, 91405 ORSAY Cedex, France.

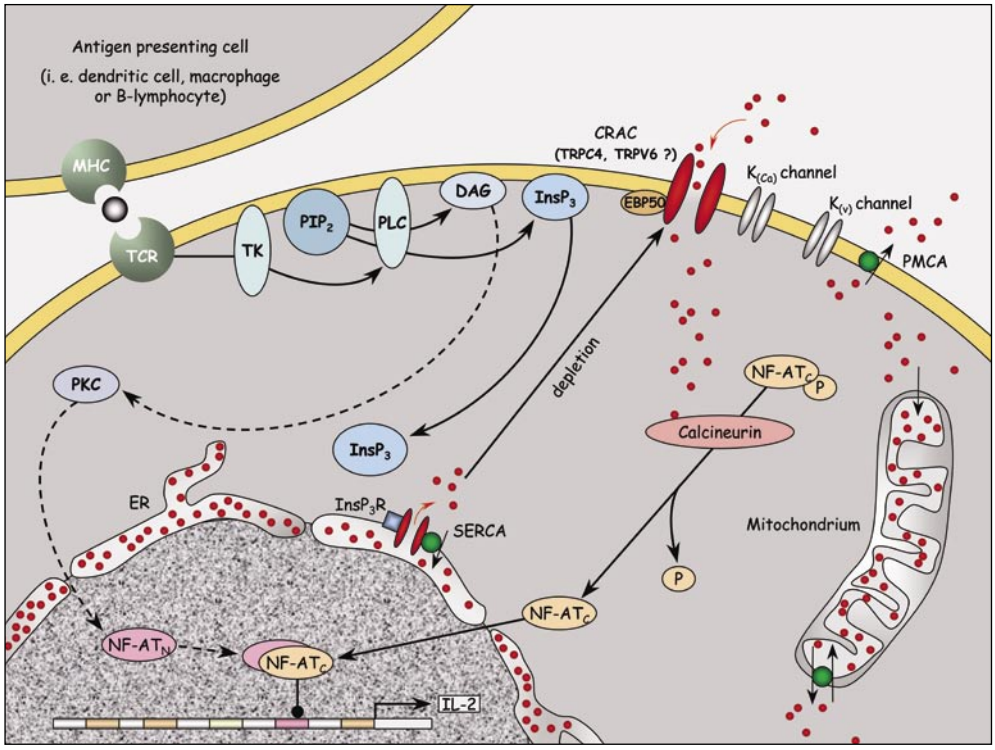

Fig. 1 The Ca^{2+} signaling cascade in T lymphocytes. Explanations are given in the text.

cascades initiated by this interaction is the phospholipase C (PLC) pathway. Active PLC hydrolyzes phosphatidyl-inositol-bisphosphate (PIP_2) to release inositol trisphosphate ($InsP_3$) and diacylglycerol (DAG). DAG is the physiological activator of protein kinase C (PKC) and is required for the *de-novo* synthesis of the nuclear subunit of Nuclear Factor of Activated T cells ($NF\text{-}AT_N$), a key transcriptional regulator of cytokine genes in the nucleus. At the same time, $InsP_3$ causes Ca^{2+} release from intracellular stores, depleting the Ca^{2+} content of the endoplasmic reticulum (ER). This depletion generates a signal which in turn activates store-operated CRAC (Ca^{2+} release-activated Ca^{2+}) channels (Hoth and Penner 1992, Lewis 2001, Parekh and Penner 1997, Zweifach and Lewis 1993) in the plasma membrane (Fig. 1). CRAC channels are highly selective for Ca^{2+} over other cations with permeation properties very similar to voltage gated Ca^{2+} channels of electrically excitable cells (Hoth 1995). Following activation of CRAC channels, long-lasting complex cytosolic Ca^{2+} signals are generated. The pattern of the cytosolic Ca^{2+} signals range from steady elevations to complex oscillations depending on the interplay of several transport mechanisms including CRAC channels (Dolmetsch and Lewis 1994), Ca^{2+} pumps (PMCA, SERCA) (Bautista et al. 2002), Ca^{2+} uptake and release from stores (mitochondria, ER) (Hoth et al. 2000), and potassium channels which control the electrical driving force for Ca^{2+} entry (Fanger et al. 2001). The cytosolic Ca^{2+} signals are necessary for the activation of several transcription factors including the cytosolic subunit of the Nuclear Factor of Activated T-cells ($NF\text{-}AT_C$). $NF\text{-}AT_C$ is transported from the cytosol into

the nucleus upon dephosphorylation by the Ca^{2+}-dependent phosphatase calcineurin (Fig. 1). There, NF-AT_C binds to NF-AT_N to form a complete and functional transcription factor which, in conjunction with other transcription factors activated by this signal transduction cascade, promotes the transcription of many genes important for T cell activation including IL-2 (Lewis 2001). Whereas regulation and modulation of the channel is well understood, neither its activation mechanism nor the composition of the channel itself are known on the molecular level. Currently, members of the TRP protein family are considered good candidates to be part of the Ca^{2+} channel in T cells (Lewis 2001).

2. TRP Proteins as a Molecular Basis for CRAC Channels

It is the eyes of the fruitfly *Drosophila melanogaster* that have shed some light on the potential molecular identity of CRAC Ca^{2+} channels. The light response of *Drosophila* has been intensively studied during more than 30 years and flies have been screened for mutations in proteins involved in this signaling cascade that transforms the energy of even single photons of light into electrical signals. Phototransduction in *Drosophila* is a G protein-coupled, phospholipase C-mediated signaling cascade that results in the depolarization of the receptor membrane and influx of Na^+ and Ca^{2+} into the cell. One of the mutants originally described by Cosens and Manning (1969) shows a receptor potential that cannot sustain a steady state depolarization during prolonged light stimulation. This results in premature termination of the light-activated conductance. The mutant was thus named Transient Receptor Potential (TRP). The trp gene was cloned in 1989 and was found to encode a 143 kDa protein with structural similarity to vertebrate voltage-gated ion channels (Montell and Rubin 1989). However, the charged residues believed to act as voltage sensors in the S4 domain of voltage-gated channels are missing in TRP. This is consistent with the hypothesis that the light-activated conductance is activated by an intracellular signal and not by changes in membrane potential. TRP channels were found to be important for Ca^{2+} influx following stimulation of *Drosophila* photoreceptors (Hardie and Minke 1992).

In 1995 and 1996, the first papers were published reporting cloning and functional expression of mammalian TRP homologues (Philipp et al. 1996, Wes et al. 1995, Zhu et al. 1995, 1996, Zitt et al. 1996). The family of mammalian TRP proteins consists so far of more than 20 related proteins that can be grouped in three families, TRPC (classic TRPs), TRPV (vanilloid-like TRPs), and TRPM (melastatin-like TRPs) (Montell et al. 2002). TRP proteins have been shown to form Ca^{2+}/cation channels with very different properties and some of the TRPs are considered to underlie store-operated Ca^{2+} entry through CRAC channels in T lymphocytes. The two best candidates at present are probably TRPC4 and TRPV6 (Fig. 1). The biophysical properties of TRPV6 make this channel an excellent candidate to be part of the CRAC channel, however, its mode of activation is controversial. While some reports indicate that TRPV6 can be activated following depletion of internal Ca^{2+} stores, others do not confirm this observation. Expression of TRPC4 in human embryonic kidney (HEK-293) cells provided evidence for the channel to form Ca^{2+} selective store-operated ion channels. HEK cells have endogenous CRAC currents which can be enhanced by TRPC4 (Philipp et al. 1996). The role of TRPC4 mediating CRAC channel activity is strongly supported by data from the TRPC4-deficient mouse (Freichel et al. 2001). CRAC currents were found to be reduced in endothelial cells derived from TRPC4-deficient mice when compared to CRAC currents from the same tissue of wild

type mice. The importance of TRPC4 for CRAC currents is also evident in a bovine adrenal cortex (SBAC) cells, in which the protein level of TRPC4 could be linked to the activity of CRAC channels (Philipp et al. 2000), further strengthening the possibility that TRPC4 is part of endogenous CRAC channels. However, expression of TRPC4 cDNA also led to appearance of store-independent cation currents (Schaefer et al. 2000, McKay et al. 2000). These contradictions could be due to the background of TRP proteins present in all expression systems because it can not be excluded that heteromultimeric channels with very different properties are formed between recombinant and endogenous channels.

Considering the results described above one can hypothesize that CRAC channels are composed of different TRP proteins. It is therefore of great importance to investigate potential interactions of the TRP proteins which are candidates to underlie CRAC channel activity. Recently it has been shown that TRPV6 can form heteromultimers with its closest related TRP protein, TRPV5 (Hoenderop et al. 2003, Van De Graaf et al. 2003). Interactions of TRPC4 with TRPC1 and TRPC5 have also been described *in vitro* (Strubing et al. 2001, Hofmann et al. 2002). At the moment it is not clear if the potential interactions of TRPV6 and TRPC4 with their partners have any physiological implications or resemble endogenous channels.

Another recently described partner of TRPC4 appears to be very important for its cellular localization. The PDZ protein EBP50 (for Ezrin/Radixin/Moesin binding phosphoprotein 50) binds to the last three amino acids (TRL) of TRPC4 (Mery et al. 2002, Tang et al. 2000). This interaction is extremely important for the correct plasma membrane localization of TRPC4 and is thus also regulating its activity (Mery et al. 2002). Immunofluorescence analysis of expressed proteins showed that a TRPC4 mutant lacking the TRL motif accumulated into cell outgrowths and exhibited a punctate distribution pattern whereas the wild type channel was evenly distributed on the cell surface. Deletion of the PDZ-interacting domain also decreased the expression of TRPC4 in the plasma membrane by 2.4 fold, as assessed by cell surface biotinylation experiments. Finally, in a large percentage of cells co-expressing TRPC4 and an EBP50 mutant lacking the ERM-binding site, TRPC4 was not present in the plasma membrane but co-localized with the truncated scaffold in a perinuclear compartment (most likely representing the Golgi apparatus) and in vesicles associated with actin filaments. As EBP50 is highly expressed in T lymphocytes it could well be involved in the localization of TRPC4 and potentially in the correct assembly and localization of CRAC channels (Fig. 1).

3. Physiological and Pathophysiological Role of Ca^{2+} Transport

The physiological and pathophysiological importance of CRAC channels is readily evident from measurements in T lymphocytes of patients with a severe-combined immunodeficiency (SCID). The absence of CRAC channel activity in the T cells of those patients does not allow a proper immune response (Feske et al. 2001, Le Deist et al. 1995, Partiseti et al. 1994) making the patients therefore very susceptible to dangerous opportunistic infections. Additional evidence for the importance of Ca^{2+} transport *via* CRAC channels is provided by the analysis of Ca^{2+} transport defective mutant T cell lines (Fanger et al. 1995). Mutants of the T cell line Jurkat specifically defective in NF-AT activation were generated by using a suicide gene attached to the minimal IL-2 promoter. By repeated stimulation of such cells following mutagenesis, several cell lines were derived with decreased activation of an NF-AT-sensitive reporter gene. Patch-clamping of the mutant cells revealed severe defects in the current through

CRAC channels, but extensive testing showed few other significant abnormalities. The level of NF-AT reporter gene expression in the mutant cells is well-correlated with the level of CRAC channel activity in each mutant line. Furthermore, these mutant cells can be restored to normal activation of gene transcription by "forcing" Ca^{2+} into the cytosol with ionomycin and high external Ca^{2+} concentrations. Thus, influx of Ca^{2+} through CRAC channels is absolutely necessary for T cell activation.

4. Perspectives

Reactivity towards antigen greatly varies between T cell populations and also between T lymphocytes from different organs, for instance between hyper-reactive blood helper T cells and hypo-reactive intestinal helper T cells. The close link between Ca^{2+} transport across the plasma membrane and T cell activity raises the question if this could be a common mechanism in T lymphocytes for varying their degree of activity. To test this hypothesis, it is necessary to fully understand the molecular basis of CRAC channels in T lymphocytes.

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Prof. Dr. Markus Hoth
Institut für Physiologie
Gebäude 58
Universität des Saarlandes
66421 Homburg/Saar
Germany
Phone: +49 6841 1626266
Fax: +49 6841 1626060
E-Mail: markus.hoth@uniklinik-saarland.de

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Analyses of Rare Genetic Diseases Give Insight in Magnesium Homeostasis

Martin Konrad[1,2], Karl Peter Schlingmann[1], and Thomas Gudermann[3]

With 3 Figures and 1 Table

Abstract

Many biological processes such as protein synthesis, nucleic acid stability or neuromuscular excitability depend on magnesium. Its extracellular concentration is tightly regulated by modulation of intestinal absorption and renal excretion. The exact mechanisms mediating transepithelial magnesium transport are still not fully understood. Recently, the molecular analysis of inherited diseases related to a disturbed magnesium handling revealed several new proteins along with already known molecules unexpectedly involved in renal epithelial magnesium transport, e.g. paracellin-1 which is involved in paracellular reabsorption of divalent cations in the thick ascending limb or the γ-subunit of the Na^+K^+ ATPase in the distal convoluted tubule. In this review, we focus on TRPM6, a member of the "transient receptor potential" (TRP) protein family, which is mutated in primary hypomagnesemia with secondary hypocalcemia, a combined defect of intestinal magnesium absorption and renal magnesium conservation.

Zusammenfassung

Magnesium spielt eine wichtige Rolle bei verschiedenen biologischen Vorgängen, wie z. B. der Proteinsynthese, für die Stabilität von Nukleinsäuren oder für die neuromuskuläre Erregbarkeit. Durch Regulation sowohl der intestinalen Resorption wie auch der renalen Exkretion wird die extrazelluläre Magnesiumkonzentration nahezu konstant gehalten. Allerdings ist die molekulare Physiologie, die diesen epithelialen Transportvorgängen zugrunde liegt, noch weitgehend unbekannt. Durch die Aufklärung der molekularen Pathogenese einiger hereditärer Magnesiumverlusterkrankungen konnten in den letzten Jahren verschiedene Proteine identifiziert werden, die für den epithelialen Magnesiumtransport in der Niere von großer Bedeutung sind; so z. B. Paracellin-1 als wichtige Komponente der parazellulären Magnesium- und Calciumreabsorption im Bereich der Henleschen Schleife oder die γ-Untereinheit der Na^+K^+ ATPase im distalen Konvolut des Tubulus. Diese Übersicht legt den Schwerpunkt auf TRPM6, einen Ionenkanal der „transient receptor potential"(TRP)-Familie. Mutationen dieses Kanals verursachen einen kombinierten Defekt der intestinalen Absorption von Magnesium und der renalen Magnesiumreabsorption und führen zum Krankheitsbild der primären Hypomagnesiämie mit sekundärer Hypokalziämie.

1. Magnesium Physiology

Most of the total body magnesium is stored in bone, muscle and soft tissues, only less than one percent is circulating in the blood (Elin 1994). Serum magnesium levels are kept in a narrow

1 University Children's Hospital Philipps-University, Marburg, Germany.
2 University Children's Hospital, Inselspital, Bern, Switzerland.
3 Institute of Pharmacology and Toxicology, Philipps-University, Marburg, Germany.

range (0.7 to 1.1 mmol/L). Its homeostasis depends on intestinal absorption and renal excretion. Within physiologic ranges, diminished magnesium intake leads to enhanced magnesium absorption in intestine and reduced renal excretion. These transport processes are under tight hormonal control (Kerstan and Quamme 2002, Quamme and De Rouffignac 2000).

The main site of magnesium absorption is the small intestine where it occurs *via* two different pathways: a saturable active transcellular transport and a nonsaturable paracellular passive transport (Fine et al. 1991, Kerstan and Quamme 2002) (Fig. 1*A*). At low luminal concentrations magnesium is absorbed primarily *via* the active transcellular route and with rising concentrations *via* the paracellular pathway, yielding a curvilinear function for total absorption (Fig. 1*B*).

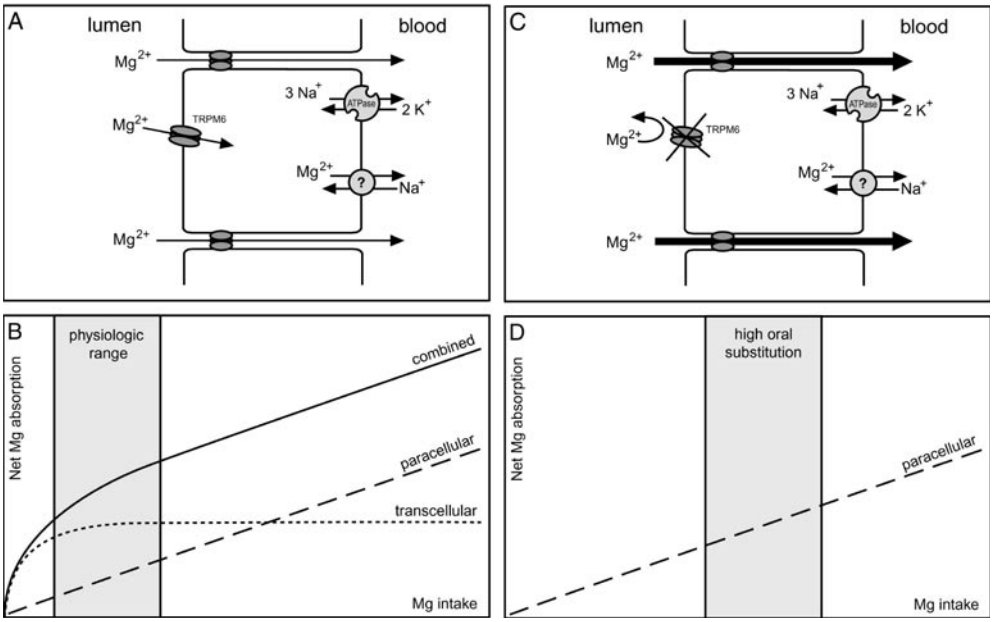

Fig. 1 (*A*) Intestinal magnesium absorption. (*B*) Intestinal magnesium absorption versus intake. The curvilinear function (solid line) results from a non-saturable paracellular and a saturable transcellular transport. (*C*) In hypomagnesemia with secondary hypocalcemia, all magnesium is absorbed *via* the paracellur pathway because TRPM6 mutations lead to a disruption of the transcellular route. (*D*) In hypomagnesemia with secondary hypocalcemia high oral magnesium substitution allows a more effective use of the paracellular magnesium absorption mechanism.

In the kidney, roughly 80 % of magnesium is filtered in the glomeruli with more than 95 % being reabsorbed along the nephron. In adult kidneys, 15 – 20 % are reabsorbed in the proximal tubule. About 70 % is reabsorbed in the loop of Henle, especially in the cortical thick ascending limb (TAL). Transport in this segment is passive and paracellular, driven by the lumen-positive transepithelial voltage (Fig. 2*A*), although only 5 – 10 % of the filtered magnesium is reabsorbed in the distal convoluted tubule (DCT). As there is no significant reabsorption of magnesium in the collecting duct, this part of the nephron is crucial for the fine adjustment of renal excretion. In the DCT, magnesium transport is active and transcellular in nature (Fig. 2*B*). Apical entry

into DCT cells is mediated by a magnesium channel driven by a favorable transmembrane voltage (Dai et al. 2001). The mechanism of basolateral transport into the interstitium is unknown. Most physiologic studies favor a sodium-dependent exchange mechanism (Quamme 1997). Magnesium transport in the distal tubule has been recently reviewed in detail (Dai et al. 2001). Finally, three to five percent of the filtered magnesium is excreted in the urine.

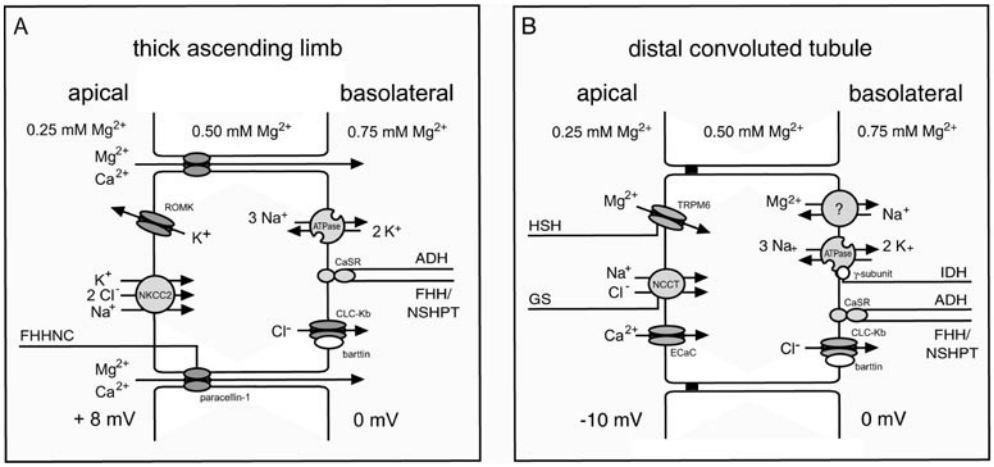

Fig. 2 (*A*) Magnesium reabsorption in the thick ascending limb of the loop of Henle. Driving force for the reabsorption against a concentration gradient is a lumen-positive voltage gradient generated by the reabsorption of NaCl. (*B*) Magnesium reabsorption in the distal convoluted tubule. Active transcellular transport mediated by an apical entry through a magnesium channel and a basolateral exit, presumably *via* a Na^+/Mg^{2+} exchange mechanism.

The evidence for the magnesium transport pathways described above mainly evolved from physiological studies. During recent years, the analysis of disease phenotypes characterized by disturbances in magnesium handling turned out to be very helpful for a better understanding of magnesium homeostasis (Tab. 1; for review see Cole and Quamme 2000, Konrad and Weber 2003).

Tab. 1 Inherited disorders or magnesium handling

Disorder	Inheritance	Locus	Gene	Protein
Familial hypomagnesemia with hypercalciuria/nephrocalcinosis	AR	3q28	*CLDN16*	paracellin-1, tight junction protein
Isolated dominant hypomagnesemia with hypocalciuria	AD AD	11q23 ?	*FXYD2* ?	γ-subunit of the Na^+-K^+-ATPase ?
Isolated recessive hypomagnesemia with normocalciuria	AR	?	?	?
Gitelman variant of Bartter syndrome	AR	16q13	*SLC12A3*	NCCT, Na^+Cl^- cotransporter
Hypomagnesemia with secondary hypocalcemia	AR	9q22	*TRPM6*	TRPM6, cation channel

AR autosomal recessive, AD autosomal dominant

The first example was the identification of mutations in *CLDN16* encoding paracellin-1 in familial hypomagnesemia with hypercalciuria and nephrocalcinosis (FHHNC) in 1999 (Simon et al. 1999). As paracellin-1 is almost exclusively expressed in the TAL, these findings confirmed the hypothesis of Rodriguez-Soriano and Vallo who predicted a defective magnesium and calcium reabsorption in this nephron segment as the primary defect in FHHNC (Rodriguez-Soriano and Vallo 1994). Paracellin-1 is a member of the claudin family which is involved in tight junction formation. From the disease phenotype, it was concluded that paracellin-1 might regulate the paracellular transport of magnesium and calcium ions by contributing to a selective paracellular conductance by building a pore permitting paracellular fluxes of magnesium and calcium down their electrochemical gradients (Simon et al. 1999, Wong and Goodenough 1999).

An unexpected finding was the identification of a trafficking mutation in the γ-subunit of the Na^+K^+ ATPase as the cause of isolated dominant hypomagnesemia (IDH) (Meij et al. 2000). Co-expression with the mutant γ-subunit abolished the correct routing of the entire Na^+K^+ ATPase complex to the plasma membrane. However, another group observed an isolated trafficking defect of the mutant γ-subunit (with normal membrane insertion of α- and β-subunits) (Pu et al. 2002). Their results indicated that a failure of the mutant γ-subunit to modulate the kinetics of the Na^+K^+ ATPase may lead to a decrease in pump activity and to a secondary reduction in transcellular magnesium reabsorption. However, the precise cellular mechanism of decreased magnesium reabsorption remains to be determined as well as the concomitant finding of hypocalciuria in IDH.

The most frequent hereditary tubular disorder affecting renal magnesium handling is the Gitelman variant of Bartter syndrome (GS). This primary salt wasting disorder is caused by mutations in the Na^+Cl^- cotransporter (NCCT) of the DCT (Simon et al. 1996). A conclusive explanation for the hypomagnesemia regularly observed in these patients is still lacking. An increased rate of apoptosis, as shown in rats after chronic thiazide administration (Loffing et al. 1996), might reduce the absorptive surface area of the DCT, and thereby compromise magnesium absorption in GS.

The most recent example of a genetic approach yielding a new molecule involved in epithelial magnesium transport is the characterization of TRPM6 mutations in primary hypomagnesemia with secondary hypocalcemia which allowed the identification of the first component of intestinal magnesium absorption (Schlingmann et al. 2002, Walder et al. 2002).

2. Hypomagnesemia with Secondary Hypocalcemia

Hypomagnesemia with secondary hypocalcemia (HSH) is a rare autosomal-recessive disorder that manifests in early infancy with generalized convulsions or other symptoms of increased neuromuscular excitability as first described in 1968 (Paunier et al. 1968). Failure of early diagnosis or non-compliance with treatment can be fatal or result in permanent neurological damage.

Biochemical abnormalities include extremely low serum magnesium and low serum calcium levels. The mechanism leading to hypocalcemia is still not completely understood. Severe hypomagnesemia results in an impaired synthesis and/or release of PTH (Anast et al. 1972). Consistently, PTH levels in HSH patients were found to be inappropriately low. The hypocalcemia observed in HSH is resistant to treatment with calcium or vitamin D. Relief of

clinical symptoms, normocalcemia, and normalization of PTH levels can only be achieved by administration of high doses of magnesium (SHALEV et al. 1998).

Transport studies in HSH patients pointed to a primary defect in intestinal magnesium absorption (MILLA et al. 1979). However, in some patients an additional renal leak for magnesium was suspected (MATZKIN et al. 1989).

By linkage analysis, a gene locus (*HOMG1*) for HSH had been mapped to Chr 9q22 in 1997 (WALDER et al. 1997). Recently, two independent groups identified *TRPM6* on Chr 9q22 and reported presumable loss of function mutations, mainly truncating mutations, as the underlying cause of HSH (Fig. 3) (SCHLINGMANN et al. 2002, WALDER et al. 2002). *TRPM6* encodes a new member of the transient receptor potential (TRP) family of cation channels. TRPM6 protein is homologous to TRPM7, which was characterized as a calcium and magnesium permeable ion channel regulated by Mg-ATP (NADLER et al. 2001). TRPM6 expression could be demonstrated along the entire small intestine and colon but also in kidney in distal tubule cells. Immunofluorescence studies with an antibody generated against murine TRPM6 could localize TRPM6 to the apical membrane of the DCT (VOETS et al. 2004). The detection of TRPM6 expression in the DCT confirms the hypothesis of an additional role of renal magnesium wasting for the pathogenesis of HSH (COLE and QUAMME 2000). This was also supported by intravenous magnesium loading tests in HSH patients, which disclosed a considerable renal magnesium leak albeit still being hypomagnesemic (WALDER et al. 2002).

The observation that in HSH patients the substitution of high oral doses of magnesium achieves at least subnormal serum magnesium levels supports the theory of two independent intestinal transport systems for magnesium. TRPM6 probably represents a molecular component of active transcellular magnesium transport. An increased intraluminal magnesium concentration (by increased oral intake) enables to compensate for the defect in active transcellular transport by increasing absorption *via* the passive paracellular pathway (Fig. 1*C*, *D*).

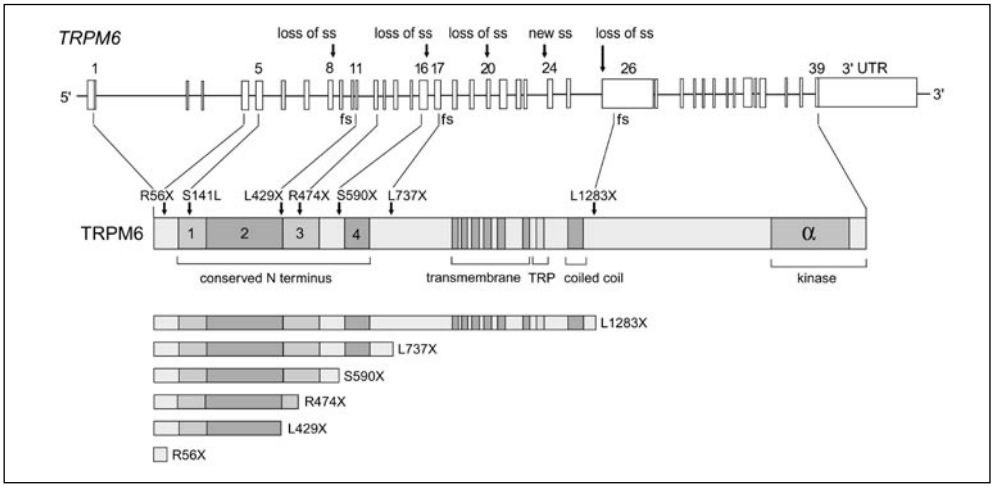

Fig. 3 Schematic model of TRPM6. Genomic organization (*top*) and mRNA structure of TRPM6 (*bottom*). Functional domains are deduced from the TRPM7 model described by NADLER et al. (2001). All mutations reported are indicated (SCHLINGMANN et al. 2002, WALDER et al. 2002). ss: splice site, fs: frameshift, trp: transient receptor potential

3. TRP Cation Channels

The "transient receptor potential" (TRP) protein superfamily comprises more than 20 related cation channels playing important roles in various physiological processes, e.g. phototransduction, sensory physiology and regulation of smooth muscle tone (Montell et al. 2002). *Drosophila* flies carrying the *trp* mutation are inflicted with impaired vision because of the lack of a specific Ca^{2+} influx pathway in the photoreceptors (Hardie et al. 2001). The identification of the *trp* gene product as a cation channel and the rewarding search for TRP homologs in other species led to the discovery of a new family of cation channels.

TRP proteins are allocated to the structural superfamily of six-transmembrane ion channels encompassing most voltage-gated K^+ channels, the cyclic nucleotide-gated channel family, and single transmembrane cassettes of voltage-activated Ca^{2+} and Na^+ channels. Both N- and C-termini of TRP proteins are thought to be located intracellularly, and a putative pore-forming region is bordered by transmembrane domains 5 and 6. Four TRP protein subunits assemble to form a functional channel complex (Hofmann et al. 2002).

TRP proteins can be subdivided into 3 subfamilies: TRPC, TRPV, and TRPM. TRPM proteins display the structural hallmark of exceptionally long intracellular N- and C-termini. The founding member, TRPM1 (melastatin), was detected as a potential tumor suppressor in malignant melanoma cells, probably mediating Ca^{2+} entry (Xu et al. 2001). TRPM3 has also been shown to represent a Ca^{2+} permeable ion channel (Lee et al. 2003). TRPM4 and TRPM5 highlight a novel facet of the functional properties of TRPM proteins in that both proteins give rise to Ca^{2+}-activated cation channels permeable for monovalent cations and mediating cell membrane depolarization (Hofmann et al. 2003, Launay et al. 2002, Nilius et al. 2003). TRPM5 is enriched in taste receptor cells and provides for the receptor potential underlying sweet, amino acid and bitter taste perception (Zhang et al. 2003). A different role in sensory physiology has been ascribed to TRPM8 which is expressed in sensory neurons and prostate carcinoma cells and activated by menthol, icillin and cool temperatures (summarized in Montell et al. 2002).

Three members of the TRPM family, i.e. TRPM2, TRPM6, and TRPM7, are set apart from other known ion channels because they harbor enzyme domains in their respective C-termini and thus represent prototypes of an intriguing new protein family of enzyme-coupled ion channels. TRPM2 is C-terminally fused to an ADP-pyrophosphatase and has found to be activated by one of the products of NAD hydrolysis, ADP-ribose (Perraud et al. 2001). TRPM6 as well as TRPM7 contain protein kinase domains in their C-termini, which bear sequence similarity to elongation factor 2 (EF-2) serine/threonine kinases and other proteins which contain an α-kinase domain (Runnels et al. 2001). Despite the lack of detectable sequence homology to classical eukaryotic protein kinases, the crystal structure of TRPM7 kinase surprisingly revealed striking structural similarity to the catalytic core of eukaryotic protein kinases as well as to metabolic enzymes with ATP-grasp domains (Yamaguchi et al. 2001). TRPM7 is widely expressed, and targeted disruption of the channel gene in cell lines proved to be lethal, underpinning a salient and non-redundant role in cell physiology (Nadler et al. 2001). Interestingly, TRPM7 exhibits significant Mg^{2+} permeation, a rather unusual feature of other cation channels, and is inhibited by cytosolic Mg^{2+} as well as Mg-ATP. A systematic analysis of the permeation properties of TRPM7 revealed that the latter channel has the unique property to conduct a wide range of divalent trace metal ions, some of which with detrimental consequences for the cell upon intoxication (Monteilh-Zoller et al. 2003). In light of its broad

expression pattern and its constitutive activity TRPM7 may provide a general mechanism for the entry of divalent cations into cells. However, recent data suggest that TRPM7 represents a primarily magnesium permeable ion channel required for the cellular uptake of magnesium (Schmitz et al. 2003). The magnesium permeability seems to be modulated by a functional coupling between TRPM7's ion channel and kinase domains indicated by coordinated changes in phosphotransferase activity and ion flow. By the phosphorylation of yet unidentified target proteins, the kinase domain might thus be involved in negative feedback mechanism which inhibits a further uptake of magnesium in the presence of rising intracellular magnesium concentrations (Schmitz et al. 2003).

TRPM6 is closely related to TRPM7 and represents the second TRP protein being fused to a C-terminal α-kinase domain. The TRPM6 gene is composed of 39 exons coding for a total of 2022 amino acid residues. TRPM6-mRNA shows a more restricted expression pattern than TRPM7 with highest levels along the intestine (duodenum, jejunum, ileum, colon) and the DCT of the kidney (Schlingmann et al. 2002). Immunohistochemistry shows a complete colocalization with the sodium-chloride co-transporter NCCT (also serving as a DCT marker) but also with parvalbumin and calbindin-D_{28K}, two cytosolic proteins that putatively act as intracellular (calcium and) magnesium buffers (Voets et al. 2004).

The functional expression of TRPM6 in HEK cells revealed large outwardly rectifying whole cell currents strongly resembling the currents observed for TRPM7 by Nadler and colleagues with a reversal potential near 0 mV (Voets et al. 2004). Permeation characteristics with currents almost exclusively carried by divalent cations with a higher affinity for Mg^{2+} than Ca^{2+} support the role of TRPM6 as the apical Mg^{2+} influx pathway. Furthermore, TRPM6 – in analogy to TRPM7 – exhibits a marked sensitivity to intracellular Mg^{2+}. Thus one might speculate about an inhibition of TRPM6-mediated Mg^{2+} uptake by rising intracellular Mg^{2+} concentrations as a possible mechanism of a regulated intestinal and renal Mg^{2+} (re-)absorption. This inhibition might in part be mediated by intracellular Mg-ATP as shown for TRPM7 by Nadler and colleagues who suggested a possible link to the cellular energy metabolism (Nadler et al. 2001).

In contrast to wildtype-TRPM6, transfection of two TRPM6 mutants found in HSH patients yielded no detectable currents compared to non-transfected controls (Voets et al. 2004). However, both mutants analyzed lead to an early truncation of the TRPM6 protein lacking the pore-forming transmembrane domains. Certainly, the analysis of point mutations will be more helpful in elucidating functional aspects of the TRPM6 ion channel disturbed in HSH. In conclusion, the genetic analysis of HSH patients together with the expression studies and the functional channel characteristics highlight a crucial role of TRPM6 for epithelial Mg^{2+} transport in intestine and kidney. However, considering the tetrameric structure of TRP channels, a participation of other members of the TRP family in the formation of the physiologically active apical Mg^{2+} channel in intestine and kidney cannot be excluded.

In summary, careful clinical observation in combination with molecular genetic analysis considerably enlarged the current understanding of epithelial magnesium transport. It might be expected that the characterization of other disease phenotypes associated with disturbed magnesium handling will lead to the identification of additional proteins involved in magnesium homeostasis. Hopefully, this knowledge will provide starting points for the development of new therapeutic strategies in these rare diseases.

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Dr. Martin Konrad
University Children's Hospital, Inselspital
3010 Bern
Switzerland
Phone: +41 31 6329512
Fax: +41 31 6329420
E-Mail: martin.konrad@insel.ch

Uwe Pörksen:
Was spricht dafür, das Deutsche als Naturwissenschaftssprache zu erhalten?

Vortrag in der Sitzung der Deutschen Akademie der Naturforscher Leopoldina
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Die atemberaubende Entwicklung auf zahlreichen Feldern der Naturwissenschaft und der Medizin hat zu beeindruckenden Ergebnissen, aber ebenso auch zu Ängsten in Teilen der Bevölkerung, insbesondere den dem modernen Wissenschaftsbetrieb ferner stehenden Kreisen geführt. Nicht nur das Unverständnis für die fachlichen Details, sondern auch die immer häufigere Verwendung des Englischen als die *lingua franca* der Naturwissenschaften, erschweren die Propagierung von neuen wissenschaftlichen Erkenntnissen und das Verständnis für den erreichten wissenschaftlichen Fortschritt. Auf der einen Seite belegen Statistiken eindeutig den Vormarsch und die Vorzüge der englischen Sprache als Naturwissenschaftssprache. Andererseits erscheint die von Uwe Pörksen diskutierte Frage: »Was spricht dafür, das Deutsche als Naturwissenschaftssprache zu erhalten?« durchaus als berechtigt. Der Autor unternahm in einem öffentlichen Vortrag vor der Deutschen Akademie der Naturforscher Leopoldina im Dezember 2000 den Versuch, die verschiedenen Argumente mit Augenmaß und unter Aufzeigung der gegensätzlichen Standpunkte zu diskutieren und zu wichten.

Die Wissenschaftsgeschichte beweist: Zweisprachigkeit oder Mehrsprachigkeit war der Normalfall: das Gegenüber von Deutsch und Latein (800–1800), Deutsch und Französisch (1600–1800), Deutsch und Englisch (1960–?) belegt diese Aussage. Nur zwischen 1800 und 1960 war Deutsch die allein vorherrschende Sprache unserer Naturwissenschaft.

Die Dominanz des Englischen in den Naturwissenschaften führt zu einer Verwendung dieser Sprache als einer schmalen Funktionssprache (Englisch II), die verbunden mit der visuellen und mathematischen Zeichensprache als ein rasches internationales Verständigungsmittel fungiert. Unter der aktuellen ökonomisch-technischen Entwicklung und der jüngsten elektronischen Medienrevolution hat dieser Code beachtliche Vorteile. Trotzdem sind der Anwendung von Englisch II bei anspruchsvollen wissenschaftlichen Aufgaben Grenzen gesetzt, da im Ergebnis »blinde Flecken« bleiben und der Selektion von Denkmodellen aufgrund der reduzierten Ausdrucksmöglichkeiten Vorschub geleistet wird. Das Fazit des Germanisten Pörksen kann deshalb nur heißen: »Nur das differenzierte Englisch (Englisch I) und eine differenzierte Ausarbeitung der Naturwissenschaften in der eigenen Muttersprache, entwickelte Ein-, Zwei- und Mehrsprachigkeit dürften der wachsenden Komplexität der Forschungsaufgaben und Erkenntnisprobleme gewachsen sein. Die Naturwissenschaftssprache ist nicht nur Erkenntnisinstrument, sondern ebenso ein öffentliches, ein kulturelles, gesellschaftliches Phänomen.«

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TRPV1 to TRPV4: Four Temperature Sensitive, Multimodal Channels Expressed in Epithelia

Christopher D. Benham (Harlow)

With 2 Figures and 1 Table

Abstract

The past year has provided the first reports on the properties of TRPV3, possibly the final member of the mammalian TRPV family to be described. We now know that TRPV1, 2, 3, and 4 are all heat sensitive ion channels. These channels characteristically have Q_{10} values of > 10 above the thermal threshold, very different from the Q_{10} values of 1.5–2.0 seen in most ion channels. These properties make the channels efficient thermosensors. Cells expressing TRPV1 show similar temperature sensitivity to small capsaicin sensitive nociceptor neurons, consistent with these neurons expressing homomers of TRPV1. A-δ-fibers exhibit properties that may be explained by TRPV2 containing channels, found in large diameter sensory neurons that do not express TRPV1. TRPV3 has a lower temperature threshold and may contribute to warm sensitive channels together with TRPV1. Warm sensation may also be transduced by TRPV4 expressing neurons.

The most studied channel, TRPV1, is also a multimodal channel activated by various chemical ligands. In addition, the temperature threshold of the channel is dependent on its phosphorylation state so that it may be activated at physiological body temperature. Recent exploration of the properties of TRPV4 indicates that this channel also has intracellular chemical ligands. It seems likely that activation by chemical ligands is a general property of this group of TRPV channels allowing them to sub-serve a variety of functions when expressed in cells that are not exposed to fluctuating temperatures.

Zusammenfassung

Im letzten Jahr sind erste Berichte von den Eigenschaften von TRPV3 erschienen, womöglich das letzte Mitglied der TRPV-Familie, das entdeckt wurde. Wie wir jetzt wissen, sind TRPV1, 2, 3 und 4 Ionenkanäle, die alle auf Wärme reagieren. Diese Kanäle haben üblicherweise Q_{10}-Werte von 10 über den normalen thermischen Grenzwert, im Gegensatz zu Werten von 1 – 2 in den meisten anderen Kanälen. Aufgrund dieser Eigenschaft sind diese Kanäle effiziente Thermosensoren. Zellen, die TRPV1 produzieren, haben eine ähnliche Temperatur-Empfindlichkeit wie kleine Capsaicin-empfindliche Nozizeptor-Neuronen. Dies deutet darauf hin, daß diese Neuronen homomere TRPV1-Kanäle produzieren. A-δ-Nerven dagegen haben Eigenschaften, die durch TRPV2 erklärt werden können. Diese Kanäle sind in Sinnesneuronen anzutreffen, die kein TRPV1-Protein produzieren. TRPV3 hat einen niedrigen Temperatur-Grenzwert und könnte zusammen mit TRPV1 zu Wärme-empfindlichen Kanälen beitragen. Wärme könnte auch durch Neuronen transduziert werden, die TRPV4 produzieren.

Der am ausführlichsten untersuchte Kanal, TRPV1, ist ein multimodaler Kanal, der durch verschiedene chemische Liganden aktiviert wird. Außerdem ist sein Temperatur-Grenzwert von seinem Phosphorylierungszustand abhängig, so daß er bei physiologischer Körpertemperatur aktiviert werden könnte. Studien an den Eigenschaften von TRPV4 zeigen, daß dieser Kanal auch intrazelluläre chemische Liganden besitzt. Es ist wahrscheinlich, daß chemische Aktivierung eine allgemeine Eigenschaft aller TRPV-Kanäle ist. So könnten sie eine Reihe von Funktionen in Zellen erfüllen, die keinen Temperaturschwankungen ausgesetzt sind.

1. Introduction TRPV Channels

Temperature sensing is important in all animals. Mammals require precise assessment of body temperature for setting internal thermoregulation, while cold blooded animals need to sense internal body temperature and to sense warm and cool surroundings to regulate their behavior in seeking warming or cooling environments. In addition, all animals depend on the rapid sensation of noxious heat to activate rapid avoidance reflexes.

The TRPV channels are a sub-group of the TRP family of cation channels (Clapham et al. 2001, Gunthorpe et al. 2002). Structurally, these channels share homology with potassium channels. Each protein subunit has six transmembrane domains and like potassium channels the functional channels are likely composed of tetramers (Kedei et al. 2001). Four of these channels (TRPV1-4) are thermosensitive (Benham et al. 2003). Localization studies of the temperature sensitive TRPV family of ion channels reveal expression of these channels in epithelial cells as well as sensory nerve endings. These recent data suggest that epithelial cells and sensory nerve endings may be components of thermosensitive and mechanosensitive signaling units.

2. Activation of TRPV Channels

The expression cloning of the capsaicin sensitive vanilloid receptor (Caterina et al. 1997) paved the way for subsequent work on the molecular basis of the other temperature sensitive TRP channels. TRPV1 is a Ca^{2+} permeable cation channel activated by exogenous vanilloids such as capsaicin, but also by endogenous lipid signaling molecules such as anandamide (Zygmunt et al. 1999) and eicosanoids (Hwang et al. 2000). As suspected, given the co-location of vanilloid sensitivity and noxious heat gated currents in small sensory neurons and the knowledge that capsaicin evokes a "hot" sensation in humans, VR1 or TRPV1 can be activated by mildly noxious heat with a threshold of about 43 °C in mammals (Caterina et al. 1997, Tominaga et al. 1998, Hayes et al. 2000). Rapid temperature jumps show that TRPV1 is activated relatively rapidly with currents reaching a plateau after less than 1 second (Hayes et al. 2000).

TRPV2 was the second family member cloned, and the initial description provided a strong rationale for a role in noxious heat sensation (Caterina et al. 1999). In *in-vivo* experiments, heating the skin beyond 45 °C at which TRPV1 expressing polymodal nociceptors are excited, successively recruits myelinated A-δ-fibers at thresholds of about 46 and 53 °C, the latter having a similar threshold to TRPV2. So far no endogenous or exogenous chemical ligands have been identified for this channel so that the function of this channel in tissues that are not subject to noxious heat remains speculative. A clue may lie in the recent description of mechanosensitive properties of this channel in vascular smooth muscle cells (Muraki et al. 2003).

Further genome analyses lead to the cloning of a third heat sensitive channel, TRPV3, that is mainly expressed in the CNS and in sensory neurons (Smith et al. 2002, Peier et al. 2002, Xu et al. 2002). This channel is activated at lower temperatures than TRPV1 (Tab. 1) and unlike TRPV1 and 2 shows sensitization on heat activation, such that repeated heat challenges evoke larger currents (Benham et al. 2003). This distinctive property provides a direct sensitization mechanism in contrast to the indirect sensitization of TRPV1 as a result of tissue damage and resultant inflammation. Like TRPV2, there are as yet no chemical ligands of this channel, nor has a TRPV3 channel current been described in a native system yet. Interestingly this channel protein appears to form functional heteromers when co-expressed with TRPV1 (Smith et al. 2002). Further work is needed to understand the functional significance of this.

Tab. 1 Activation of TRPV channels by thermal, mechanical (osmotic) and chemical gating

Channel expressed Pseudonyms	TRPV1 VR1	TRPV2 VRL-1	TRPV3 VRL-3	TRPV4 VRL-2, VR-OAC, OTRPC4, trp12
High expression	DRG, TG	DRG, TG	DRG, TG, Skin	TG
TRPV heteromers	1 and 3	not 2 and 1	3 and 1	?
p_{Ca}/p_{Na}	9.6	2.9	12.1 2.6	6.3
Heat threshold (°C)	>43 °C	>53 °C	>31 °C >35 °C >39 °C	>24 °C >33 °C
Effect of prior heating	sensitizes/ desensitizes	strongly sensitizes	strongly sensitizes	desensitizes
Lipid activation	Anandamide HPETE	?	?	5'6' EET
Hypotonicity	no effect	activates	no effect	activates
Ca^{2+}_i	desensitizes	?	desensitises	blocks IC_{50} 0.4 μM

TRPV4 was originally described as an osmosensing channel (Strotmann et al. 2000, Liedtke et al. 2000), although it is now clear that TRPV4 also acts as a thermosensor and may play important roles in homeostatic temperature regulation. This is suggested because its temperature sensitivity spans mammalian body temperatures (Guler et al. 2002, Watanabe et al. 2002). Expression in the hypothalamus in thermosensitive neurons is consistent with a role in central control of body temperature. The functional activation of TRPV4 expressed in vascular endothelial cells (Watanabe et al. 2002) suggests that this channel may have a role in local vascular responses to changes in temperature. The physiological functions of TRPV4 are being more rapidly explored thanks to the availability of chemical ligands and the recent identification of a candidate endogenous ligand, 5'6' epoxyeicosatrienoic acid or 5'6' EET (Watanabe et al. 2003). The activation properties of the four channels are summarized in Table 1.

3. TRPV Channels in Epithelia

3.1 Bladder Urothelium

The urinary bladder is richly innervated with afferent nerves that express TRPV1. A large body of data has suggested that capsaicin activated afferent nerves have important functional roles in detecting irritant stimuli in the bladder wall. The recent description of the expression of TRPV1 in bladder urothelial cells (Birder et al. 2001) has added a new dimension to the mechanism of afferent signal transduction in the bladder (Fig. 1). Functional studies described by Birder et al. (2001) show that vanilloids induce calcium elevation in urothelial cells and the release of nitric oxide and that these responses are absent in TRPV1 –/– cells. Urothelial cell membrane ion channels can directly sense bladder contents and so may provide a sensitive monitor to feed forward to the sensory nerve endings. Differing intracellular modulation of TRPV1 in urothelia

and sensory terminals could allow complex refinement of signals in inflammatory conditions. A further subtlety is the co-expression of TRPV2 in urothelia. The recent demonstration that this channel behaves as a stretch activated channel in vascular smooth muscle (Muraki et al. 2003) immediately suggests a role in sensing bladder wall stretch.

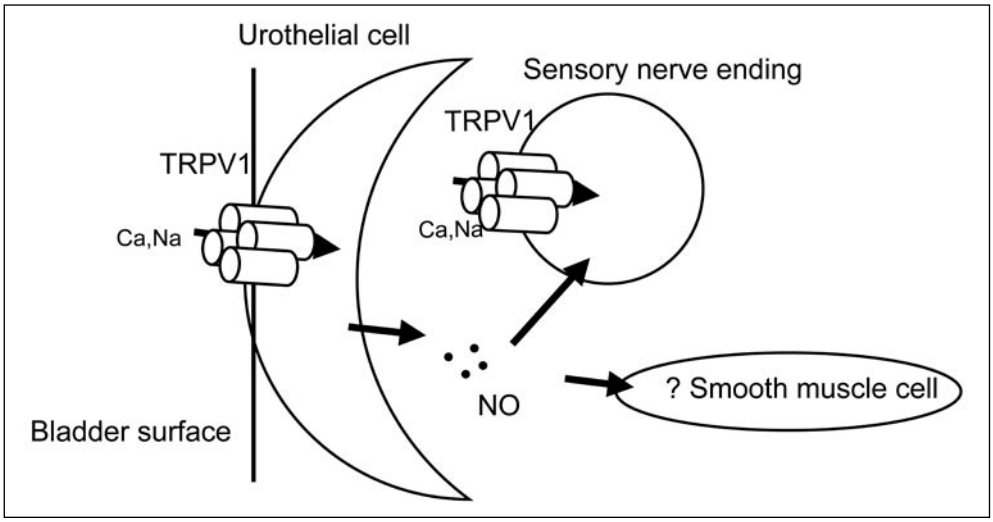

Fig. 1 Urothelial cell and sensory nerve signaling unit in bladder wall

3.2 Skin Keratinocytes

Detailed analysis of the localization of TRPV3 protein using polyclonal antibodies to TRPV3 peptides revealed expression of TRPV3 in basal keratinocytes in the skin epidermis (Peier et al. 2002). Most sensory nerves terminate more deeply in the dermis but some that also express TRPV3 ramify into the epidermis. This has led (Peier et al. 2002) to hypothesize that keratinocytes may form a signaling complex with these sensory nerve endings (Fig. 2). The position of keratinocytes more superficially in the skin makes them better placed as warm transducers, more sensitive to the external environment and less affected by differential heating from the body mediated by changes in blood flow.

There is no direct data to support the signaling complex proposal at present, but the hypothesis neatly explains the absence of purely warm sensitive DRG neurons in isolated culture. A possible transmitter between keratinocyte and nerve ending is ATP, that is known to be released by keratinocytes, signaling through $P2X_3$ purinergic channels. Support for this lies in the observation of deficits in warm sensation in $P2X_3$ knockout mice (Souslova et al. 2000, Cockayne et al. 2000). Further support for these proposals will be provided when functional TRPV3 currents can be recorded from keratinocytes.

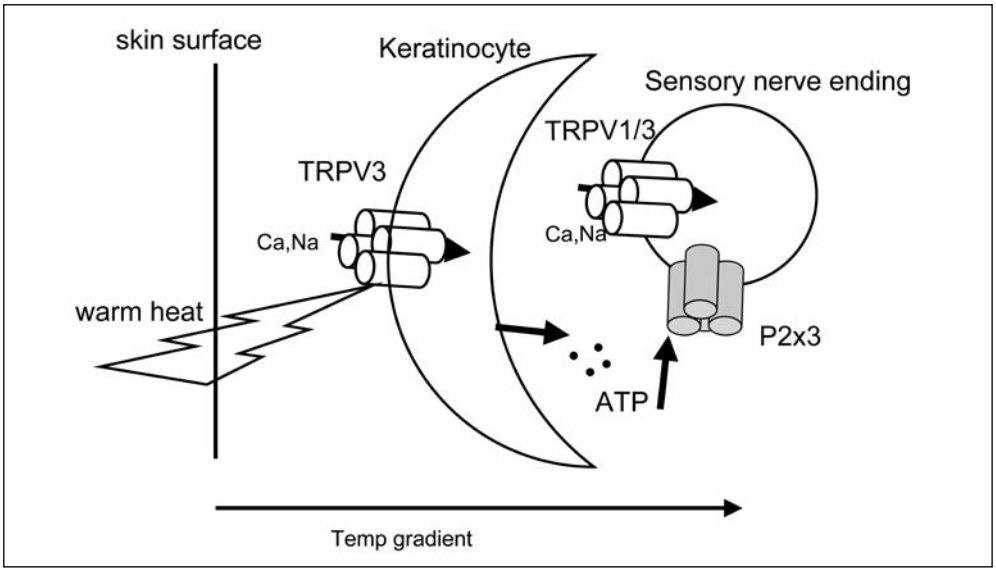

Fig. 2 Putative keratinocyte sensory nerve signaling unit in skin epidermis

4. Conclusions: TRPVs and Endogenous Heat Sensation

We most likely now have the complete set of cation channels with which to fully explore the molecular basis for thermosensation in mammalian cells. To understand their role in physiology we also need to integrate in the polymodal features of their activation properties. A further emerging complexity is the co-localization of these channels in different but adjacent cell types in tissues, illustrating that we must start to explore whole tissue physiology. Data from the study of mice in which TRPV2, TRPV3 or TRPV4 have been deleted are also eagerly awaited.

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Christopher D. Benham, PhD
Neurology & GI Centre of Excellence for Drug Discovery
GlaxoSmithKline Research and Development Ltd
New Frontiers Science Park (North)
Third Avenue
Harlow, Essex, CM19 5AW
UK
E-Mail: Christopher.D.Benham@gsk.com

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The Epithelial Calcium Channel Family

Joost G. J. Hoenderop[1], Bernd Nilius[2], and René J. M. Bindels[1]

With 1 Figure

Abstract

The epithelial calcium channels, TRPV5 and TRPV6, have been extensively studied in epithelial tissues controlling the Ca^{2+} homeostasis and exhibit a range of distinctive properties that distinguish them from other transient receptor potential (TRP) channels. These two novel members of the superfamily of TRP channels were cloned from vitamin D-responsive epithelia kidney, small intestine, and placenta. This review covers the unique properties of these highly Ca^{2+}-selective channels and highlights the implications for the process of transepithelial Ca^{2+} transport.

Zusammenfassung

Die epithelialen Calcium-Kanäle, TRPV5 und TRPV6, sind ausführlich in Epithelgeweben, die die Ca^{2+}-Homöostase kontrollieren, untersucht worden und zeigen eine Reihe besonderer Eigenschaften, die sie von anderen transitorischen Rezeptor-Potential-Kanälen unterscheiden. Diese zwei neuen Mitglieder der Superfamilie der TRP-Kanäle wurden aus den Vitamin-D-empfindlichen Epithelien Niere, Dünndarm und Plazenta geklont. Dieser Übersichtsartikel behandelt die einzigartigen Eigenschaften dieser für Ca^{2+} hoch selektiven Kanäle und hebt die Folgerungen für den Prozeß des transepithelialen Ca^{2+}-Transport hervor.

1. Identification of Two Unique TRP Members, TRPV5 and TRPV6

The maintenance of the body Ca^{2+} balance is of crucial importance for many vital physiological functions including neuronal excitability, muscle contraction and bone formation, and is tightly controlled by a concerted action of kidney, intestine and bone. Ca^{2+} transport occurring in these Ca^{2+}-absorbing tissues is realized by Ca^{2+} entry across the apical plasma membrane; cytosolic diffusion of Ca^{2+} bound to calbindin-D; and extrusion across the basolateral membrane (Fig. 1) (Hoenderop et al. 2002b). In the last decade a plurality of Ca^{2+}-permeable cation channels have been identified as relevant Ca^{2+} influx pathways, the most of which belong to the superfamily of transient receptor potential (TRP) channels (Montell et al. 2002). The molecular nature of the luminal Ca^{2+} influx pathway has been identified by our group and baptized epithelial Ca^{2+} channel 1 (recently renamed as TRPV5) (Hoenderop et al. 1999). Subsequently, a homologous

1 Department of Cell Physiology, Nijmegen Center for Molecular Life Sciences, University Medical Center Nijmegen, P.O. Box 9101, 6500 HB Nijmegen, the Netherlands.

2 Department of Physiology, Campus Gasthuisberg, KU Leuven, Belgium.

isoform was identified from rat intestine that was named calcium transporter 1 (recently renamed as TRPV6) (Peng et al. 1999). TRPV5 and TRPV6 represent two novel highly homologous members within the transient receptor potential (TRP) superfamily (Montell et al. 2002). These channels consist of six transmembrane domains including a putative pore-forming region between transmembrane segments 5 and 6 including large intracellular amino and carboxyl tails. They belong to the TRPV (vanilloid) subfamily, which is one of the three subfamilies (TRPC [canonical], TRPV [vanilloid] and TRPM [melastatin]) comprising this superfamily (Montell et al. 2002). The genes of TRPV5 and TRPV6 are juxtaposed on human chromosome 7q35 and on mouse chromosome 6. The distinct genes comprise 15 exons, encoding proteins of about 730 amino acids. The tissue distribution of TRPV5 and TRPV6 has been studied extensively by Northern blot, RT-PCR analysis and immunohistochemistry (Hoenderop et al. 2002b). In human, both channels are co-expressed in the organs that mediate transcellular Ca^{2+} transport such as duodenum, jejunum, colon, and kidney, but also in exocrine tissues such as pancreas, prostate, mammary gland, sweat gland, and salivary gland.

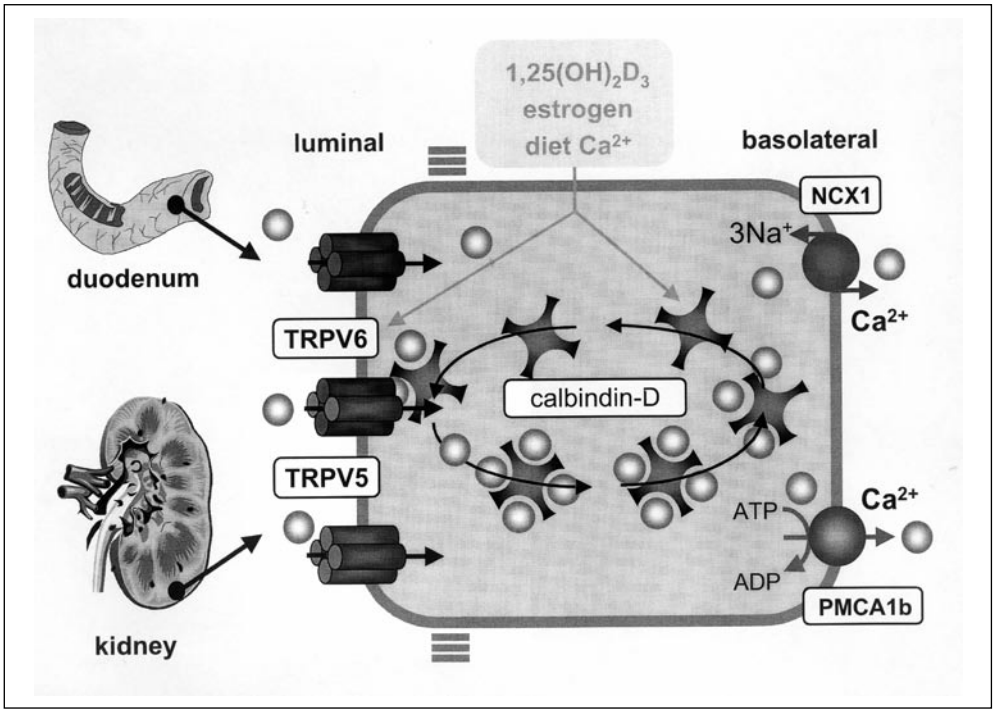

Fig. 1 Transcellular Ca^{2+} transport takes place in the kidney and intestine and is carried out as a three-step process. Following entry of Ca^{2+} through the (hetero)tetrameric epithelial Ca^{2+} channels, TRPV5 and TRPV6, cytosolic Ca^{2+} is buffered by the calbindins. At the basolateral membrane, Ca^{2+} is extruded *via* a Ca^{2+} ATPase (PMCA1b) and a sodium-calcium exchanger (NCX1).

2. Functional Properties of TRPV5 and TRPV6

When expressed in *Xenopus laevis* oocytes and human embryonic kidney (HEK) 293 cells, TRPV5 and TRPV6 mediate a saturable Ca^{2+} uptake and manifest distinct electrophysiological features including hyperpolarization-dependent Ca^{2+} entry and Ca^{2+}-dependent inactivation. TRPV5 or TRPV6-expressing HEK 293 cells displayed large inward currents that are strongly dependent on extracellular Ca^{2+} and reversed at high positive membrane potentials (VENNEKENS et al. 2000). The current-voltage relationship showed prominent inward rectification. Thus, under unstimulated physiological conditions when the membrane potential is typically around -70 mV in the distal part of the nephron, TRPV5 constitutes a substantial Ca^{2+} conductance permitting basal Ca^{2+} influx. The characteristic pore region of TRPV5 and TRPV6 is unique for its high Ca^{2+} selectivity. A single aspartic residue in the pore region at position number 542 (D542) is crucial for Ca^{2+} permeation. This amino acid is completely conserved in TRPV6. Mutation of D542 into an alanine abolishes Ca^{2+} permeation, but does not affect the permeation of monovalent cations (NILIUS et al. 2001). From an electrophysiological point of view TRPV5 and TRPV6 are highly similar in line with the 75 % homology at the amino acid level between these channels. The main sequence differences are located in the N- and C-terminal tails. However, detailed analysis demonstrated three distinctive differences that include kinetics of Ca^{2+}-dependent inactivation and recovery from inactivation, Ba^{2+} conductance and pharmacological block by the potent channel inhibitor ruthenium red. Differences were found in the Ca^{2+}-dependent feedback mechanism, which may reflected mainly the Ca^{2+}-dependent inactivation of currents in response to a hyperpolarizing voltage step. TRPV6 inactivation consists of a fast initial phase followed by a slower phase of inactivation, whereas TRPV5 displays only this slow inactivation behavior. Furthermore, an intriguing difference is the Ba^{2+} selectivity resulting in a significant higher Ba^{2+}/Ca^{2+} current ratio for TRPV5 compared to TRPV6. Importantly, a pharmacological distinction can be made between TRPV5 and TRPV6 with ruthenium red, for which TRPV6 has a 100 fold lower affinity compared to TRPV5.

3. Regulation of TRPV5 and TRPV6

Ca^{2+} (re)absorption is primarily regulated by vitamin D_3 metabolites. Alterations in these regulatory processes are present in many (patho-)physiological states including idiopathic hypercalciuric syndromes, chronic renal failure, aging-related Ca^{2+} malabsorption and vitamin D intoxication. The active hormonal form of vitamin D, 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$), is well established in humans and animal models as having a significant role in the regulation of Ca^{2+} (re)absorption in Ca^{2+}-transporting epithelia. Recent studies indicated that TRPV5 and TRPV6 are tightly controlled by $1,25(OH)_2D_3$ (HOENDEROP et al. 2002a, VAN CROMPHAUT et al. 2001). The first evidence for this vitamin D sensitivity was obtained in *in-vivo* studies in which rats were depleted for vitamin D_3 and subsequently repleted. This was accompanied by normalization of the plasma Ca^{2+} concentration and an increase in the amount of TRPV5 mRNA and protein expression. This observed vitamin D-dependent regulation of TRPV5 and TRPV6 was confirmed using different animal models and cell lines including in vitamin D receptor (VDR) and in 25-hydroxyvitamin D_3-1α-hydroxylase (1α-OHase) knockout mice. Intriguingly, the reduced expression level of renal TRPV5 in the 1α-OHase$^{-/-}$ mice could also be restored by high dietary Ca^{2+} intake and accompanied by normalization of the plasma

Ca^{2+} concentration. Importantly, this effect was observed in the absence of 1α-OHase-activity. In line with the $1,25(OH)_2D_3$-dependent regulation dietary Ca^{2+} controls also the other Ca^{2+} transport proteins. In contrast, this Ca^{2+}-enriched rescue diet reduced the expression of renal TRPV5 in 1α-OHase$^{+/-}$ mice that exhibit normal serum vitamin D and Ca^{2+} levels. It is known that under physiological conditions, plasma Ca^{2+} acts *via* a negative feedback mechanism that eventually leads to suppression of the 1α-OHase-activity that decreases Ca^{2+} reabsorption and expression of Ca^{2+} transport proteins.

4. Molecular Architecture of the TRPV5/6 Channel

The oligomerization of TRPV5 and TRPV6 channels has recently been unraveled (Hoenderop et al. 2003). Cross-linking studies, co-immuno precipitations and molecular mass determination of TRPV5/6 complexes using sucrose gradient sedimentation showed that TRPV5 and TRPV6 form homo- and heterotetrameric channel complexes. As described above TRPV5 and TRPV6 are co-expressed in several tissues, which allow oligomerization of these channels *in vivo*. Heteromeric complex formation has been shown to modify the activity of members of the TRPC family. For TRPC1 and TRPC3 it has been shown that hetero-oligomers of these channels possess distinctive properties than that of either channel alone. Likewise, oligomerization of TRPV5 and TRPV6 might influence the functional properties of the Ca^{2+} channel. As TRPV5 and TRPV6 exhibit different channel kinetics with respect to Ca^{2+}-dependent inactivation, Ba^{2+} selectivity and sensitivity for inhibition by ruthenium, the influence of the heterotetramer composition on channel properties was investigated. Concatemers were constructed consisting of four TRPV5 and TRPV6 subunits configured in a head-to-tail fashion. A different number of TRPV5 and TRPV6 subunits in these concatemers showed that the phenotype resembled the mixed properties of TRPV5 and TRPV6. An increased number of TRPV5 subunits in such a concatemer displayed more TRPV5-like properties, indicating that the stoichiometry of TRPV5/6 heterotetramers influences the channel properties (Hoenderop et al. 2003). Consequently, regulation of the relative expression levels of TRPV5 and TRPV6 may be a mechanism to fine-tune the Ca^{2+} transport kinetics in TRPV5/6-expressing tissues.

The tetrameric organization of TRPV5/6 resembles that of the Shaker potassium channel, which is composed of four tandemly associated homologous domains. The clustering of four subunits is assumed to create an aqueous pore centered at the four-fold symmetry axis. This tetrameric architecture of TRPV5/6 implies that four of the aspartic residues (D542) form a negatively charged ring that functions as a selectivity filter for Ca^{2+} in analogy with voltage-gated Ca^{2+} channels.

5. Conclusion

The new epithelial Ca^{2+} channels, TRPV5 and TRPV6, comprise a unique couple of highly Ca^{2+}-selective channels mainly expressed in epithelial tissues. The molecular properties of these channels have been extensively studied and include a high selectivity for Ca^{2+}, regulation by calciotropic hormones and Ca^{2+}, and operation as homo- and heterotretrameric channel complex.

Acknowledgements

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Dr. René J. M. Bindels
160 Cell Physiology
University Medical Center Nijmegen
P.O. Box 9101
NL-6500 HB Nijmegen
The Netherlands
Phone: +31 24 3614211
Fax: +31 24 3616413
E-Mail: r.bindels@ncmls.kun.nl

Parasitismus, Commensalism, Symbiosis – Common Themes, Different Outcome

Leopoldina-Symposium,
gemeinsam veranstaltet von der Deutschen Akademie der Naturforscher Leopoldina in Kooperation mit dem Forschungszentrum für Infektionskrankheiten der Universität Würzburg und der Deutschen Gesellschaft für Hygiene und Mikrobiologie (DGHM) vom 24. bis 26. Juli 2002 in Würzburg

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Herausgegeben von Roy Gross, Matthias Frosch, Werner Goebel, Jörg Hacker, Ute Hentschel, Jürgen Kreft, Markus Riederer, Michael Steinert und Volker ter Meulen (Würzburg)
(2004, 198 Seiten, 23 Abbildungen, 19 Tabellen, 21,80 Euro,
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Die Charakterisierung der komplexen Interaktionen zwischen pathogenen, kommensalen und symbiontischen Bakterien und ihren Wirtsorganismen hat durch die Entwicklung der modernen molekularbiologischen Techniken wie Polymerasekettenreaktion, Genomsequenzierung und *In-situ*-Hybridisierung (»Genomics«) große Fortschritte verzeichnen können. Die neuen Erkenntnisse über die Genomorganisation der Mikroorganismen erlauben nun auch bei bisher nicht kultivierbaren Bakterien interessante Einblicke in deren Biologie und Phylogenie. Vor allem lassen sich die verschiedenen Strategien der Wechselwirkung von Mikroorganismen mit ihren Wirten aus der Pflanzen- oder Tierwelt aufklären. Darüber hinaus werden in den Beiträgen die Evolutionsvorgänge im Reich der Mikroorganismen im Lichte der Genomik-Ära betrachtet. Die genetische Variabilität als Grundlage der Evolution beruht in Prokaryoten im wesentlichen auf horizontalem Gentransfer, Umlagerungen der DNA und auf Punktmutationen. Die Beiträge reichen von der Analyse von Selektionsprozessen bei bioaktiven Molekülen bis hin zu pathogenetischen Prozessen bei Interaktionen von Mikroben mit Pflanzen, Vertebraten und Nichtvertebraten. Dabei werden an ausgewählten Beispielen Prozesse der Symbiose, des Kommensalismus und der Pathogenese beschrieben. Der aktuelle Wissensstand zu Fragen der bakteriellen Pathogenität und Symbiose wird aus der Sicht verschiedener Wissenschaften (Evolutionsbiologie, Mikrobiologie, Infektionsbiologie, Biochemie, Biophysik, Molekularbiologie, Genetik, Zoologie und Botanik) an einer Reihe von Beispielen diskutiert.

In Kommission bei Wissenschaftliche Verlagsgesellschaft mbH Stuttgart

Nova Acta Leopoldina NF *89*, Nr. 336, 103–106 (2004)

Phosphorylation on Tyr161 by Src Kinase and Dephosphorylation by Protein Tyrosine Phosphatase 1B Is a Candidate Mechanism for Regulation of the TRPV6 (CaT1) Calcium Channel

Thomas Magg[1], Irene Schulz[2], and Hans Werner Hofer[1]

With 3 Figures

Abstract

The Ca^{2+}-selective calcium transport protein TRPV6 is phosphorylated on tyrosine when expressed together with Src tyrosine kinase in yeast or HEK293 cells. TRPV6 interacts with the protein tyrosine phosphatase PTP1B in a yeast two-hybrid system and interaction is enhanced by co-expression of Src kinase. Interaction is mediated by the catalytic moiety of the phosphatase located within the N-terminal part of the phosphatase. We showed by site-directed mutagenesis of several candidate tyrosine residues that phosphorylation occurs in the region of the two adjacent tyrosine residues 161 and 162.

Zusammenfassung

Das Ca^{2+}-selektive Calcium-Transportprotein TRPV6 wird an Tyrosin phosphoryliert, wenn es zusammen mit Src-Tyrosinkinase entweder in Hefezellen oder in HEK293-Zellen exprimiert wird. Im Hefe-2-Hybrid-System interagiert TRPV6 mit der Tyrosinphosphatase PTP1B. Die Interaktion wird durch Koexpression von Src-Kinase verstärkt. Die Interaktion wird durch den N-terminal gelegenen katalytischen Abschnitt der Phosphatase vermittelt. Durch gezielte Mutation von Tyrosinresten wurde gezeigt, daß die Phosphorylierungsstelle im Bereich der benachbarten Tyrosinreste 161 und 162 liegt.

Observations that Ca^{2+}-release-activated Ca^{2+} (CRAC) influx into certain cells is attenuated by inhibitors of tyrosine kinases (among others tyrphostin and genistein, Pfeiffer et al. 1995) and enhanced by inhibitors of tyrosine phosphatases (see contribution by Hsu et al.) were a first trigger of our study. It was further guided by reports that the Ca^{2+}-selective *Ca*lcium *T*ransport Protein CaT1 (TRPV6, Peng et al. 2000) exhibited properties of a CRAC channel (Hoth and Penner 1992) when expressed in CHO-K1 (Yue et al. 2001) or HEK293 cells (Schindl et al. 2002, see also the abstract by Hsu et al.). TRPV6 is a member of the "Transient *R*eceptor *P*otential" (TRP) family of cation channels which are characterized by a variable number of ankyrin domains within the intracellular domain located at the N-terminal side of transmembrane domains forming the ion channels.

We here report that the N-terminal intracellular domain of TRPV6 (amino acids 1–326) from rat (the clone was kindly provided by M. Hediger) is phosphorylated on tyrosine when

1 Department of Biology, University of Konstanz, 78547 Konstanz, Germany.
2 Institute of Physiology, University of the Saarland, 66421 Homburg/Saar, Germany.

expressed in *S. cerevisiae* cells in the presence of co-expressed Src kinase. No tyrosine phosphorylation occurred in the absence of co-expressed tyrosine kinase. Tyrosine phosphorylation was also observed when the rTRPV6(1–326) domain was expressed in HEK293 cells and was strongly stimulated by the co-expression of Src suggesting that this tyrosine kinase is a candidate enzyme that may be involved in secondary modification of the protein (Fig. 1).

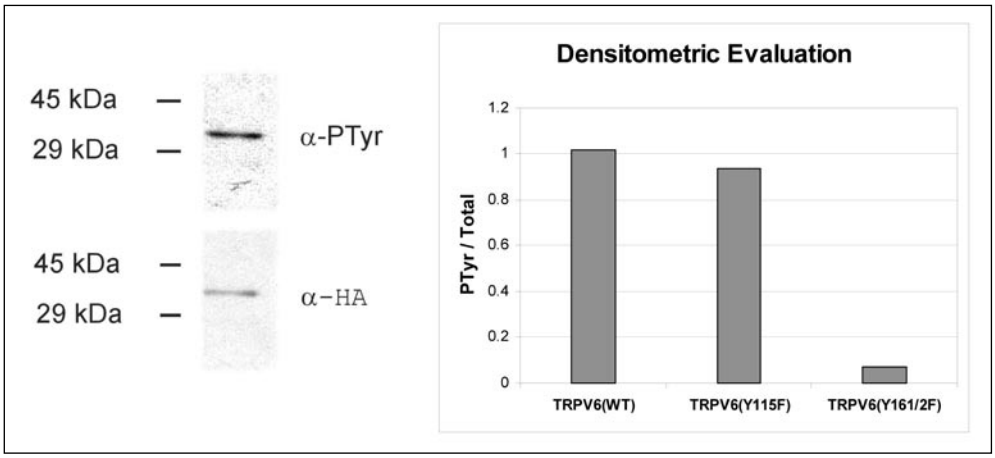

Fig. 1 The N-terminal intracellular domain of TRPV6 is phosphorylated when transfected into HEK293 cells. TRPV6(1–326) was cloned into the pCDNA3 vector in N-terminal fusion with a HA epitope and co-expressed with Src kinase. (*A*) Immunoblots of immunoprecipitated TRPV6(1–326) protein probed with anti-phosphotyrosine (*upper panel*) and anti-HA (*lower panel*). (*B*) Blots were made in analogous manner of wild type (wt) TRPV6(1–326) and mutants in which tyrosines 115 and 161/2 were replaced by phenylalanine (Y115F and Y161/2F). The areas on blots probed with anti-phosphotyrosine and anti-HA were referred to each other and plotted.

A yeast two-hybrid interaction assay (Fig. 2), based on the β-galactosidase reporter system, was employed in the search for phosphatases that are able to reverse phosphorylation of rTRPV6(1-326). Co-expression of full-length human protein tyrosine phosphatase 1B (PTP1B) fused to the DNA-binding domain of LexA with rTRPV6(1–326) fused to the B42 activation domain did not lead to any activation of the reporter system. However, rapid activation of the reporter was observed when Src kinase was also co-expressed in the yeast cells. This was not the case when either PTP1B or rTRPV6(1–326) was absent from the yeast colonies suggesting that the presence of phosphorylated tyrosine was in fact a prerequisite of protein-protein interactions between the phosphatase and the intracellular domain of rTRPV6.

Full length PTP1B is mainly located on membranes of the endoplasmic reticulum but proteolytic cleavage of its hydrophobic C-terminus generates a soluble form of the phosphatase (Haj et al. 2002). A construct devoid of the hydrophobic region exhibited weak interaction with the N-terminal moiety of theTRPV6 protein even in the absence of Src, but interaction was again enhanced by co-expression of the tyrosine kinase (Fig. 2, rows 3 and 4). Thus, the hydrophobic domain of the tyrosine phosphatase was not required for interaction with TRPV6(1–326). Therefore, following release from the endoplasmic reticulum, PTP1B would be also able to interact with TRPV6 in the plasma membrane.

Fig. 2 Two-Hybrid assays for interactions between protein tyrosine phosphatase 1B (PTP1B) and TRPV6(1–326) in *S. cerevisiae*. Positive reaction is indicated by blue colonies. *Row 1*: PTP1B(wt) and TRPV6(1–326); *row 2*: PTP1B(wt) and TRPV6(1–326) plus Src kinase; *row 3*: PTP1B(ΔTM), devoid of its C-terminal part, and TRPV6(1–326) minus Src; *row 4*: PTP1B(ΔTM) and TRPV6(1–326) plus Src kinase.

There are three tyrosine residues in rTRPV6(1–326) that may serve as potential phosphorylation sites of Src kinases. Two of these residues (Y115 and Y161) are conserved in the protein from various sources. The third (Y162) is replaced by a conservative exchange to phenylalanine in the human protein (Fig. 3, lower panel). Mutation of the corresponding tyrosine residues and co-expression of the mutants with Src in HEK293 cells revealed that the rTRPV6(1–326)(Y115F) mutant still underwent phosphorylation on tyrosine, whereas a rTRPV6(1–326)(Y161F,Y162F) mutant no longer did, suggesting that one or two tyrosine residue(s) positioned at the N-side of the third out of five ankyrin domains (Fig. 3, upper panel) of rTRPV6 comprising amino acids 162–191 are targets for reversible tyrosine phosphorylation. Since the tyrosine equivalent to Y161 in TRPV6 is highly conserved in TRP proteins we suspect it to be an ideal target for phosphorylation-dependent modulation of the functions of this family of channel proteins.

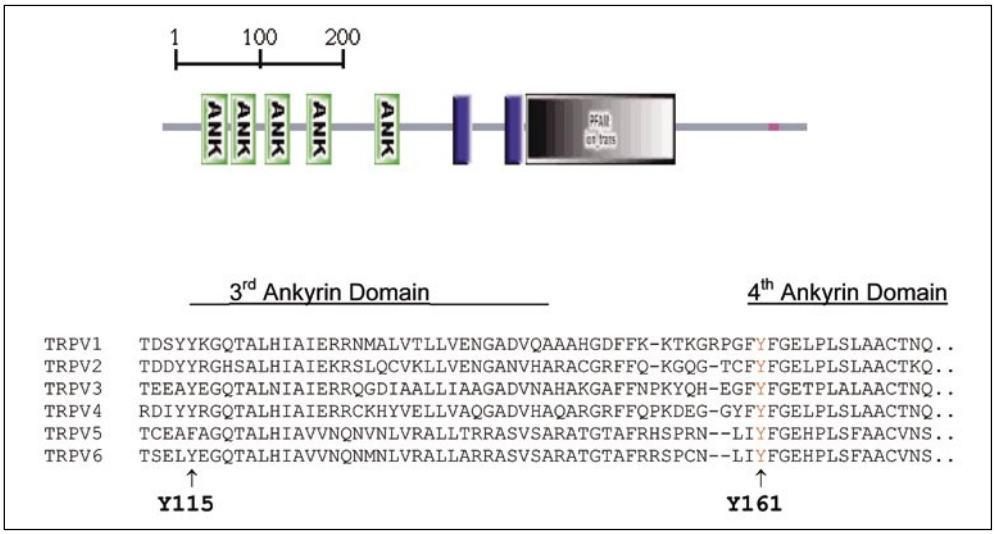

Fig. 3 *Upper panel*: Schematic representation of the domain structure of TRPV6 as evaluated by SMART (Schultz et al. 1998). *Lower panel*: Comparison of the amino acid sequences of human TRPV proteins in the area of the 3rd and 4th ankyrin domains of TRPV6. The positions of Tyr115 and Tyr161 of TRPV6 are marked. Tyr161 is phosphorylated by Src kinase.

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Prof. Dr. Werner Hofer
Universität Konstanz
Fakultät für Biologie
Universitätsstraße 10
78464 Konstanz
Germany
Phone: +49 75 31884028
Fax: +49 75 31882903
E-Mail: werner.hofer@uni-konstanz.de

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Tyrosine Phosphatase PTP1B Modulates Ca^{2+}-Release-activated Ca^{2+}-(CRAC) Influx

Shyuefang Hsu[1], Andreas Schmid[1], Lutz Sternfeld[1], Ines Anderie[1], Anna Latas[1], Elmar Krause[1], Hans Werner Hofer[2], and Irene Schulz[1]

With 4 Figures

Abstract

Depletion of inositol 1,4,5-trisphosphate (IP3)-sensitive calcium stores leads to "capacitative" calcium influx into pancreatic acinar cells (Putney 1986). In mast cells (Hoth and Penner 1992) and different other cell types (Parekh and Penner 1997, Putney et al. 2001) these "Ca^{2+} release-activated Ca^{2+} channels" (CRAC) have been electrophysiologically characterized. However, the molecular identity of these Ca^{2+} channels has remained largely unknown and the mechanism of CRAC-channel activation is also not yet completely understood. Since evidence suggests that CRAC-channel activation and -inactivation can be modulated by both protein kinases and protein phosphatases (Pfeiffer et al. 1995, Rosado et al. 2000, Parekh and Penner 1995) we have studied modulation of "Ca^{2+} release-activated Ca^{2+} influx" by tyrosine phosphatase in the pancreatic acinar cell line AR42J and in HEK293 cells. Inhibition of tyrosine phosphatases by bis-N,N-dimethyl-hydroxamido hydrooxovanadate (DMHV) leads to an increase in Ca^{2+}-release-activated Ca^{2+} entry into these cells. This Ca^{2+} influx can be completely blocked in the presence of 2-aminoethyl diphenyl borate (2-APB), an inhibitor of store-operated Ca^{2+} influx. Furthermore, overexpression of the human wild type tyrosine phosphatase PTP1B in HEK293 cells leads to inhibition of CRAC influx. Transfection with the substrate trapping mutant of PTP1B (D181A) on the other hand moderately increases Ca^{2+} influx. It also decreases enzymatic activity of PTP1B as compared to non-transfected cells. Our data therefore suggest that CRAC-influx is modulated by tyrosine phosphorylation and dephosphorylation which involves the tyrosine phosphatase PTP1B.

Several members of the Trp-family of cation channels, which are related to the *Drosophila* transient receptor potential (trp) gene product (Harteneck et al. 2000, Montell 2001, Montell et al. 2002b) have been considered to be candidates for the CRAC-channel (Zitt et al. 1996, Zhu et al. 1996, Philipp et al. 1996, 1998). Recently, the epithelial Ca^{2+} transporter (CaT1), now termed TRPV6 (Montell et al. 2002a,b) has been implicated in Ca^{2+} release activated Ca^{2+} influx (Yue et al. 2001, Schindl et al. 2002).

Rat TRPV6, transfected into HEK293 cells, enhances Ca^{2+} release activated Ca^{2+} influx. The blocker of endogenous CRAC-channels 2-APB inhibits store-operated Ca^{2+} influx in control but not in TRPV6-transfected cells. Therefore, TRPV6 is unlikely to be the CRAC-channel itself. However, since overexpression of TRPV6 induces properties of the CRAC-channel, TRPV6 either interacts with the endogenous CRAC-channel to induce store-operated Ca^{2+} influx even in the presence of 2-APB, or it is a CRAC-channel without being inhibited by 2-APB in an overexpressed system.

Zusammenfassung

Die Entleerung von Inositol 1,4,5-trisphosphat-sensitiven Calcium-Speichern führt zu einem „kapazitativen" Ca^{2+}-Einstrom in Pankreas-Azinuszellen (Putney 1986). In Mast-Zellen (Hoth und Penner 1992) und vielen anderen Zelltypen (Parekh und Penner 1997, Putney et al. 2001) ist dieser „Ca^{2+} release-activated" Ca^{2+} channel (CRAC) elektrophysiologisch charakterisiert worden. Die molekulare Identität diese Kanals ist jedoch noch nicht bekannt, und der Mechanismus der Kanal-Aktivierung ist auch noch nicht verstanden worden. Da es Hinweise dafür gibt, daß CRAC-Aktivierung und -Inaktivierung durch Proteinkinasen und Proteinphosphatasen moduliert werden kann (Pfeiffer et al. 1995, Rosado et al.2000, Parekh und Penner 1995) haben wir die Modulation des „Ca^{2+} release"-aktivierten Ca^{2+}-Einstromes durch Tyrosinphosphatasen in der Pankreaszell-Linie AR42J und in HEK293-Zellen untersucht.

1 Institute of Physiology, Saarland University, Faculty of Medicine, 66421 Homburg/Saar, Germany.
2 Department of Biology, University of Konstanz, 78547 Konstanz, Germany

Hemmung der Tyrosinphosphatasen durch N,N-dimethyl-hydroxoamido-hydroxooxovanadat (DMHV) führte zu einem Anstieg des „Ca^{2+} release"-aktivierten Ca^{2+}-Einstromes in diese Zellen. Dieser Ca^{2+}-Einstrom konnte vollständig durch 2-Aminoethyl-diphenyl-borat (2-APB), einen Hemmer des „speichergesteuerten" bzw. „Ca^{2+} release"-aktivierten Ca^{2+}-Kanals, gehemmt werden. Weiterhin führte die Überexpression der humanen Wild-Typ Tyrosin-Phosphatase PTP1B in HEK293-Zellen zu einer Hemmung des Ca^{2+}-Einstromes. Transfektion mit der Substrat-bindenden Mutante PTP1B(D181A) erhöhte etwas den Ca^{2+}-Einstrom und hemmte die endogene PTP1B-Aktivität im Vergleich zu nicht transfizierten Zellen. Unsere Daten weisen deshalb darauf hin, daß der CRAC-Einstrom durch Tyrosin-Phosphorylierung und -Dephosphorylierung, die die Tyrosinphosphatase PTP1B involviert, moduliert werden kann.

Einige Mitglieder der TRP-Familie der Kationen-Kanäle, die mit dem „Drosophila transient receptor potential"(trp)-Genprodukt verwandt sind (Harteneck et al. 2000, Montell 2001, Montell et al. 2002b) wurden als Kandidaten für den CRAC-Kanal vorgeschlagen (Zitt et al. 1996, Zhu et al. 1996, Philipp et al. 1996, 1998). Vor kurzem wurde der epitheliale Ca^{2+}-Transporter (CaT1), jetzt TRPV6 genannt (Montell et al. 2002a,b), mit „Ca^{2+} release"-aktiviertem Ca^{2+}-Einstrom in Zusammenhang gebracht (Yue et al. 2001, Schindl et al. 2002).

Ratten-TRPV6, das in HEK293 Zellen transfiziert wurde, erhöhte den „Ca^{2+} release"-aktivierten Ca^{2+}-Einstrom. Der Blocker endogener CRAC-Kanäle, 2-APB, hemmte diesen Ca^{2+}-Einstrom in Kontroll-, aber nicht in TRPV6-transfizierten Zellen.

Wir schließen aus diesen Untersuchungen, daß TRPV6 Eigenschaften des endogenen CRAC-Kanals zeigt, aber nicht mit ihm identisch ist.

1. Introduction

Depletion of inositol 1, 4, 5-trisphosphate (IP_3)-sensitive calcium stores leads to "capacitative" calcium influx into pancreatic acinar cells (Putney 1986). In mast cells (Hoth and Penner 1992) and different other cell types (Parekh and Penner 1997) these "$\underline{C}a^{2+}$ release-$\underline{a}$ctivated $\underline{C}a^{2+}$ channels" (CRAC) have been electrophysiologically characterized. However, the molecular identity of these Ca^{2+} channels has remained largely unknown, and the mechanism of CRAC channel activation is also not yet completely understood.

Several members of the Trp family of cation channels, which are related to the *Drosophila* transient receptor potential (trp) gene product (Harteneck et al. 2000, Montell et al. 2002a) have been considered to be candidates for the CRAC-channel. Recently the epithelial Ca^{2+} transporter (CaT1), now termed TRPV6 has been implicated in Ca^{2+} release-activated Ca^{2+} influx (Yue et al. 2001, Schindl et al. 2002).

Since evidence suggests that CRAC channel activation and inactivation can be modulated by both protein kinases and protein phosphatases (Parekh and Penner 1995, Pfeiffer et al. 1995) we have studied modulation of "Ca^{2+} release-activated Ca^{2+} influx" by tyrosine phosphatase in the pancreatic acinar cell line AR42J and in HEK293 cells. Inhibition of tyrosine phosphatases by bis-N,N-dimethyl-hydroxamido hydrooxovanadate (DMHV) leads to an increase in Ca^{2+} release-activated Ca^{2+} entry into these cells. This Ca^{2+} influx can be completely blocked in the presence of 2-aminoethyl diphenyl borate (2-APB), an inhibitor of store-operated Ca^{2+} influx (Ma et al. 2000). Furthermore, overexpression of the human wild type tyrosine phosphatase PTP1B in HEK293 cells leads to inhibition of CRAC-influx. Transfection with the substrate trapping mutant of PTP1B (D181A) on the other hand moderately increases Ca^{2+} influx and decreases enzymatic activity of PTP1B as compared to non-transfected cells. Our data therefore suggest that CRAC-influx is modulated by tyrosine phosphorylation and dephosphorylation which involves the tyrosine phosphatase PTP1B (Hsu et al. 2003).

Rat TRPV6, transfected into HEK293 cells, enhances Ca^{2+} release-activated Ca^{2+} influx. The blocker of endogenous CRAC channels 2-APB inhibits store-operated Ca^{2+} influx in control but not in TRPV6-transfected cells. It therefore seems to be unlikely that the endogenous CRAC channel is identical to TRPV6.

However, in a TRPV6 overexpressed system, it is possible that TRPV6 interacts with the endogenous CRAC channel to enhance Ca^{2+} release-activated Ca^{2+} influx. Our recent data suggest that tyrosine phoshorylation of TRPV6 increases Ca^{2+} release-activated Ca^{2+} influx in TRPV6-transfected cells (Sternfeld et al. 2004).

2. Cell-culture

AR42J cells were purchased from ATCC (CRL-1492) and maintained in DMEM/Ham's F-12 (1:1) medium (PAA laboratories, Germany) supplemented with 2 mM L-glutamine and 10 % FCS. 2 to 3 d prior to experiments, AR42J cells were induced to differentiate using 100 nM dexamethasone. Approximately 2 to 3 h before calcium measurements, cells were removed from the culture flask using accutase (Innovative Cell Technologies, CA) and replated onto cover glasses. HEK 293 cells were also cultured in DMEM/ Ham's F-12 (1:1) medium with 10 % FCS and 2 mM L-glutamine. 24 h prior to experiments, HEK 293 cells were plated onto cover slides at a confluency of around 75 % and allowed to adhere for 12–24 h.

3. Transfection of HEK 293 Cells

Cells were treated overnight with either wild type PTP1B (accession no M31723), constructed by PCR using human wild type PTP1B in pLexA (Clontech) as template or the human trapping hPTP1B (D181A) in pcDNA3 vector (Invitrogen) using LT1 reagent (Panvera, Madison WI). This procedure resulted in a transfection efficiency of > 80 % of the cells when checked with a construct of the fluorescent CFP-PTP1B fusion protein.

4. Fluorescence Imaging

For measurement of Ca^{2+} fluxes by fura2 fluorescence imaging (T.I.L.L. Photonics) HEK293 cells grown on glass coverslips in media at 37 °C, 95 % O_2, 5 % CO_2, (100 % moisturized) were transfected with pTracer-CMV2 (Invitrogen). The pTracer-CMV2 vector permits the translation from a single bicistronic mRNA of both the gene of interest (rTRPV6, received from Matthias Hediger/Boston) and the gene of the green fluorescence protein (GFP). 18–24 h after transfection, the cells were loaded with the membrane permeating fluorescent Ca^{2+} indicator fura2-AM (1 µM) for 15 min at room temperature. Fluorescence measurements were performed with an inverted microscope (Axiovert 135, Zeiss) equipped with an oil-immersion 40× objective.

Transfected cells were visually identified by their green fluorescence. GFP-negative cells from the same image were used as controls. In separate experiments cells transfected with the pTracer-CMV2-vector without TRPV6 were used as control cells. The free Ca^{2+}-concentration in the cytosol of the cell ($[Ca^{2+}]_{cyt}$) was determined by the ratio of fura2- fluorescence intensities collected at 510 nm at excitation wavelengths of 340 and 380 nm, respectively. Calibration and calculation of $[Ca^{2+}]_{cyt}$ was carried out as described by Grynkiewicz et al. (1985).

5. Confocal Microscopy

For confocal microscopy, AR42J and HEK 293 cells growing on cover glasses were loaded with 4 μM Fluo-3/AM or Fluo-4/AM at 37 °C for 30 – 40 min. Then, the dye-AM-ester was washed off with a standard bath solution containing (in mM): 140 NaCl, 4.7 KCl, 1.3 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, 10 glucose, pH 7.4. During calcium measurements continuous superfusion was used. Fluorescence images (excitation: 488 nm; emission: > 515 nm) were taken with a confocal laser scanning microscope (MRC-1024; Bio-Rad) every 3–10 s. Fluorescence signals from several cells and from several experiments were averaged and presented as mean ± SE.

6. Measurement of Tyrosine Phosphatase Activity in Cell Homogenates

To determine the activity of tyrosine phosphatase, cultured cells were scrapped into ice cold "homogenization buffer" (in mM: mannitol 280, Hepes 5, KCl 10, $MgCl_2$ 1, benzamidine 1, leupeptin 0.2; supplemented with trypsin inhibitor 20 μg/ml, PMSF 1 μM, 0.25 % TritonX-100, pH 7) and then broken by sonication. PTP1B activity was assayed using reduced, carboxymethylated, and maleylated lysozyme (RCML) as substrate. RCML had been phosphorylated with [γ-^{32}P]ATP in the presence of type II protein tyrosine kinase purified from porcine spleen (Batzer et al. 1990) following the procedure described by Tonks et al. (1988).

7. Results and Discussion

7.1 The Effect of DMHV on Store-operated Ca^{2+} Influx in AR42J Cells

Stimulation of cultured pancreatic AR42J cells with the secretagogue bombesin (10 nM) caused a rapid rise in the cytosolic calcium concentration followed by a slow decline to values close to the resting calcium concentration (Fig. 1*A*). Pre-incubation of the cells with 10 μM DMHV for 5 min changed this pattern and produced a prolonged calcium signal in the presence of extracellular calcium (Fig. 1*B*). When cells were stimulated with bombesin in the absence of extracellular calcium only a short calcium spike could be detected due to Ca^{2+} release from intracellular stores. Subsequently the intracellular calcium concentration rapidly decayed to values slightly lower than under resting condition before agonist application (Fig. 1*C*). In these store-depleted cells addition of calcium to the bath solution led to calcium re-entry. The calcium re-entry trace showed a biphasic behavior, with a rapid rise and a slow decline. This changed when cells were pre-incubated with 10 μM DMHV. Then calcium re-entry into store-depleted cells remained high (Fig. 1*D*).

To decide if the effect of DMHV on calcium entry involves a process upstream or downstream from calcium store depletion, AR42J cells were stimulated with thapsigargin which depletes calcium stores without receptor activation. Treatment of AR42J cells with 1 μM thapsigargin in the presence of extracellular calcium caused a rise in the cytosolic calcium concentration which slowly declined to values close to resting calcium concentration within 10 min. When cells were pre-treated with DMHV, again a prolonged calcium signal could be observed. Furthermore, in thapsigargin-stimulated cells which had almost recovered resting calcium

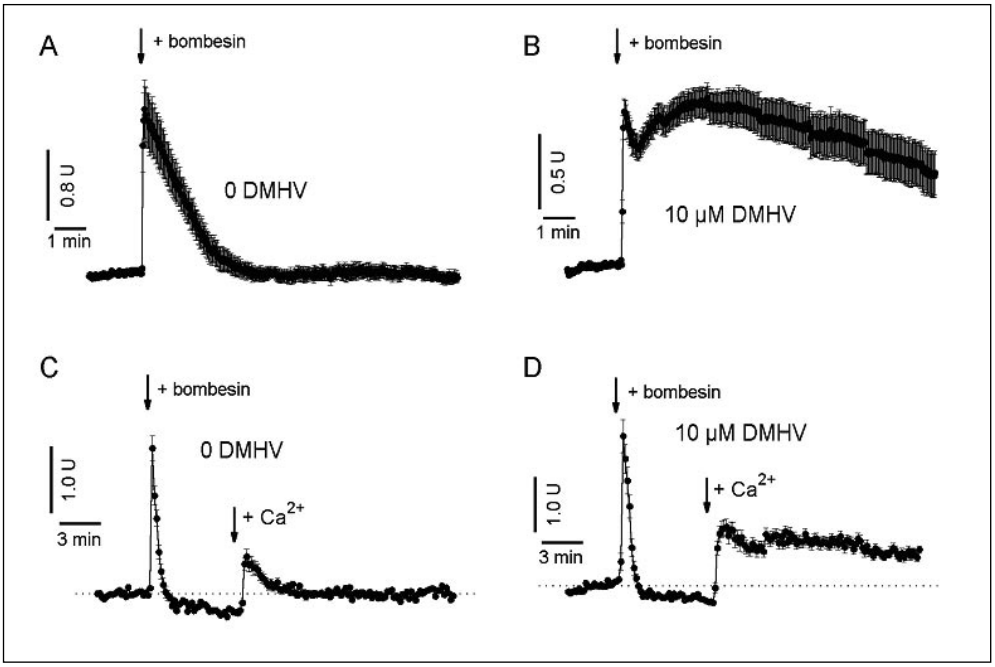

Fig. 1 Effect of DMHV on bombesin-triggered calcium signals in AR42J cells. Stimulation of AR42J cells with 10 nM bombesin in the presence of extracellular calcium causes a rapid rise in the cytosolic calcium concentration. In the absence of DMHV the cytosolic calcium signal recovers close to baseline within 300 s after hormone application (*A*). When cells are pre-incubated with 10 µM DMHV for 5 min there is a prolonged rise in the cytosolic calcium signal (*B*). When calcium is omitted from the bath solution, stimulation with 10 nM bombesin causes a transient cytosolic calcium signal which rapidly declines below the pre-stimulation resting levels, both in the absence (*C*) and presence (*D*) of DMHV. Addition of extracellular calcium to the store-depleted cells causes calcium re-entry, which in the presence of DMHV produces enhanced and prolonged calcium signals.

concentrations, a second rise in the cytosolic calcium concentration could be observed after addition of DMHV (not shown). These data indicate that DMHV exerted its effect on a regulatory mechanism downstream to pool depletion (Hsu et al. 2003).

7.2 The Effect of DMHV on Store-operated Ca^{2+} Influx in HEK 293 Cells

The effect of DMHV on store-operated calcium influx was also investigated in HEK 293 cells. We used this cell line for further experiments since HEK 293 cells, in contrast to AR42J cells, can easily be transfected with vectors containing the DNA for the tyrosine phosphatase PTP1B or its mutant. All basic findings concerning the effect of DMHV on thapsigargin-induced calcium signaling in AR42J cells could be reproduced in experiments on HEK 293 cells.

As shown in Figure 2*A* and *B*, 2-APB at a concentration of 100 µM rapidly blocked the DMHV-enhanced store-operated calcium influx into the cells. Similar data were obtained with 20 µM 2-APB. Furthermore, DMHV failed to induce any augmentation of thapsigargin-induced capacitative calcium entry in the presence of 2-APB (Fig. 2*C*). These data suggest that both DMHV and 2-APB exert antagonistic effects on the same calcium entry mechanism.

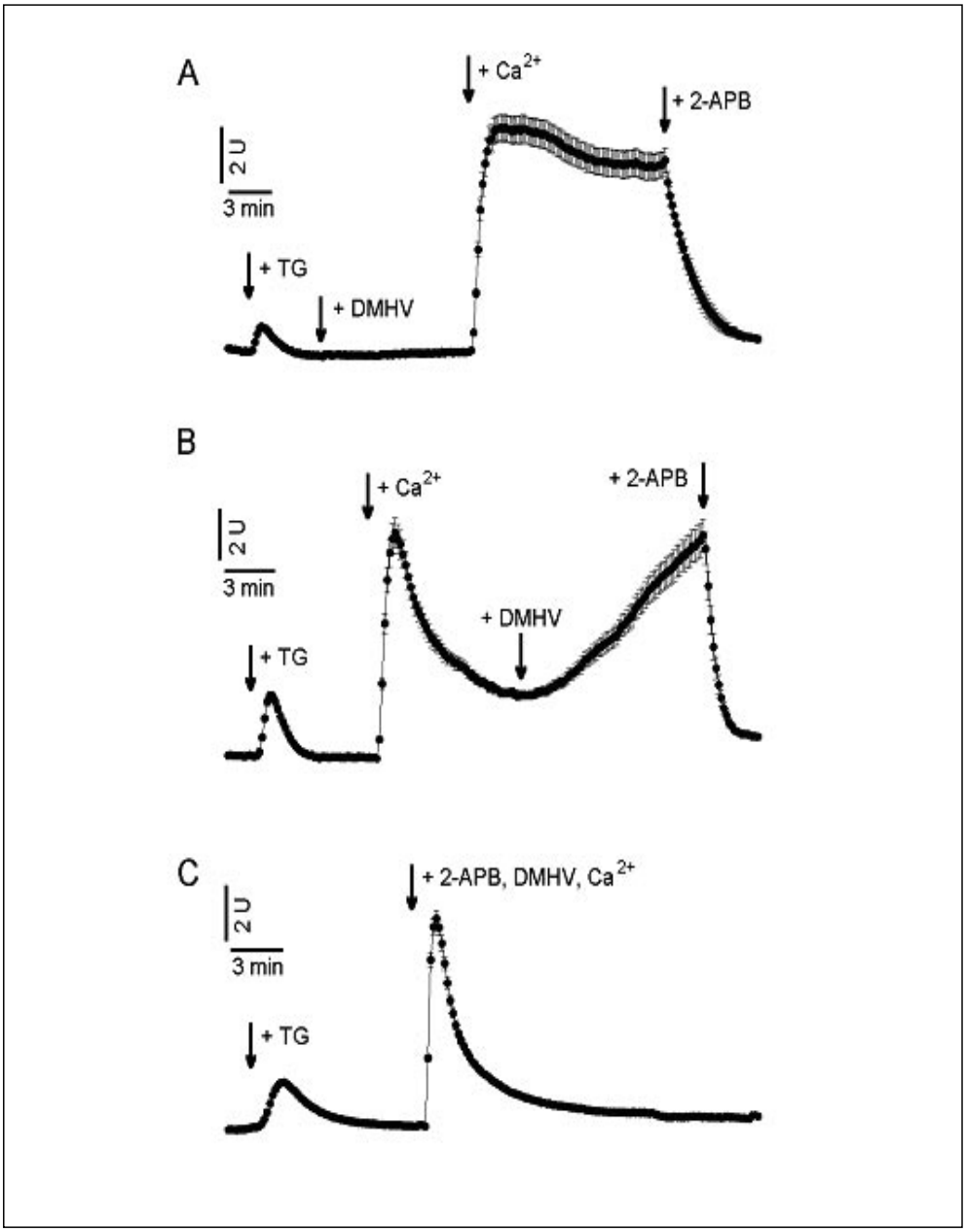

Fig. 2 2-APB inhibits DMHV-enhanced store-operated calcium entry in HEK 293 cells. HEK293 cells were store-depleted with 1 μM thapsigargin (TG) in the absence of extracellular calcium. Re-addition of calcium to the bath solution in the presence of DMHV (27 μM) caused sustained calcium influx which could rapidly be inhibited by 100 μM 2-APB (*A*). Application of DMHV (30 μM) after calcium re-addition caused the typical long-lasting rise in calcium influx which was also inhibited by 100 μM 2-APB (*B*). When 2-APB (100 μM) together with DMHV (18 μM) was added before calcium re-addition, inhibition of store-operated calcium entry by 2-APB predominated and calcium influx was completely inhibited (*C*).

7.3 Expression of Wild Type and Substrate-trapping Mutant of PTP1B in HEK Cells

In a more direct approach to determine whether the tyrosine phosphatase PTP1B is indeed involved in modulation of the capacitative Ca^{2+} influx, we performed experiments on cells which had been transfected with either the wild-type PTP1B or the inactive substrate-trapping mutant PTP1B (D181A). We found that in HEK 293 cells transfected with wild type PTP1B, thapsigargin-induced calcium entry was reduced as compared to non-transfected cells (Fig. 3). The kinetics of the calcium entry signal in PTP1B-transfected cells were similar as in non-transfected cells, showing a rapid rise and a slow decline. The calcium signal produced by intracellular calcium release from thapsigargin-sensitive stores was not affected by transfection with wild type PTP1B. Transfection of cells with the substrate-trapping PTP1B mutant D181A showed a slight increase in store-operated calcium influx as compared to non-transfected cells.

7.4 Measurement of PTP1B Activity

PTP1B activity was measured in homogenates from AR42J and HEK 293 cells using radioactive labeled RCML as substrate. The specific PTP1B activity was 2.2 ± 0.2 nmol/(mg protein × min) in AR42J cells (n = 3) and 1.4 ± 0.5 nmol/(mg protein × min) in HEK 293 cells (n = 17). Transfection of HEK 293 cells with wild type PTP1B (n = 8) caused an about 17-fold increase in PTP1B activity to 23.7 ± 9.3 nmol/(mg protein × min) whereas transfection with

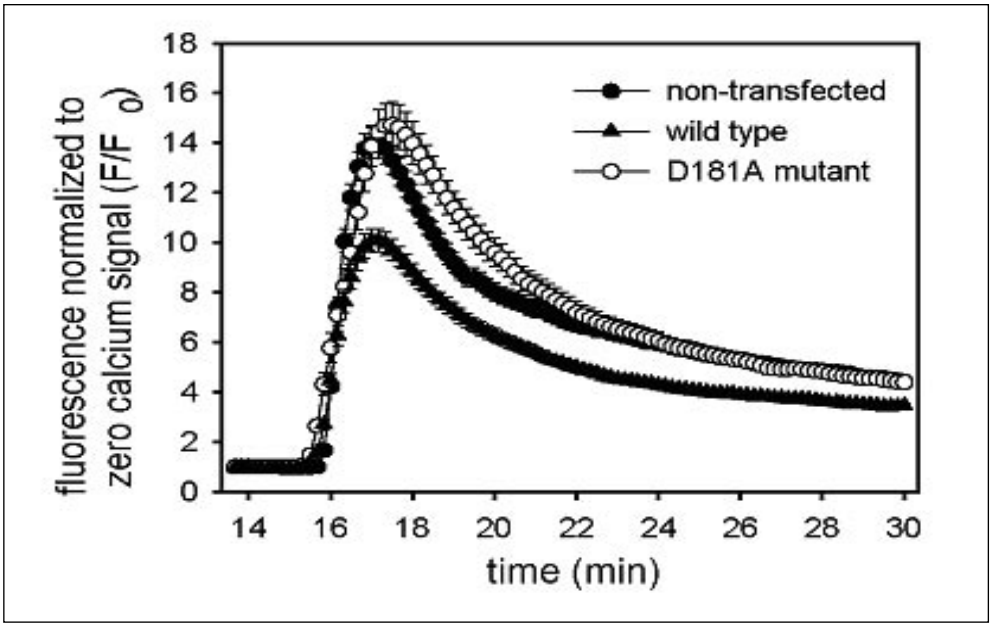

Fig. 3 Capacitative calcium entry in non-transfected HEK 293 cells and cells transfected with PTP1B wild-type and D181A mutant. Cells were store-depleted by thapsigargin (1 μM) in a calcium free bath solution for 10 min. Readdition of calcium to the bath solution caused a long-lasting rise in the intracellular calcium concentration (n = 199 cells). The capacitative calcium entry was reduced in cells tranfected with wild type PTP1B (n = 230 cells), whereas a slight increase could be observed in cells transfected with the D181A mutant (n = 266 cells). Fluorescence signals were normalized to fluorescence signals in extracellular calcium free solution (F_0).

the substrate trapping mutant D181A led to a decrease in endogenous PTP1B activity to 0.7 ± 0.5 nmol/(mg protein × min) (n = 3). Ten µM DMHV inhibited PTPase activity by ~80 % in both, non-transfected HEK 293 cells and HEK 293 cells transfected with wild type PTP1B. The IC_{50} value for inhibition of PTP activity with DMHV was about 0.2 µM (Hsu et al. 2003). We have also measured the effect of 2-APB on PTP activity. 2-APB, in the concentration range used in our experiments on calcium signaling (20–100 µM), had no significant effect on PTP activity. However, at concentrations > 100 µM there was inhibition of tyrosine PTPase activity (Hsu et al. 2003).

7.5 Ca^{2+} Influx in TRPV6-transfected HEK293 Cells

Following Ca^{2+} store depletion with thapsigargin Ca^{2+} influx was higher in TRPV6-transfected than in non-transfected cells. Whereas 2-APB inhibited Ca^{2+} influx completely in non-transfected cells, in TRPV6-transfected cells inhibition was not complete (see Fig. 4).

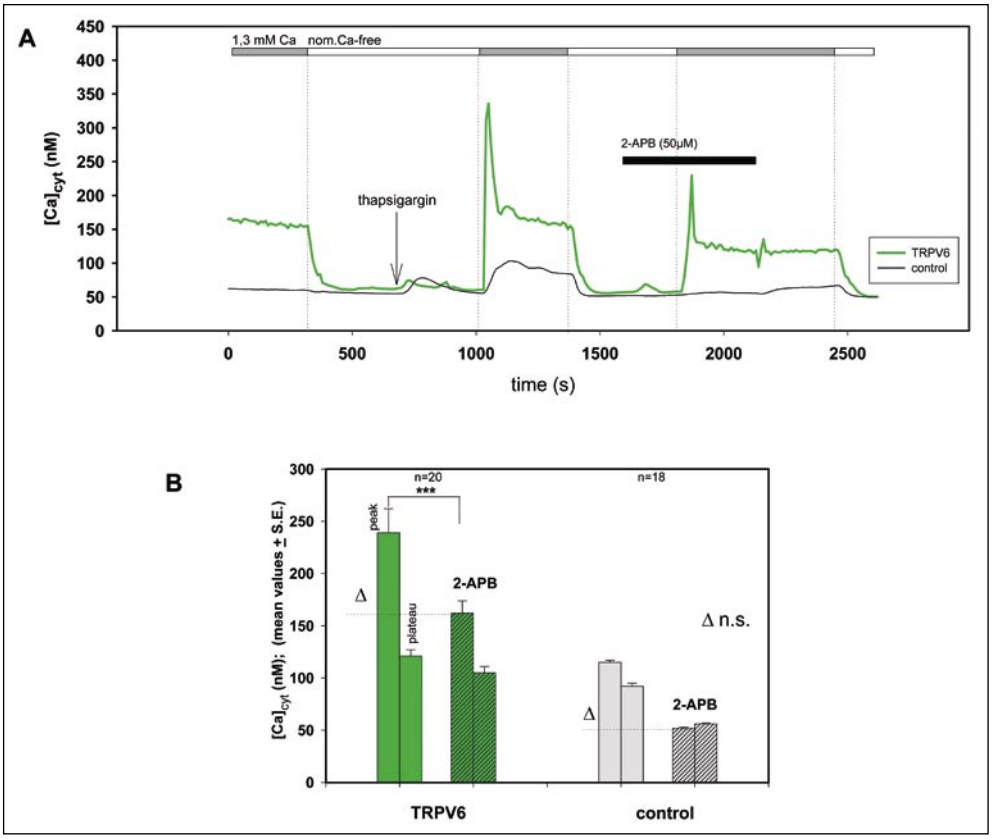

Fig. 4 Inhibition of Ca^{2+} influx by 2-aminoethyldiphenyl borate (2-APB). After depletion of calcium stores in the presence of thapsigargin, replacement of nominal Ca^{2+} free buffer by Ca^{2+}-containing buffer was followed by an elevation of cytosolic [Ca^{2+}] representing the calcium influx through the endogenous CRAC and in TRPV6-transfected cells also through TRPV6 (*A*). In the presence of 2-APB, 50 µM, Ca^{2+}-influx was completely abolished in control

It therefore appears that in TRPV6-transfected cells the Ca^{2+} influx through the endogenous CRAC-channels was inhibited by 2-APB, whereas TRPV6-mediated Ca^{2+} influx it was not inhibited by 2-APB. We therefore conclude that TRPV6 shows properties of the CRAC channel although it is not identical to it in HEK293 cells. Alternatively TRPV6 interacts with CRAC to induce increased Ca^{2+} influx with loss of 2-APB inhibitability. If tyrosine phosphorylation/-dephosphorylation of TRPV6 (see MAGG et al. this volume) has any consequences on CRAC activity remains to be investigated.

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cells. TRPV6-transfected cells exhibited a significantly reduced peak. The difference between the peak before and after the inhibition with 2-APB in transfected cells corresponds to the mean calcium influx in controls which could be completely blocked by 2-APB (*B*). We therefore assume that the reduction in calcium entry in transfected cells was due to the blockade of endogenous CRAC.

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Prof. Dr. Irene Schulz
Institute of Physiology
Saarland University Medical School
Building 58
66421 Homburg (Saar)
Germany
Tel.: +49 6841 1626450
Fax: +49 6841 1626655
E-Mail: irene.schulz@uniklinik-saarland.de

Epithelial Transporters of Electrolytes and Ion Channels

Nova Acta Leopoldina NF *89*, Nr. 336, 119–124 (2004)

Regulation of the Epithelial Sodium Channel (ENaC)

Christoph Korbmacher (Erlangen)

With 1 Figure

Abstract

The appropriate regulation of the amiloride-sensitive epithelial Na^+ channel (ENaC) in the aldosterone sensitive distal nephron is essential for the maintenance of body sodium balance and hence for long-term regulation of arterial blood pressure. Since abnormal activity of ENaC may cause arterial hypertension the investigation of ENaC regulation at the molecular level has become an area of intense research interest. This article briefly reviews some recent data regarding a possible functional interaction of ENaC with the cystic fibrosis transmembrane conductance regulator (CFTR) and the renal secretory potassium channel ROMK (Kir1.1). Furthermore, the role of regulatory proteins is discussed, in particular of Nedd4 and of a novel Nedd4-interacting protein, N4WBP5A.

Zusammenfassung

Die angemessene Regulation des Amilorid-empfindlichen epithelialen Na^+-Kanals (ENaC) im Aldosteron-regulierten Teil des distalen Nephrons ist entscheidend für die Aufrechterhaltung des Natriumhaushalts des Körpers und damit für die Langzeitkontrolle des arteriellen Blutdrucks. Da eine Überaktivität von ENaC eine arterielle Hypertonie verursachen kann, hat sich die Untersuchung der ENaC-Regulation auf molekularer Ebene zu einem wichtigen Forschungsgebiet entwickelt. Dieser Artikel gibt einen kurzen Überblick über aktuelle Befunde zu einer möglichen funktionellen Interaktion von ENaC mit dem „Cystic fibrosis transmembrane conductance regulator" (CFTR) und dem renalen sekretorischen Kaliumkanal ROMK (Kir1.1). Weiterhin wird die Rolle von Regulatorproteinen diskutiert, insbesondere die von Nedd4 und von einem kürzlich identifizierten, mit Nedd4 interagierenden Protein, N4WBP5A.

1. Introduction

The amiloride-sensitive epithelial Na^+ channel (ENaC) is the rate-limiting step for sodium absorption in a variety of epithelia including the renal collecting duct. The appropriate regulation of this channel is essential for the maintenance of body sodium balance and hence for long-term regulation of arterial blood pressure. This is evidenced by gain of function mutations of ENaC which cause a rare hereditary form of arterial hypertension called Liddle's syndrome. In contrast, loss of function mutations of ENaC cause pseudohypoaldosteronism type 1 (PHA1) with salt wasting and arterial hypotension (Rossier et al. 2002). These findings suggest that minor abnormalities in the pathways that regulate ENaC function may be critically involved in the pathophysiology of essential hypertension, the most common form of arterial hypertension that affects about 20–30 % of the adult population.

Aldosterone is the most important hormonal regulator of ENaC but vasopression (ADH) and various other mediators are also involved in its regulation (Garty and Palmer 1997). At

the molecular level ENaC channel activity and its surface expression appear to be controlled and modified by a range of regulatory proteins including the ubiquitin-protein ligases Nedd4, the serum and glucocorticoid-inducible kinase SGK1, components of the SNARE machinery (e.g. syntaxin 1A) and cytoskeletal elements (e.g. actin) (Kamynina and Staub 2002, Gormley et al. 2003). However, the complex interdependence and relative importance of these regulatory interactions are not yet fully understood.

2. Functional Interaction of ENaC with CFTR and ROMK (Kir1.1)

Membrane transport proteins do not operate in isolation, and ENaC may be functionally coupled to other ion channels present in the same membrane. Activation of cystic fibrosis transmembrane conductance regulator (CFTR) chloride channels by cAMP has been shown to inhibit ENaC channels in recombinant expression systems (Stutts et al. 1995) and in various epithelial tissues including respiratory epithelia (Mall et al. 1998), colon (Ecke et al. 1996) and cultured renal cortical collecting duct cells (Letz and Korbmacher 1997). In the lungs of cystic fibrosis (CF) patients the failure of defective CFTR to inhibit ENaC is thought to cause hyperabsorption of Na^+ and fluid, possibly contributing to the formation of dry sticky mucus typical of pulmonary CF pathophysiology. This pathophysiological concept has generated intense research efforts to study the regulatory relationship between CFTR and ENaC (Kunzelmann et al. 2000).

At present the molecular mechanism of this reciprocal regulation of ENaC and CFTR and its physiological relevance remain unclear. For example, it is a matter of debate whether co-expression of CFTR per se reduces ENaC currents as reported in some studies (Hopf et al. 1999, Ji et al. 2000) or whether cAMP-mediated activation of CFTR is necessary for its inhibitory effect on ENaC as suggested by others (Chabot et al. 1999, Jiang et al. 2000, Konstas et al. 2003). Moreover, it is unclear whether ENaC and CFTR physically interact as suggested by results from yeast two hybrid analysis (Kunzelmann et al. 1997) or whether their regulatory relationship is of a more indirect nature. Recently, it has been reported that CFTR inhibits ENaC through an increase in the intracellular chloride concentration (König et al. 2001). However, in the *Xenopus laevis* oocyte expression system an inhibitory effect of CFTR activation on ENaC has been observed at a continuous holding potential of −60 mV with CFTR-mediated inward currents corresponding to chloride efflux. These findings do not support the hypothesis that an increase in intracellular chloride is essential for the inhibitory effect of activated CFTR on ENaC. Interestingly, the inhibitory effect of CFTR appears to be due to a decrease in channel open probability of ENaC without a reduction of its surface expression (Konstas et al. 2003). The complexity of ENaC-CFTR interaction is further demonstrated by co-expression studies suggesting that ENaC may have a stimulatory effect on CFTR activity (Chabot et al. 1999, Ji et al. 2000, Jiang et al. 2000). Finally, ENaC enhances the suface expression of the secretory K^+ channel ROMK (Kir1.1) in a CFTR-dependent manner (Konstas et al. 2002a). Thus, CFTR may provide a mechanistic link that mediates the coordinated upregulation of ROMK during the stimulation of ENaC by hormones such as aldosterone or antidiuretic hormone.

3. Regulation of ENaC by Nedd4 and by N4WBP5A, a Novel Nedd4-interacting Protein

ENaC consists of α, β and γ subunits and the carboxyl terminus of each ENaC subunit contains a PPxY motif which is believed to be important for interaction with the WW domains of the ubiquitin-protein ligase Nedd4. Disruption of this interaction, as in Liddle's syndrome where mutations delete or alter the PPxY motif of either the β or γ subunits, has been shown to result in increased ENaC activity and arterial hypertension (Kellenberger and Schild 2002, Hummler 2003). In mouse and human there are at least two Nedd4 isoforms which have distinct characteristics with respect to ENaC regulation (Kamynina et al. 2001a, b). Nedd4-2 is believed to be the physiologically important isoform regarding ENaC regulation in the kidney but Nedd4 may also play a role. Two differentially spliced Nedd4-2 transcripts have been identified in mouse and humans and both isoforms have the ability to down-regulate ENaC currents expressed in *Xenopus laevis* oocytes. Moreover, among the four WW domains of Nedd4-2 the WW3 and WW4 seem to be particularly important for ENaC binding (Fotia et al. 2003).

The PY motif-WW interaction brings the ubiquitin-protein ligase Hect domain of Nedd4 into close proximity with ENaC, leading to channel ubiquitination, endocytosis and lysosomal/proteosomal degradation (Staub et al. 2000, Malik et al. 2001). Clathrin-mediated endocytosis has also been shown to be involved in regulated ENaC retrieval from the plasma membrane (Shimkets et al. 1997). The interdependence of Nedd4-mediated ubiquitination and clathrin-mediated endocytosis is not yet fully understood. A possible mechanism could be that Nedd4 binds to the PY motif of βENaC and induces a conformational change exposing the sequence recognized by the endocytic machinery (Gormley et al. 2003). Recent studies suggest that SGK-mediated phosphorylation of Nedd4-2 reduces the binding of Nedd4-2 to ENaC resulting in a reduction of ENaC ubiquitination and hence a stabilization of ENaC in the plasma membrane (Debonneville et al. 2001, Snyder et al. 2002). Thus, the stimulatory effect of aldosterone through activation of SGK may at least in part be mediated *via* Nedd4. Moreover, sodium feedback regulation of ENaC also requires the presence of Nedd4 (Dinudom et al. 1998, Harvey et al. 1999).

Recently, additional substrates for Nedd4 have been identified by far Western screen using the WW domains of Nedd4 as probe (Jolliffe et al. 2000). One of these, N4WBP5, is a novel Golgi-associated protein containing two PY motifs in the amino-terminal domain and three putative transmembrane domains in the carboxy-terminal half of the protein (Harvey et al. 2002). A closely related protein, N4WBP5A, identified in data base searches also contains two PY motifs and the three transmembrane domains. This novel protein N4WBP5A interacts with Nedd4-2 in addition to Nedd4. Most importantly, in *Xenopus laevis* oocytes N4WBP5A was found to increase surface expression of ENaC by reducing the rate of ENaC retrieval (Konstas et al. 2002b). In contrast, N4WBP5A has no stimulatory effect on CFTR or ROMK1 (Kir1.1a) which are apically located channel proteins that are co-expressed with ENaC in renal collecting duct principal cells. This suggests that the stimulatory effect of N4WBP5A is rather specific for ENaC. Furthermore, N4WBP5A prevents sodium feedback inhibition of ENaC possibly by interfering with the xNedd4-2 mediated regulation of ENaC.

As N4WBP5A binds Nedd4/Nedd4-2 *via* PY motif/WW domain interactions and appears to be associated with specific intracellular vesicles, N4WBP5A probably regulates Nedd4/Nedd4-2 availability and trafficking. Thus, N4WBP5A enhances ENaC surface expression most likely by preventing Nedd4-2-mediated channel retrieval from the plasma membrane. The effect of

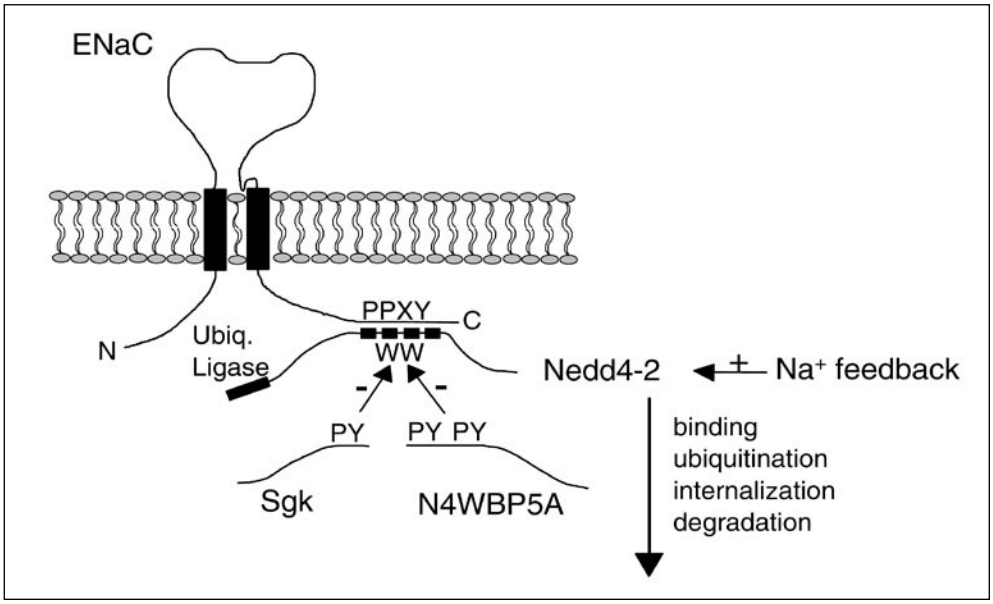

Fig. 1 Schematic view of ENaC regulation by N4WBP5A, serum- and glucocorticoid-regulated kinase (Sgk) and Na^+ feedback *via* stimulation (+) or inhibition (–) of Nedd4-2-mediated channel retrieval (from Konstas et al. 2002b; with permission from the American Society for Biochemistry and Molecular Biology).

N4WBP5A is reminiscent of the action of SGK which also increases surface expression of ENaC at least in part by PY-dependent binding to Nedd4-2 and its phosphorylation (Debonneville et al. 2001, Snyder et al. 2002). As illustrated schematically in Figure 1, Nedd4-2 seems to be an integrator of various pathways regulating ENaC activity. Since N4WBP5A is highly expressed in native renal collecting duct and other tissues that express ENaC (Konstas et al. 2002b), it is a likely candidate to modulate ENaC function *in vivo* by interfering with ENaC/Nedd4 interaction. It remains a challenge for future research to demonstrate the physiological and pathophysiological relevance of N4WBP5A in different ENaC regulatory pathways.

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Prof. Dr. Christoph Korbmacher
Friedrich-Alexander-Universität Erlangen-Nürnberg
Institut für Zelluläre und Molekulare Physiologie
Waldstraße 6
91054 Erlangen
Germany
Phone: +49 9131 8522300
Fax: +49 9131 8522770
E-Mail: christoph.korbmacher@physiologie2.med.uni-erlangen.de

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Epithelial Na^+ Channels and their Regulation

David Ian Cook, Anuwat Dinudom, and John Atherton Young (Sydney)

Abstract

This paper considers regulation of epithelial Na^+ channels with a particular focus on the role of PY motifs and ubiquitin-protein ligases. Issues that will be addressed include the role of PY motifs in regulating epithelial Na^+ channels, the identity of the proteins that interact with the PY motifs in these channels, and the role of phosphorylation in regulating these interactions.

Zusammenfassung

Der Beitrag behandelt die Regulation von epithelialen Na^+-Kanälen unterer besonderer Berücksichtigung der Rolle des PY-Motivs und der Ubiquitin-Protein-Ligasen. Zu den angesprochenen Themenkreisen gehören die Rolle des PY-Motivs in der Regulation epithelialer Na^+-Kanäle, die Identität der Proteine, die mit den PY-Motiven in diesen Kanälen interagieren und die Rolle der Phosphorylierung in der Regulation dieser Wechselwirkungen.

1. The Role of PY Motifs in Regulating Epithelial Na^+ Channels

Epithelial Na^+ channels contain 3 subunits, α, β and γ, each of which contains a proline rich (PY) motif in its C-terminal, cytosolic domain. Deletion or mutation of these motifs in the β- or the γ-subunits leads to the autosomal dominant, hereditary form of hypertension called Liddle's syndrome and causes an increase in the number and the open probability of Na^+ channels in the cell membrane (Snyder 2002).

The marked channel stimulation observed on removing the PY motifs suggests that they are critical to a tonically active negative feedback system. In particular, the Na^+ feedback system, which inhibits the channels when intracellular Na^+ increases (Komwatana et al. 1996), appears to be dependent on these motifs. Mutations of the PY motifs in Na^+ channels expresses in *Xenopus* oocytes (Konstas et al. 2002, Kellenberger et al. 1998) abolishes Na^+ feedback regulation of the channels. Furthermore, whole-cell patch-clamp studies in mouse mandibular duct cells show that the presence in the cytosol of peptides corresponding to the PY-motifs of β-ENaC and γ-ENaC, but not that of α-ENaC, also inhibits Na^+ feedback regulation of the channels (Dinudom et al. 2001). Although this distinction between the PY motifs of the three ENaC subunits is consistent with the observation that Liddle's syndrome is only caused by mutations in the β- or the γ-subunits of ENaC (Snyder 2002), it is not observed in expression studies in *Xenopus* oocytes (Schild et al. 1996) and its mechanistic basis is unclear.

The PY motifs do not, however, mediate the channel's responses to cytosolic pH (Konstas et al. 2000), activation of the cystic fibrosis transmembrane conductance regulator (Hopf et al 1999), or increased intracellular Cl^- (Kunzelmann 2003, Dinudom et al. 1998).

2. The Proteins that Interact with the PY Motifs

Although intracellular Na^+ may exert multiple effects on Na^+ channels, deletion or blockade of the PY motifs largely abolishes Na^+ feedback regulation (Kellenberger et al. 1998, Konstas et al. 2002, Harvey et al. 1999). Since cytosolic Na^+ does not act directly on the PY motifs (Kanelis et al. 2001, Dinudom et al. 1998), these effects must be mediated by binding of a protein mediator (Dinudom et al. 1998, Komwatana et al 1998) although the identity of this protein remains uncertain. Currently, there are three candidates as the physiological mediator of Na^+ feedback on epithelial Na^+ channels: Nedd4 (Staub et al. 1996, Dinudom et al. 1998), Nedd4-2 (Kamynina et al. 2001, Harvey et al. 2001) and WWP2 (McDonald et al. 2002). All three candidates contain multiple WW domains and a Hect ubiquitin protein ligase domain, consistent with their binding the PY motifs and ubiquitinating the channel (Snyder 2002, Dinudom et al. 1998). Studies in *Xenopus* oocytes and in transfected mammalian epithelia have shown that both Nedd4 and Nedd4-2 are able to decrease the activity of co-expressed Na^+ channels (Kamynina et al. 2001, Snyder 2002, Fotia et al. 2003), while whole-cell patch-clamp studies in mouse mandibular duct cells have demonstrated that the presence in the cytosol of recombinant WW domains of Nedd4 or Nedd4-2 blocks Na^+ feedback (Dinudom et al. 1998, Harvey et al. 2001, Fotia et al. 2003). Finally, non-selective antibodies against Nedd4 and Nedd4-2, when included in the pipette solution, are able to block Na^+ feedback in mouse mandibular duct cells (Dinudom et al. 1998).

These data do not permit us, however, to determine whether the physiological regulator of epithelial Na^+ channels is Nedd4 or Nedd4-2. *Xenopus* oocyte studies have suggested that mouse Nedd4-2 is more effective than mouse Nedd4 in suppressing the activity of co-expressed Na^+ channels (Kamynina et al. 2001). This difference may be due rodent Nedd4 only containing 3 WW domains, whereas rodent Nedd4-2 contains four. The missing domain corresponds to WW3 in human Nedd4 which has a high affinity for ENaC *in vitro* and its absence has been proposed to reduce the affinity or rodent Nedd4 for ENaC (Harvey et al. 1999, Farr et al. 2000, Lott et al. 2002). This explanation is excluded, however, by our finding that recombinant human WW3 does not block Na^+ feedback in mouse mandibular duct dells, either alone or in combination with WW domains from mouse Nedd4 (Harvey et al. 1999). Furthermore, our observation that mouse Nedd4 requires all three of its WW domains to inhibit Na^+ channels (Harvey et al. 1999), whereas mouse Nedd4-2 only uses WW3 and WW4 (Fotia et al. 2003) may indicate that rodent Nedd4 uses an alternative mechanism to bind to ENaC in order to overcome its lack of a high affinity WW3 domain. This alternative mechanism may involve WW1 binding to a channel associated protein, as this WW domain does not bind ENaC (Harvey et al. 1999, Farr et al. 2000).

Another means of determining whether Nedd4 or Nedd4-2 is the physiological regulator of epithelial Na^+ channels is by comparing their tissue distribution with that of ENaC. In the case of Nedd4, immunohistochemical studies show that it is present in many of the tissues that express epithelial Na^+ channels, including renal collecting duct, salivary ducts and respiratory epithelium (Dinudom et al. 1998, Staub et al. 1997). It is not, however, expressed in colonic

ville (Staub et al. 1997). Nedd4-2, on the other hand, appears to be expressed in all of the epithelia in which epithelial Na^+ channels are found including the colonic mucosa (Kamynina et al. 2001, Harvey et al. 2001).

The recent proposal that serum- and glucocorticoid-stimulated kinase (Sgk) inactivates Na^+ feedback as a consequence of the selective phosphorylation of Nedd4-2 (Snyder 2002, Debonneville et al. 2001) may also provide a means of determining whether Nedd4 or Nedd4-2 is the physiological mediator. Our studies have failed to demonstrate any effect of Sgk on the Na^+ feedback system in mouse mandibular duct cells (unpublished results) but the recent report that Sgk stimulates the insertion of channels into the membrane rather than inhibiting their removal from it (Rosa de la Alvarez and Canessa 2003) casts doubt on the physiological relevance of the reported selective phosphorylation of Nedd4-2 by Sgk.

The third candidate as a physiological regulator of epithelial Na^+ channels is WWP2 (McDonald et al. 2002). In transfected epithelial cells it inhibits the activity of heterologously expressed epithelial Na^+. Furthermore, the pattern of expression of WWP2 mRNA is similar to that for ENaC and for Nedd4-2 (McDonald et al. 2002). Given that antibodies directed against Nedd4 do not bind WWP2, the finding in mouse mandibular duct cells that these antibodies block Na^+ feedback (Dinudom et al. 1998) rules out a role for WWP2 in regulating Na^+ channels in this tissue. Furthermore, *in vitro* studies suggest that the interaction between WWP2 and ENaC is quite weak (Harvey et al. 2001), as is also apparent from the heterologous transfection studies (McDonald et al. 2002). WWP2 may, however, mediate some other regulatory system such as Na^+ feedback regulation of NHE1 in salivary endpiece cells which is known to involve an abiquitin-protein ligase other than Nedd4 or Nedd4-2 (Ishibashi et al. 1999).

3. Is the Interaction of the Ubiquitin-Protein Ligase with ENaC Regulated by Phosphorylation?

Modulation of the Na^+ feedback system by regulating the interaction of the ubiquitin-protein ligase with the Na^+ channels is an attractive explanation for integrating hormonal regulation with homeocellular regulation of epithelial Na^+ channels. Sgk has been proposed, for example, to mediate the rapid actions of growth factors such as IGF-1 and of steroid hormones such as aldosterone, on Na^+ channels, by regulating the interaction of Nedd4-2 with ENaC (Snyder 2002, Debonneville et al. 2001). This proposal, however, does not fit easily with recent findings on the actions of Sgk (Rosa de la Alvarez and Canessa 2003). The ERK kinase has also been found *in vitro* to phosphorylate a threonine close to the PY motifs in β- and γ-ENaC leading to a increased affinity of the channel for Nedd4 (Shi et al. 2002). The physiological role of ERK phosphorylation of ENaC is, however, unknown.

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Prof. Dr. David Cook
University of Sydney
Department of Physiology
Anderson Stuart Building
Sydney, NSW 2006
Australia
Phone: +61 2 93512809
Fax: +61 2 93519926
E-Mail davidc@physiol.usyd.edu.au

Nonlinear Dynamics and the Spatiotemporal Principles of Biology

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Schwerpunkte der Veranstaltung bildeten nichtlineare Dynamik im allgemeinen, stochastische Resonanz und Synchronisation. Alle diese Themen wurden mit engem Bezug zu aktuellen Anwendungen in der Biologie im Wechselspiel von Theorie und Empirie behandelt. Sobald man bei dem Versuch, ein mathematisches Modell eines biologischen Phänomens zu entwerfen, die dynamischen Variablen über lineare Flüsse miteinander verbindet, findet die Beschreibung im Rahmen der linearen Systemtheorie statt. Wenn dann nichtlineare Wechselwirkungen einbezogen werden, gelangt die Modellierung in den Bereich der nichtlinearen Dynamik. Die Beiträge behandeln als Kernthemen Oszillationen, zeitliche und räumliche Synchronisation und Rauschen. Die im Rahmen des Symposiums diskutierten Oszillationen in der Natur reichen von ultradianen zu circadianen Rhythmen, von der Dynamik einzelner Enzyme zu Zellen, Organismen und Populationen. Die Diskussion von Synchronisation reicht von springenden Brownschen Teilchen, Ionenkanälen, Mikroalgen, Wasserflöhen (Daphnien), Zellen in einem Organ, Neuronen, entwicklungsbiologischen Organisations- und Signalzentren, Fischen in einem Vortex bis hin zu Planktonpopulationen. Rauschen wird als Umwelteinfluß oder als interner Beitrag schneller Dynamiken beschrieben. Biologische Variabilität in nichtlinearen Systemen vermag, ähnlich wie Rauschen, raumzeitliche Muster zu induzieren. Mit dem Auftreten nichtlinearer Phänomene in der Biologie stellt sich die Frage eines evolutionär wirksamen Nutzens für das biologische System. So können Oszillationen etwa den evolutionären Vorteil einer Zeitreferenz und einer inneren Uhr mit sich bringen. Die Beiträge machen solche funktionellen Vorteile in der Synchronisation biologischer Prozesse und im auf Brownsche Motoren wirksamen Rauschen bzw. in der Kontrolle von Krankheiten aus. Die nichtlineare Struktur ihrer Dynamik verleiht lebenden Systemen Robustheit.
Alle Beiträge sind in englischer Sprache abgefaßt.

In Kommission bei Wissenschaftliche Verlagsgesellschaft mbH Stuttgart

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Physiology and Pathophysiology of Renal Organic Anion Transporters OAT1 and OAT3

Gerhard Burckhardt, Member of the Academy, Andrew Bahn, Nadiya Bakhiya, and Natascha A. Wolff (Göttingen)

With 3 Figures

Abstract

The organic anion transporters 1 (OAT1) and 3 (OAT3) in the basolateral membrane of renal proximal tubules take up various endogenous and exogenous compounds from the blood into the cells. The organic anion (OA)/α-ketoglutarate exchanger OAT1 utilizes the intracellular-to-extracellular concentration gradient of α-ketoglutarate as driving force for OA uptake. Here we show that human OAT3 expressed in *Xenopus laevis* oocytes exchanges intracellular glutarate for extracellular *p*-aminohippurate (PAH) and is thus also able to perform OA/dicarboxylate exchange. Urate inhibits PAH uptake by OAT3 more than uptake by OAT1, suggesting that OAT3 has the higher affinity for urate. Finally, we demonstrate that aristolochic acid, a plant constituent causing the Chinese herbs nephropathy, potently interacts with OAT1. Thus, OAT1 may be involved in proximal tubular damage caused by aristolochic acid.

Zusammenfassung

Die Transporter 1 und 3 für organische Anionen (OAT1, OAT3) in der basolateralen Membran proximaler Nierentubuli sind für die Aufnahme vieler endogener und exogener anionischer Verbindungen aus dem Blut verantwortlich. OAT1 ist ein Anionen/α-Ketoglutarat-Austauscher, der den über der basolateralen Membran bestehenden Konzentrationsunterschied von α-Ketoglutarat als Triebkraft für die Aufnahme von organischen Anionen in die Zelle nutzt. Hier zeigen wir, daß der in *Xenopus-laevis*-Oozyten exprimierte humane OAT3 intrazelluläres Glutarat gegen extrazelluläres *p*-Aminohippurat (PAH) austauscht und daher wie OAT1 als Anionenaustauscher arbeitet. Urat hemmt die PAH-Aufnahme durch OAT3 stärker als die durch OAT1, was auf eine höhere Affinität des OAT3 für dieses Stoffwechselprodukt hinweist. Schließlich zeigen wir, daß die Aristolochiasäure, ein pflanzlicher, die „Chinese herbs nephropathy" auslösender Inhaltstoff, ein potenter Hemmstoff des OAT1 ist. Damit könnte OAT1 an den durch die Aristolochiasäure verursachten Nierenschäden beteiligt sein.

1. Introduction

The kidneys eliminate potentially toxic exogenous compounds such as administered drugs and their metabolites. A considerable number of renally excreted drugs are organic anions (OA). Examples are β-lactam antibiotics, diuretics, angiotensin converting enzyme (ACE) inhibitors, and uricosurics. These drugs are secreted in the proximal tubules. As a first step, OA are taken up from the blood into the proximal tubule cells across the basolateral membrane. This uptake is coupled to the efflux of the dicarboxylate α-ketoglutarate, which is recycled into the cell by a sodium-dicarboxylate cotransporter. The sodium ions taken up by the co-transporter are pumped out by the Na^+, K^+ ATPase, and the OA are released through the apical membrane into the urine (Burckhardt et al. 2001).

Two organic anion transporters (OAT1, OAT3) possibly involved in OA uptake have been cloned from various species (van Aubel et al. 2000, Dresser et al 2001, Sekine et al. 2000). Early on, it became clear that OAT1 is able to perform OA/α-ketoglutarate exchange and to accept organic anions of diverse chemical structures, including most of the renally excreted anionic drugs (Burckhardt and Burckhardt 2003). The OAT3 accepted various organic anions, most notably sulfated steroid hormones such as estrone sulfate, and even the organic cation cimetidine (Burckhardt and Burckhardt 2003). OAT1 and OAT3 are located at the basolateral membrane of rat and human proximal tubule cells (Hosoyamada et al. 1999, Tojo et al. 1999, Cha et al. 2001, Enomoto et al. 2002, Hasegawa et al. 2002, Kojima et al. 2002).

As opposed to OAT1, the cloned OAT3 was thought not to operate as an anion exchanger, since efflux of radiolabeled estrone sulfate (ES) from OAT3 expressing *Xenopus laevis* ocytes was not *trans*-stimulated by external unlabeled ES (Kusuhara et al. 1999). If OAT3 were a uniporter rather than an exchanger, OA transported into the cells by OAT1 would flow back out through OAT3 localized in the same cells. Since such a futile cycle is highly unlikely to occur we reinvestigated the transport mode of OAT3.

The broad substrate specificity of OAT1 and OAT3 enables these transporters to interact with a variety of drugs but, unfortunately, also with toxins. A prominent example is the nephrotoxic and cancerogenic ochratoxin A which was translocated by both OATs (Jung et al. 2001). Potentially cytotoxic mercapturic acids were transported by rat OAT1 (Pombrio et al. 2001), and uremic toxins such as indoxyl sulfate and various hydroxy-hippurates by human and rat OAT1 as well as by rat OAT3 (Deguchi et al. 2002, Enomoto et al. 2002, Motojima et al. 2002). Antiviral drugs known for their nephrotoxicity are substrates of human OAT1 and OAT3 (Takeda et al. 2002). Interestingly, cytotoxicity was diminished by inhibition of expressed human OAT1 with probenecid (Ho et al. 2000). Here, we investigated whether aristolochic acid, an anionic plant constituent causing severe renal damage and urothelial cancers (reviewed in Arlt et al. 2002), is a substrate of human OAT1.

2. Methods

Human OAT1 was obtained by homology cloning (Reid et al. 1998) and expressed in *Xenopus laevis* oocytes or in COS7 cells. The cDNA of human OAT3 was obtained from the Resource Center/Primary Data Base, Berlin, Germany. After correction of two mutations in that clone hOAT3 was expressed in oocytes (Bakhiya et al. 2003). The transport activity of OAT1 and OAT3 in oocytes was assessed either by measuring the uptake of radiolabeled PAH or by determining the efflux of radiolabeled PAH or glutarate injected into the oocytes before the onset of the experiment (for details see Wolff et al. 2001). OAT1 activity transiently expressed in COS7 cells was measured by uptake of the OA fluorescein (for details see Bahn et al. 2002).

3. Results

First we investigated whether human OAT3 is able to operate as an anion exchanger similar to OAT1. We expressed OAT1 and OAT3 by injecting the respective cRNAs into *Xenopus laevis* oocytes. After three days, OAT1-expressing oocytes were microinjected with [^{3}H]PAH, and OAT3-expressing oocytes with [^{14}C]glutarate, respectively. Figure 1 shows that the efflux

of [^{3}H]PAH from OAT1-expressing oocytes was *trans*-stimulated by the presence of 1 mM glutarate in the external buffer, proving the well-known ability of OAT1 to exchange PAH for a dicarboxylate with 5 carbons (α-ketoglutarate or the non-metabolizable glutarate). In oocytes expressing OAT3, 1 mM of external PAH *trans*-stimulated [^{14}C]glutarate efflux. Thus, also OAT3 is capable of PAH/dicarboxylate exchange. *Trans*-stimulation of glutarate efflux was also seen in the presence of unlabeled α-ketoglutarate, glutarate, cimetidine, and urate in the buffer (Bakhiya et al. 2003).

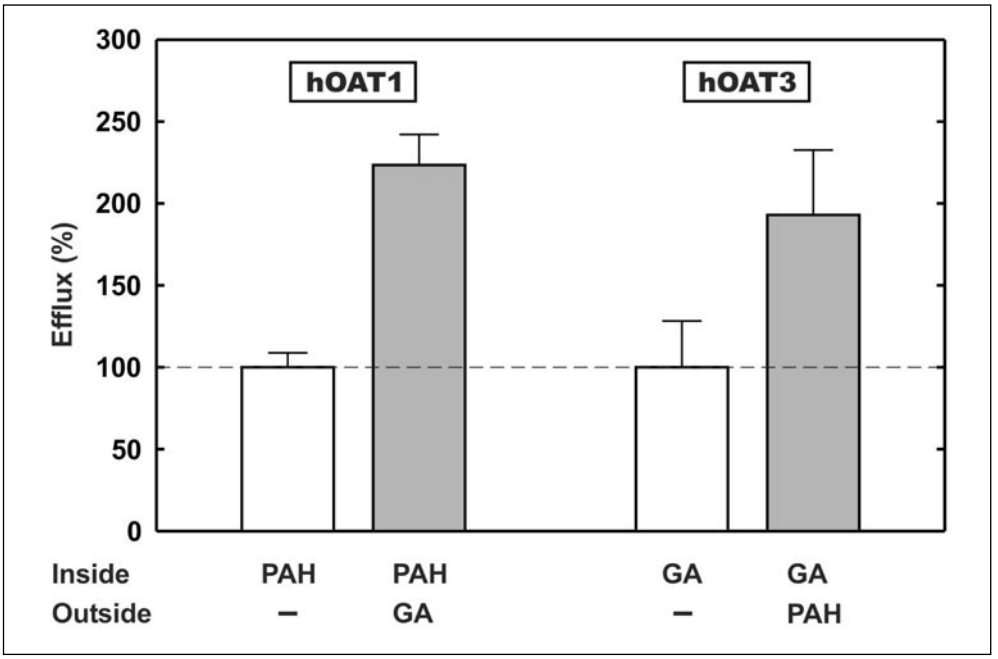

Fig. 1 OAT1 and OAT3 operate as anion exchangers. Human OAT1 and OAT3 were expressed in *Xenopus laevis* oocytes. The oocytes were loaded with 40 nCi [^{3}H]*p*-aminohippurate (PAH; 12.9 pmol in 46 nl) or 2.6 nCi [^{14}C]glutarate (GA; 84 pmol in 23 nl) by microinjection ("inside"). Then, the oocytes were transferred into Oocyte's Ringer solutions without (–) or with 1 mM unlabeled glutarate or PAH ("outside"). Since there was a highly variable leak flux during the first 2 min, the buffer was changed after that time and efflux of radioactivity assayed over the next 28 min. The efflux in the absence of outside glutarate or PAH was set to 100 %. Shown are means ± SEM from 5 oocytes assayed under each experimental condition.

Urate is an endogenous organic anion that has been reported to interact with OAT1 (Ichida et al. 2003) and OAT3 (Cha et al. 2001). Therefore, we tested unlabeled urate as an inhibitor of [^{3}H]PAH uptake into oocytes expressing OAT1 or OAT3. As shown in Figure 2, urate (0.5 or 1 mM) inhibited PAH uptake by OAT1 with moderate potency, whereas PAH uptake by OAT3 was considerably inhibited. Thus, OAT3 appears to have a greater sensitivity for urate than OAT1.

Finally, we tested aristolochic acid (AA) on fluorescein uptake by OAT1-expressing COS7 cells. Figure 3 shows, that aristolochic acid is a potent inhibitor of OAT1. In the presence of 1 µM AA, fluorescein uptake was inhibited by more than 50 %, indicating a high affinity of human OAT1 for aristolochic acid.

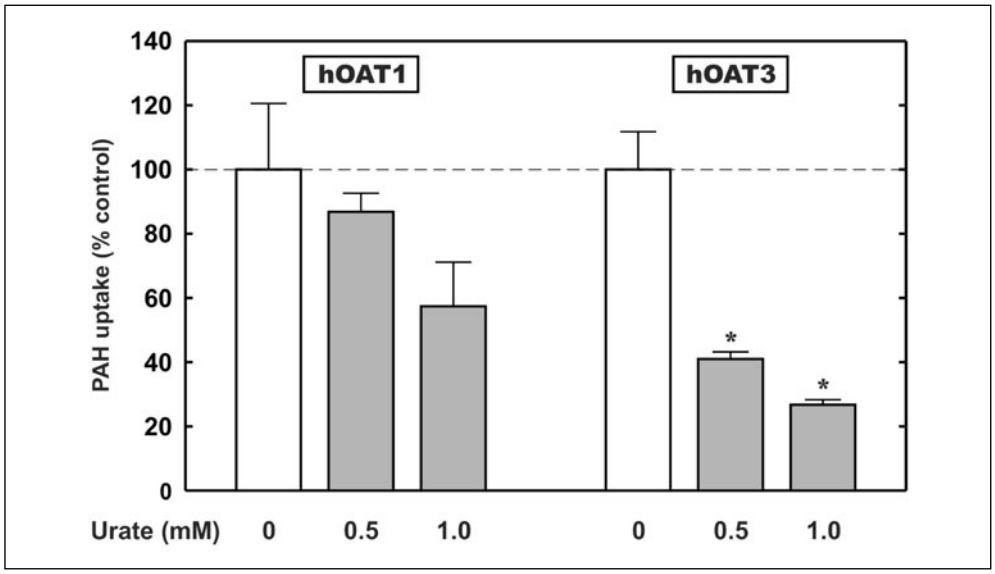

Fig. 2 OAT1 and OAT3 are inhibited by urate to different extents. Human OAT1 and OAT3 were expressed in *Xenopus laevis* oocytes. The uptake of 10 µM [^{3}H]PAH was measured for 60 min in the absence (white columns) or presence of unlabeled urate at the indicated concentrations (grey columns). Uptake in the absence of urate was set to 100 %. Shown are means ± SEM from two experiments, using 8 – 11 oocytes under each condition.

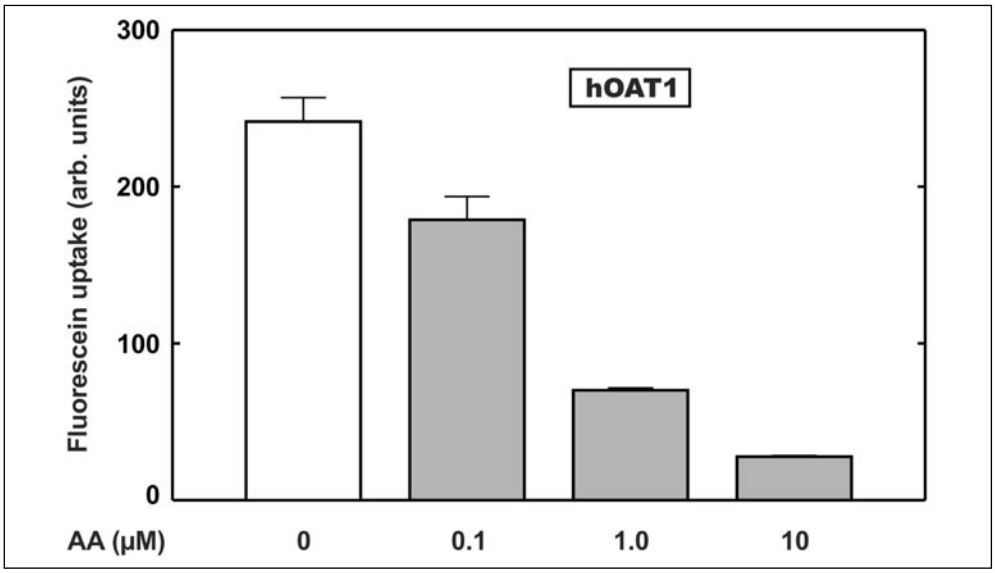

Fig. 3 OAT1 is inhibited by aristolochic acid. Human OAT1 was transiently expressed in COS7 cells. The uptake of 1 µM fluorescein was measured for 5 min in the absence or presence of unlabeled aristolochic acid at the indicated concentrations. The intracellular fluorescence as determined after disruption of the cells is shown in arbitrary units. Shown are means ± SD of 3 cell samples tested under each experimental condition.

4. Discussion

OAT1 and OAT3 are renal transporters with broad and overlapping substrate specificities (Burckhardt and Burckhardt 2003). Due to their location in the basolateral membrane of proximal tubules (Hosoyamada et al. 1999, Tojo et al. 1999, Cha et al. 2001, Enomoto et al. 2002, Hasegawa et al. 2002, Kojima et al. 2002), both transporters should operate as OA import carriers. OA import against the inside negative membrane potential requires the expense of energy, such as the coupling of OA uptake to the downhill efflux of another anion. For OAT1 there is no doubt that the efflux of α-ketoglutarate drives the OA uptake. The dicarboxylate α-ketoglutarate can be derived from metabolism or provided by the sodium-dicarboxylate cotransporter located in the basolateral membrane of proximal tubule cells (Shuprisha et al. 1999). The driving force for OA uptake by OAT3, however, was not clear. When we preloaded OAT3-expressing oocytes with the non-metabolizable α-ketoglutarate analog, glutarate, we found an accelerated efflux of glutarate in the presence of extracellular PAH (this study), α-ketoglutarate, glutarate, cimetidine, and urate (Bakhiya et al. 2003). This *trans*-stimulation is indicative of the ability of OAT3 to operate as an anion exchanger. Estrone sulfate, however, was unable to *trans*-stimulate glutarate efflux (Bakhiya et al. 2003), explaining why Cha et al. (2001) and Kusuhara et al. (1999) did not observe estrone sulfate self-exchange in rat and human OAT1-expressing oocytes. Independently of us, Sweet et al. (2003) found that rat OAT3 is an organic anion/dicarboxylate exchanger. We therefore hypothesize that OAT1 and OAT3 are energized by the in-to-out α-ketoglutarate concentration difference and operate as OA importers at the basolateral cell membrane.

The *trans*-stimulation of glutarate efflux by unlabeled urate (Bakhiya et al. 2003) indicated that urate is an endogenous substrate of OAT3. Transport of radiolabeled urate by human OAT3 was demonstrated earlier (Cha et al. 2001), and a low-affinity urate transport by OAT1 was shown most recently (Ichida et al. 2003). Our experiments suggest that, compared to OAT1, OAT3 has the higher affinity for urate and may be the predominant transporter involved in urate secretion.

OATs transport potentially cytotoxic substances and thus contribute to selective damage of proximal tubules. Transport of ochratoxin A by OATs is well documented (e.g. Jung et al. 2001). In contrast, the transport mechanism of aristolochic acid (AA) was so far unknown. AA causes the "Chinese herbs nephropathy" (CHN), which is characterized by severe interstitial renal fibrosis, quickly progressing renal failure, and by the occurrence of urothelial tumors (Arlt et al. 2002). One of the first signs of CHN is the urinary excretion of low molecular weight proteins such as α_1-microglobulin (Kabanda et al. 1995), suggesting proximal tubular damage as the initial event. We show here that AA is a potent inhibitor of human OAT1. These data suggest that OAT1 may be involved in selective AA delivery to proximal tubule cells.

Taken together, OAT1 and OAT3 take up organic anions into proximal tubule cells. Due to their broad substrate specificity they can handle chemically unrelated compounds and provide an effective means for the renal excretion of anionic drugs. The broad specificity, however, also implies that nephrotoxic compounds are accumulated in proximal tubule cells. By coadministration of drugs interacting with the OATs such as probenecid or non-steroidal anti-inflammatory drugs it should be possible to decrease the risk of proximal tubular damage during treatment with nephrotoxic agents.

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Prof. Dr. Gerhard Burckhardt
Abteilung Vegetative Physiologie und Pathophysiologie
Georg-August-Universität Göttingen
Humboldtallee 23
37073 Göttingen
Germany
Phone: +49 551 395881
Fax: +49 551 395883
E-Mail: gburckh@gwdg.de

Molecular Investigation of Metabolism and Transport of Drugs – From Animal to Human Tissue

Leopoldina-Symposium,
gemeinsam veranstaltet von der Europäischen Gesellschaft für Biochemische Pharmakologie (ESBP) und der Deutschen Akademie der Naturforscher Leopoldina vom 27. bis 29. September 2001 in Halle (Saale)

Herausgegeben von Johannes Doehmer (München), Christian Fleck (Jena), Dietrich Keppler (Heidelberg), Dieter Müller (Jena) und Karl J. Ullrich (Frankfurt/Main)
Nova Acta Leopoldina N. F., Band *87*, Nr. 329
(2003, 377 Seiten, 94 Abbildungen, 40 Tabellen, 37,80 Euro, ISBN 3-8047-2032-3)

In den vergangenen vier Jahren ist es zu einem enormen Erkenntniszuwachs sowohl auf dem Gebiet des Arzneimittelmetabolismus als auch im Zusammenhang mit dem Transport von Medikamenten im Organismus und ihrer Ausscheidung gekommen. Viele Details über Biotransformationsenzyme, die Arzneimittel abbauen, und über Carrier, die Medikamente im Organismus transportieren und über den Harn und die Galle ausscheiden, erlauben nunmehr konkrete Aussagen zu Struktur und Funktion. Die Brisanz der Thematik hatte sich erst kurz vor der Veranstaltung in der Affäre um das Lipobay® gezeigt, die hätte verhindert werden können, wenn sich die Ärzte, die die Substanz einsetzten, besser über jene jetzt bekannten Wechselwirkungen in Kombination mit anderen Pharmaka informiert hätten.
Der Band behandelt in 37 ausführlicheren Abhandlungen und 19 Kurzfassungen das wichtige Gebiet des Arzneimitteltransportes in Niere und Leber – ein Aspekt, der von wesentlicher Bedeutung auch für das Verständnis des Arzneimittelmetabolismus ist. Pharmaka müssen zunächst in die Leberzellen hinein transportiert werden, um dort metabolisiert werden zu können. Anschließend ist ein Austransport der Metabolite Voraussetzung für die endgültige Ausscheidung der Abbauprodukte über den Harn bzw. die Galle. Die Beiträge konzentrieren sich auf drei Schwerpunkte, nämlich die Biotransformation von Arzneimitteln und Fremdstoffen, den renalen und hepatischen Transport von Arzneimitteln und Fremdstoffen sowie die Interaktionen zwischen Biotransformationsprozessen und Transportvorgängen in Niere und Leber. Die Beiträge demonstrieren vor allem die Verflechtung der Biotransformationsprozesse und Transportvorgänge in Niere und Leber in mannigfaltiger Weise. Die Existenz vielfältiger Parallelen und die gegenseitige Bedingtheit von Metabolismus und Transport lieferten neue Erkenntnisse.

In Kommission bei Wissenschaftliche Verlagsgesellschaft mbH Stuttgart

Nova Acta Leopoldina NF *89*, Nr. 336, 139–143 (2004)

K_VLQT1 – a Modular K^+ Channel in Epithelia and Heart

Markus Bleich (Kiel)

Abstract

The potassium channel K_VLQT1 (KCNQ1) together with its regulatory subunit KCNE1 (minK, IsK) is responsible for the cardiac delayed rectifier current I_{Ks}. Mutations in the respective gene KCNQ1 are one cause of the congenital long QT syndrome, an arrhythmogenic disorder that entails a high risk of sudden cardiac death. Interestingly, the function of this channel complex is not only limited to the heart. Loss-of-function mutations in the human KCNQ1 gene, for example, are also related with bilateral deafness. Similarly, KCNQ1 (–/–) mice are deaf and exhibit a shaker/waltzer phenotype and the morphological consequences of a reduced endolymph production.

Meanwhile there is a body of evidence that K_vLQT1 potassium channels play important roles in almost all epithelia investigated so far. To hit the specific functional needs these channels act as modular components together with other regulatory subunits from the KCNE family to form currents which are either instantaneous or time and voltage dependent.

The discovery of K_vLQT1 potassium channels took place some time before their molecular cloning. They have been characterized by experiments in the colon. Later, two discrepancies indicated that K_VLQT1 and KCNE1 were not the only players *in vivo*: the K_VLQT1 potassium current in expression systems had a different pharmacological profile and the K_VLQT1/KCNE1 potassium current in expression systems showed kinetic properties which were completely different from those observed in colon epithelial cells. Additional subunits were the likely explanation for these observations since the channel K_VLQT1 itself was identical in colon and heart. Only the development of specific inhibitors of K_VLQT1 potassium channels, like the chromanol 293B, and the use of these inhibitors in native tissues as well as in expression systems brought the epithelial and cardiac parts of that puzzle together: K_VLQT1 co-assembles with different regulatory proteins in a tissue specific manner to form the functional potassium channel.

A variety of additional experiments have been performed to further pin down the functions of K_VLQT1 and its regulatory subunits in epithelia. Already today it is evident that K_VLQT1 is one of the key proteins for epithelial function and comprises a nice example for the concept of modular ion channel composition.

Zusammenfassung

Der Kalium-Kanal K_VLQT1 (KCNQ1), zusammen mit seiner Regulationsuntereinheit KCNE1 (minK, IsK), ist für den Herzstrom I_{Ks} verantwortlich. Mutationen im verantwortlichen Gen KCNQ1 sind ein Grund des kongenitalen langen QT-Syndroms, einer arhythmischen Störung, die ein hohes Risiko für einen plötzlichen Herztod darstellt. Interessanterweise beschränkt sich die Funktion dieses Kanalkomplexes nicht nur auf das Herz. Mutationen mit Funktionsverlust im menschlichen KCNQ1-Gen sind z. B. mit bilateraler Taubheit verbunden. Ebenso sind KCNQ1 (–/–)-Mäuse taub und weisen sowohl einen Schüttler/Walzer-Phänotyp als auch die morphologischen Folgen einer reduzierten Produktion von Endolymphe auf.

Mittlerweile gibt es Hinweise, daß K_vLQT1-Kalium-Kanäle wichtige Rollen in fast allen bisher untersuchten Epithelien spielen. Um den spezifischen funktionellen Erfordernissen gerecht zu werden, wirken diese Kanäle als modulare Komponenten mit anderen Regulationsuntereinheiten der KCNE-Familie zusammen, um Ströme auszubilden, die entweder momentan auftreten oder zeit- und spannungsabhängig sind.

Die Entdeckung der K_vLQT1-Kalium-Kanäle fand einige Zeit vor dem molekularen Klonen statt. Sie sind zunächst durch Experimente im Dickdarm charakterisiert worden. Später zeigten zwei Diskrepanzen, daß K_vLQT1 und KCNE1

nicht die einzigen beteiligten Komponenten *in vivo* waren: Der K_vLQT1-Kalium-Strom hatte in Expressionssystemen ein abweichendes pharmakologisches Profil. Der K_vLQT1/KCNE1-Kanal-Strom in Expressionssystemen zeigte kinetische Eigenschaften, die sich vollständig von denen, die in den Epithelzellen des Dickdarms beobachtet worden waren, unterschieden. Zusätzliche Untereinheiten waren die wahrscheinlichste Erklärung für diese Beobachtungen, da der Kanal K_vLQT1 selbst in Dickdarm und Herz identisch war. Nur die Entwicklung von spezifischen Inhibitoren der K_vLQT1-Kalium-Kanäle, wie Chromanol 293B, und die Anwendung dieser Inhibitoren in natürlichen Geweben und in Expressionssystemen brachten die epithelialen und kardialen Teile dieses Puzzles zusammen: K_vLQT1 setzt sich aus verschiedenen Regulationsproteinen in einer gewebespezifischen Art zusammen, um den funktionellen Kalium-Kanal zu bilden.

Eine Reihe weiterer Experimente sind durchgeführt worden, um die Funktionen von K_vLQT1 und seiner Regulationsuntereinheiten in Epithelien aufzuklären. Bereits heute ist es unzweifelhaft, daß K_vLQT1 eines der Schlüsselproteine für die Epithelfunktion und ein schönes Beispiel für das Konzept der modularen Ionenkanal-Komposition ist.

The potassium channel K_VLQT1 (KCNQ1) together with its regulatory subunit KCNE1 (minK, IsK) is responsible for the cardiac delayed rectifier current I_{Ks} (Barhanin et al. 1996, Sanguinetti et al. 1996). Mutations in the respective gene KCNQ1 are one cause of the congenital long QT syndrome, an arrhythmogenic disorder that entails a high risk of sudden cardiac death. Interestingly, the function of this channel complex is not only limited to the heart. Loss-of-function mutations in the human KCNQ1 gene, for example, are also related to bilateral deafness. Similarly, KCNQ1 (−/−) mice are deaf and exhibit a shaker/waltzer phenotype and the morphological consequences of a reduced endolymph production (Lee et al. 2000).

Meanwhile there is a body of evidence that K_vLQT1 potassium channels play important roles in almost all epithelia investigated so far. To hit the specific functional needs these channels act as modular components together with other regulatory subunits from the KCNE family to form currents which are either instantaneous, or time and voltage dependent.

The discovery of K_VLQT1 potassium channels took place some time before their molecular cloning. They have been characterized by experiments in the colon (Lohrmann et al. 1995, Warth and Bleich 2000). Later, two discrepancies indicated that K_VLQT1 and KCNE1 were not the only players *in vivo*:

(*i*) the K_VLQT1 potassium current in expression systems had a different pharmacological profile and
(*ii*) the K_VLQT1/KCNE1 potassium current in expression systems showed kinetic properties which were completely different from those observed in colon epithelial cells.

Additional subunits were the likely explanation for these observations since the channel K_VLQT1 itself was identical in colon and heart. Only the development of specific inhibitors of K_VLQT1 potassium channels, like the chromanol 293B (Bleich and Warth 2000, Gerlach et al. 2001, Greger et al. 1995), and the parallel use of these inhibitors in native tissues and in expression systems brought the epithelial and cardiac parts of that puzzle together: K_VLQT1 co-assembles with different regulatory proteins in a tissue specific manner to form the functional potassium channel.

In addition to the distinct kinetics and different sensitivities to drugs, divergent responses to changes in intracellular pH and temperature of the human K_VLQT1 were observed, depending on the presence of its regulatory subunit KCNE1. The half-activation time was temperature dependent for K_VLQT1/KCNE1 currents while there was no change in currents from K_VLQT1 alone. Similarly, the effect of mefenamic acid, originally described as an activator of I_{Ks} (K_VLQT1/KCNE1), depended on temperature and subunit composition. This indicates a complex

interaction of the subunit with the channel in generating its rather slow activation time constant. The pH dependence of K_VLQT1 might be of clinical and pathophysiological relevance in the pathogenesis of ischemic cardiac arrhythmias as well as in epithelial function. Acidification of cytosolic pH increased K_VLQT1/KCNE1 currents but decreased currents from K_VLQT1 alone. Intracellular alkalinization had the opposite effect (Unsöld et al. 2000).

Meanwhile the subunits KCNE2 and KCNE3 have been characterized for their expression and influence on the properties of K_VLQT1 currents. Interaction of KCNE2 with K_VLQT1 results in a drastic change of KCNQ1 current amplitude and gating properties. While mutations in KCNE2 are associated with cardiac arrhythmias and KCNE2 is largely expressed in gastrointestinal epithelia as well, KCNE3 does not seem to be important for cardiac function. KCNE3 is the subunit of K_VLQT1 in the colon. It changes its properties to an instantaneously opening potassium channel of very low conductance and linear current-voltage relationship.

K_VLQT1 mRNA expression is widely distributed in epithelial tissues, in the absence (stomach, large and small intestine, lung, liver, thymus) or presence (stria vascularis, kidney, exocrine pancreas) of its regulator KCNE1. The high level of K_VLQT1 expression in epithelial tissues is consistent with its potential role in maintaining the resting potential, and in regulating chloride secretion and sodium absorption. In chloride secretory epithelia, such as the colon and pancreas, this potassium channel provides the driving force for chloride exit and is located in the basolateral membrane. In the inner ear it enables luminal secretion of potassium into the endolymphatic space. The functional relevance of K_VLQT1 to epithelial function is revealed by blocking it pharmacologically or by studying the respective knockout animals.

In the stomach, H^+ secretion *via* the H^+/K^+-ATPase is coupled to the uptake of K^+. K_VLQT1 is located in tubulovesicles and apical membrane of parietal cells, where it co localizes with H^+/K^+ATPase. Blockade of K_VLQT1 current by 293B inhibits acid secretion. The putative K_VLQT1 subunits, KCNE2 and KCNE3, are strongly expressed in human stomach. KCNE1, however, is absent in stomach epithelial cells. Interestingly, the K_VLQT1/KCNE2 complex is activated by low extracellular pH which makes it perfectly suited to act in the acidic luminal milieu of gastric glands. KCNE3 and KCNE2 are both candidates to co assemble with K_VLQT1 in parietal cells. Thus, stomach- and subunit-specific inhibitors of K_VLQT1 might offer new therapeutical perspectives for peptic ulcer disease (Grahammer et al. 2001a). From findings in mice defective for K_VLQT1 this channel also might play a role in addition to acid secretion, however, the reason for the interesting finding of neck cell hyperplasia is still unknown.

In the respiratory tract, ion transport defects underlying cystic fibrosis (CF) lung disease are characterized by impaired cyclic adenosine monophosphate (cAMP)-dependent chloride conductance. Activation of chloride secretion in airways depends on simultaneous activation of luminal chloride channels and basolateral potassium channels. In native human airway epithelium CF tissues showed typical alterations of short-circuit currents with enhanced amiloride-sensitive sodium conductance and defective cAMP-mediated chloride conductance. In non-CF tissues, chloride secretion was significantly inhibited by the chromanol 293B. Inhibition was increased after cAMP-dependent stimulation. Analysis of reverse-transcribed messenger RNA from non-CF and CF airways showed expression of human K_VLQT1. Hence the potassium channel K_VLQT1 is important in maintaining cAMP-dependent chloride secretion in human airways. Activation of K_VLQT1 in CF airways in parallel with stimulation of residual CF transmembrane conductance regulator chloride channel activity or alternative chloride channels could help to circumvent the secetory defect. Recent investigations of mouse tracheal epithelium showed that KCNE1 is not expressed and that chloride secretion in these

cells is not reduced by a complete knockout of the KCNE1 gene. In contrast it was shown that KCNQ1 and KCNE3 form a basolateral potassium channel in these epithelial cells which contributes to cAMP- as well as Ca^{2+}-stimulated chloride secretion. Furthermore, it is also involved in sodium absorption *via* ENaC. At least in mice the channel complex formed by KCNQ1/KCNE3 seems to be the dominant basolateral K^+-conductance in tracheal epithelial cells (Grahammer et al. 2001b).

In the exocrine pancreas secretagogue-activated potassium conductance is indispensable for the electrogenic chloride secretion. The effect of secretin and other cAMP-mediated secretagogues acts directly *via* the slowly activating voltage-dependent potassium current (I_{Ks}). Upon depolarization, pancreatic acini showed I_{Ks} superimposed upon instantaneous background outward current. Secretin, vasoactive intestinal peptide, forskolin, isoprenaline or IBMX, increased the amplitude of I_{Ks}. At physiological concentration of secretin its effect was significantly enhanced by transient co-stimulation with carbachol. Hence in the exocrine pancreas I_{Ks} (K_VLQT1/KCNE1) is a common target for both Ca^{2+}- and cAMP-agonists. Vagal stimulation under the physiological concentration of secretin facilitates I_{Ks}, which can provide additional driving force for Cl^- secretion (Kim et al. 2001, Kim and Greger 1999, Warth et al. 2002).

Currently a variety of additional experiments are performed to further pin down the functions of K_VLQT1 and its regulatory subunits in epithelia. Already today it is evident that K_VLQT1 is one of the key proteins for epithelial function and comprises a nice example for the concept of modular ion channel composition.

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Prof. Dr. Markus Bleich
Christian-Albrechts-Universität Kiel
Ohlshausenstraße 40
24098 Kiel
Germany
Phone: +49 431 8802961
Fax: +49 431 8804580
E-Mail: m.bleich@physiologie.uni-kiel.de

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Ion Exchangers Mediating Na^+, HCO_3^- and Cl^- Transport in the Renal Proximal Tubule

Peter S. Aronson (New Haven)

With 1 Figure

Abstract

The majority of the Na^+, Cl^- and HCO_3^- filtered by the kidney is reabsorbed in the proximal tubule. Our focus has been on defining the molecular identities and the physiological contributions of apical membrane ion exchangers in this nephron segment. Evidence suggests that NHE3 is the principal Na^+-H^+ exchanger isoform involved in mediating acid secretion across the apical membrane. NHE8 is a newly identified isoform also expressed in the proximal tubule, but its function is yet to be defined. Cl^- absorption across the apical membrane of proximal tubule cells takes place at least in part by Cl^--formate exchange operating in parallel with Na^+-H^+ exchange and H^+-coupled formate recycling, and by Cl^--oxalate exchange in parallel with Na^+-sulfate cotransport and sulfate-oxalate exchange. CFEX (SLC26A6) is a recently identified anion exchanger that is expressed on the apical membrane of proximal tubule cells and that is capable of mediating many of the anion exchange activities described at this site, including Cl^--formate exchange, Cl^--oxalate exchange, sulfate-oxalate exchange and Cl^--HCO_3^- exchange.

Zusammenfassung

Die Mehrheit der Na^+-, Cl^-- und HCO_3^--Ionen, die durch die Niere herausgefiltert werden, werden im proximalen Tubulus wieder zurückgewonnen. Wir fokussierten unsere Bemühungen darauf, die molekulare Identität und die physiologischen Beiträge der einzelnen Ionenaustauscher in der apikalen Membran dieses Nephron-Segments aufzuklären. Die Befunde belegen, daß NHE3 die Hauptisoform der Na^+ – H^+-Austauscher ist, die in der Säure-Sekretion durch die Apikalmembran mitwirken. NHE8 ist eine neu identifizierte Isoform, die auch im proximalen Tubulus exprimiert wird, deren Funktion jedoch noch nicht aufgeklärt ist. Die Cl^--Aufnahme durch die Apikalmembran der Proximal-Tubulus-Zellen findet letztendlich zumindest zum Teil durch Cl^- – Formiat-Austausch, parallel zum Na^+ – H^+-Austausch und zum H^+-gekoppelten Formiat-Recycling, und durch Cl^- – Oxalat-Austausch, parallel mit Na^+ – Sulfat-Cotransport und Sulfat – Oxalat-Austausch, statt. CFEX (SLC26A6) ist ein erst kürzlich identifizierter Anion-Austauscher, der in der Apikalmembran der proximalen Tubuluszellen exprimiert wird und der in der Lage ist, viele der Anionen-Austausch-Aktivitäten, die hier beschrieben worden sind, einschließlich Cl^- – Formiat-Austausch, Cl^- – Oxalat-Austausch, Sulfat – Oxalate-Austausch und Cl^- – HCO_3^--Austausch, zu vermitteln.

1. Introduction

The majority of the Na^+, Cl^- and HCO_3^- filtered by the kidney is reabsorbed in the proximal tubule. Our initial studies on this subject used isolated brush border membrane vesicles as an experimental system to define transport activities physiologically. In recent years, we have sought the molecular identities of the apical membrane transporters responsible for these transport activities, and we have attempted to assess the contributions of these molecularly defined pathways to mediating net electrolyte transport in the proximal tubule under physiologic conditions.

2. Na^+-H^+ Exchange

The presence of Na^+-H^+ exchange activity in brush border vesicles isolated from rat renal cortex was first demonstrated by Murer and colleagues over 25 years ago (Murer et al. 1976). We used a similar preparation of microvillus membrane vesicles isolated from rabbit renal cortex to confirm and extend these findings (Kinsella and Aronson 1980). We demonstrated that this transporter is sensitive to inhibition by amiloride and that it can function in additional modes such as to mediate Na^+-NH_4^+ exchange (Kinsella and Aronson 1981a, b). Apical membrane Na^+-H^+ exchange operating in series with basolateral Na^+-HCO_3^- cotransport would most obviously play a role in mediating HCO_3^- reabsorption in the proximal tubule, as shown in Figure 1.

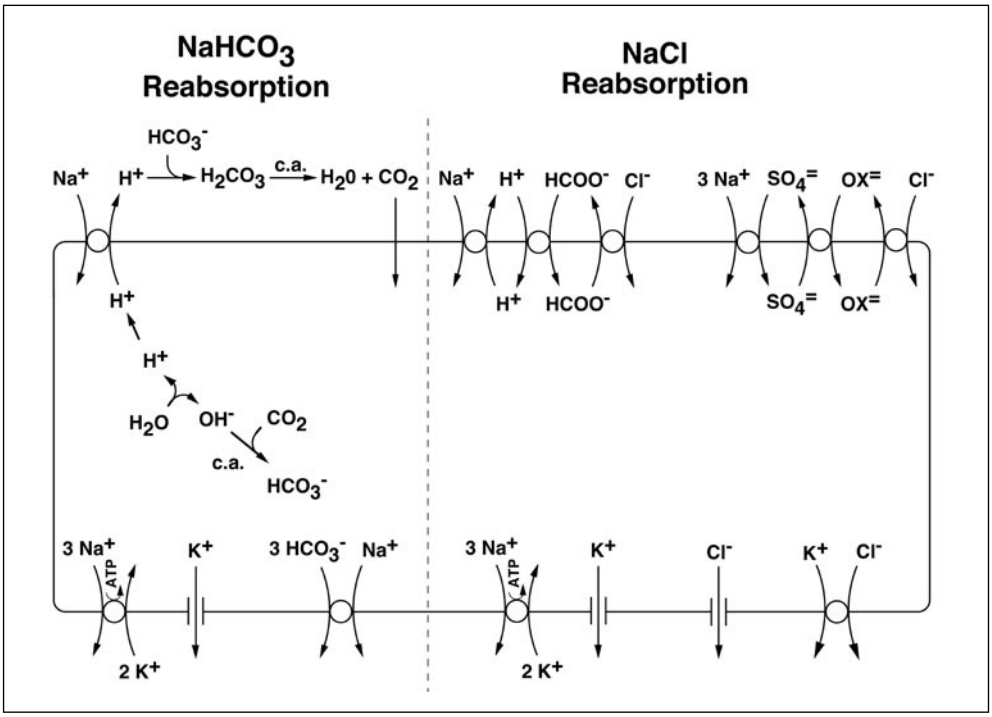

Fig. 1 Principal mechanisms mediating transcellular $NaHCO_3$ and NaCl reabsorption in the proximal tubule.

The key breakthrough in the molecular identification of Na^+-H^+ exchange proteins was the cloning by Pouyssegur and colleagues of NHE1 (Sardet et al. 1989). By homology cloning, additional isoforms were then identified by the groups of Shull and Donowitz (Orlowski et al. 1992, Tse et al. 1992, 1993). As a strategy to identify which NHE isoform(s) are expressed in proximal tubule cells, we raised polyclonal and/or monoclonal antibodies to unique regions of different NHE isoforms (Biemesderfer et al. 1992, 1993, 1997). By immunofluorescence microscopy and immunoblotting of membrane fractions, we identified expression of at least two NHE isoforms in proximal tubule cells. Expression of NHE1 was detected along the basolateral membrane in the proximal tubule and in multiple other nephron segments (Biemesderfer et al.

1992). In contrast, NHE3 was observed on the apical surface in the proximal tubule as well as the loop of HENLE (BIEMESDERFER et al. 1993, 1997).

Having identified NHE3 expression on the apical membrane of proximal tubule cells, we then used different approaches to assess the contribution of NHE3 to mediating apical membrane Na^+-H^+ exchange and transtubular HCO_3^- absorption in this segment of the nephron. One approach took advantage of the observation that different NHE isoforms have unique patterns of sensitivity to inhibitors (COUNILLON et al. 1993, SCHWARK et al. 1998). The profile of observed inhibitor sensitivity suggested that NHE3 accounts for virtually all of the measured Na^+-H^+ exchange activity in isolated brush border membrane vesicles (WU et al. 1996). Similarly, the profile of sensitivity to inhibitors suggested a major role for NHE3 in mediating volume and HCO_3^- absorption in microperfused proximal tubules (WANG et al. 2001). Interestingly, the pattern of inhibitor sensitivity suggested that NHE2 rather than NHE3 mediates HCO_3^- absorption in the microperfused distal convoluted tubule (WANG et al. 2001), in accord with immunocytochemical studies indicating that NHE2 rather than NHE3 is expressed on the apical membrane in this portion of the nephron (CHAMBREY et al. 1998).

Another approach to evaluate the role of NHE3 in proximal tubule physiology was to make use of NHE3 null mice generated by SHULL's group (SCHULTHEIS et al. 1998). We found that the rates of fluid and HCO_3^- absorption were reduced by about 60 % in microperfused tubules of NHE3 null compared to wild-type mice (SCHULTHEIS et al. 1998), confirming a major role for NHE3 in mediating $NaHCO_3$ reabsorption in the proximal tubule. The component of HCO_3^- reabsorption that persists in NHE3 null mice was completely insensitive to the Na^+-H^+ exchange inhibitor ethylisopropylamiloride (EIPA) (WANG et al. 1999), arguing against the involvement of NHE2, the only other NHE isoform known to be expressed on the apical membrane of epithelial cells. However, the vacuolar H^+ATPase inhibitor bafilomycin caused about 60 % inhibition of the HCO_3^- reabsorption that persists in NHE3 null mice, indicating a small but significant role for primary active H^+ secretion in the proximal tubule (WANG et al. 1999). Although we could not detect any component of transtubular HCO_3^- absorption inhibited by EIPA in NHE3 null mice, BAUM and colleagues observed significant EIPA-sensitive, Na^+-dependent acid extrusion across the apical membrane of proximal tubule cells in mice null for both NHE3 and NHE2, raising the possibility that a novel NHE isoform may be expressed at this site (CHOI et al. 2000).

To identify additional NHE isoforms that might be expressed in the proximal tubule, we searched DNA databases for partial sequences with homology to, but not identity with, known NHE isoforms. We were successful in identifying a partial length cDNA encoding a novel NHE isoform, and RACE PCR was used to obtain the complete coding sequence from mouse kidney cDNA (GOYAL et al. 2003). The sequence encoded a predicted protein of 576 amino acids, which we named NHE8. NHE8 has only 25 – 29 % overall amino acid identity to known mammalian NHEs 1 – 7, but, surprisingly, has greatest homology with *Drosophila* "NHE1" (54 % identity) and a related gene product in *Anopheles* (58 % identity). Thus, NHE8 most likely represents the mammalian orthologue of this invertebrate NHE isoform. Hydropathy analysis of the encoded NHE8 protein predicted that NHE8 has a ~450 amino acid N-terminal hydrophobic domain with 10 – 12 transmembrane domains as found in other NHE isoforms, but has a much shorter C-terminal hydrophilic tail (~100 amino acids) compared to other isoforms (200 – 350 amino acids). Nevertheless, the C-terminal domain may be involved in regulation of NHE8 as it contains a possible protein kinase C-epsilon phosphorylation site, as well as putative SH2, SH3, ERK and PDZ type III binding domains (GOYAL et al. 2003).

Northern analysis indicated that NHE8 is ubiquitously expressed in mouse tissues, with highest levels in the kidney as well as in testis, skeletal muscle and liver. We then utilized *in situ* hybridization to determine the cellular sites of NHE8 transcript expression in mouse kidney. We found that the NHE8 message is expressed most intensely in a region corresponding to the outer stripe of the outer medulla, with lower but significant expression diffusely throughout the renal cortex. No NHE8 expression was detected in the inner stripe or the inner medulla. Silver grains corresponding to NHE8 message were observed within tubules possessing a brush border, confirming expression in proximal tubules (Goyal et al. 2003).

Having successfully identified NHE8 as a novel NHE isoform expressed in proximal tubule cells, we next examined its subcellular localization. We generated an anti-NHE8 antibody for Western blot analysis of rat renal cortical membrane fractions. We observed that the enrichment of NHE8 in brush border membranes relative to the starting homogenate was similar to that observed for NHE3, indicating that NHE8 is either a brush border protein like NHE3 or resides in a membrane compartment that co-purifies with brush border membranes (Goyal et al. 2003). Future studies will be directed at confirming apical membrane expression of NHE8 in proximal tubules by immunocytochemistry, and characterizing its ion transport properties by functional expression experiments.

3. Cl^--base Exchange

Although a major component of Cl^- reabsorption in the proximal tubule is passive and paracellular, several lines of evidence indicated an important contribution of transcellular mechanisms (Aronson and Giebisch 1997). We therefore used rabbit renal brush border membrane vesicles to test for the presence of mechanisms for apical Cl^- entry that had previously been found in other nephron segments and in other epithelia. We could not detect significant Na^+-Cl^- or Na^+-K^+-$2Cl^-$ cotransport, or Cl^--OH^- or Cl^--HCO_3^- exchange (Seifter et al. 1984). But we did find appreciable Cl^--formate and Cl^--oxalate exchange activities in renal brush border membrane vesicles (Karniski and Aronson 1985, 1987). We demonstrated that Cl^--oxalate exchange is electrogenic, whereas Cl^--formate exchange is electroneutral (Karniski and Aronson 1987).

Given the submillimolar concentrations of formate and oxalate in biologic fluids, we reasoned that Cl^--formate exchange and Cl^--oxalate exchange could only mediate substantial quantities of Cl^- absorption across the apical membrane of proximal tubule cells if there were mechanisms available to recycle these organic anions from the lumen back into the cell and thereby to continuously replenish their intracellular concentrations.

When we screened for possible mechanisms of lumen to cell formate transport, we could not demonstrate direct Na^+-formate cotransport, but instead found that imposing an inward H^+ gradient markedly stimulated formate uptake and caused its transient uphill accumulation (Karniski and Aronson 1985, Saleh et al. 1996). The kinetics of this pH-dependent formate transport suggested a mediated process of H^+-formate cotransport or OH^--formate exchange rather than simple nonionic diffusion (Saleh et al. 1996). These findings suggested a model by which formate might facilitate NaCl entry across the apical membrane of proximal tubule cells (Karniski and Aronson 1985), as illustrated in Figure 1. The inward H^+ gradient generated by apical membrane Na^+-H^+ exchange would drive filtered formate into the cell by H^+-coupled transport, shown in Figure 1 as H^+-formate cotransport. The outward formate gradient would then drive Cl^- entry by Cl^--formate exchange.

We similarly evaluated possible mechanisms to mediate lumen to cell transport of oxalate. Although we did not detect significant Na^+-oxalate cotransport, we did find that oxalate is capable of exchanging for sulfate in renal brush border vesicles (Kuo and Aronson 1996). These findings suggested a model by which oxalate might facilitate NaCl entry across the apical membrane of proximal tubule cells (Kuo and Aronson 1996), as also illustrated in Figure 1. Uphill sulfate absorption by Na^+-sulfate cotransport would generate an outward sulfate gradient that could then drive oxalate uptake by sulfate-oxalate exchange. The outward oxalate gradient would then drive Cl^- entry by Cl^--oxalate exchange.

It was important to test the models of NaCl transport shown in Figure 1 and to determine whether formate and oxalate actually promote transtubular NaCl reabsorption. Indeed, we found that addition of formate or oxalate strongly stimulated the rate of transtubular NaCl absorption when tubules were perfused with a low HCO_3^-, high Cl^- solution as occurs physiologically in the late proximal tubule (Schild et al. 1987, Wang et al. 1992). The increments in NaCl absorption induced by formate and oxalate were completely inhibited by luminal addition of the anion exchange inhibitor DIDS, which had no effect on the baseline rate of NaCl absorption (Wang et al. 1992).

We also conducted experiments to test the proposed mechanisms of formate and oxalate recycling that are illustrated in Figure 1 (Wang et al. 1996). Luminal application of the Na^+-H^+ exchange inhibitor EIPA abolished stimulation of Cl^- absorption by formate, but had no effect on stimulation by oxalate. Conversely, oxalate stimulation of Cl^- absorption required the presence of sulfate in the perfusion solutions, but stimulation by formate was sulfate-independent. These observations strongly supported the models for NaCl reabsorption in Figure 1 in which formate recycling is dependent on Na^+-H^+ exchange, and oxalate recycling occurs by Na^+-sulfate cotransport in parallel with sulfate-oxalate exchange. Further supporting these models were the findings that formate-stimulated Cl^- absorption was abolished in NHE3 null mice, whereas oxalate-stimulated transport was unaffected by the absence of NHE3 (Wang et al. 2001).

The molecular identification of the apical membrane anion exchanger(s) mediating proximal tubule Cl^- reabsorption has been elusive. An important advance toward this goal was the discovery by Karniski and coworkers that pendrin (SLC26A4), a member of the SLC26 "sulfate transporter" family, actually is a monovalent anion exchanger with the ability to mediate Cl^--formate exchange (Scott et al. 1999, Scott and Karniski 2000). We therefore evaluated pendrin as a candidate to mediate apical membrane Cl^--formate exchange in the proximal tubule. We generated an anti-pendrin antibody to study immunocytochemical localization of pendrin in the kidney. We could detect no staining for pendrin in proximal tubule cells, but found pendrin expression on the apical membrane of a subpopulation of cells in the collecting tubule (Knauf et al. 2001). A more detailed analysis of pendrin kidney expression was performed by Royaux and coworkers who identified this cell population as non-alpha intercalated cells mediating HCO_3^- secretion in the cortical collecting tubule (Royaux et al. 2001). A significant functional role for pendrin in mediating proximal tubule Cl^- absorption was effectively ruled out by our studies with Karniski indicating that there is no reduction in either brush border membrane Cl^--formate exchange or formate-stimulated NaCl absorption in pendrin null mice (Karniski et al. 2002).

As a strategy to identify additional candidate anion exchangers capable of mediating Cl^- reabsorption in the proximal tubule, we screened the EST database for homologues of pendrin (Knauf et al. 2001). We isolated a cDNA encoding a pendrin homologue expressed in kidney

that appeared to be the mouse orthologue of human SLC26A6, a putative anion exchanger of then unknown function (Lohi et al. 2000, Waldegger et al. 2001). We raised antibodies against the encoded protein and demonstrated its expression in the kidney by Western blot analysis and on the brush border membrane of proximal tubule cells by immunocytochemistry (Knauf et al. 2001).

We functionally expressed the transporter successfully in *Xenopus* oocytes (Knauf et al. 2001). When we found that it could mediate DIDS-sensitive Cl^--formate exchange we tentatively named it CFEX. But our subsequent studies demonstrated that CFEX (SLC26A6) can mediate additional anion exchange activities previously described in renal brush border membrane vesicles including electrogenic Cl^--oxalate exchange, and oxalate-sulfate exchange (Jiang et al. 2002). We (Jiang et al. 2002) and other groups (Wang et al. 2002, Xie et al. 2002) also found that SLC26A6 can mediate Cl^--HCO_3^- exchange. Measurements of intracellular pH had indicated that Cl^--HCO_3^- exchange activity is present on the apical membrane of proximal tubule cells (Kurtz et al. 1994, Sheu et al. 1995), although there is nephron heterogeneity in this regard (Sheu et al. 1995). Thus, SLC26A6 may account for this transport activity. However, we found that affinity of SLC26A6 for oxalate was the highest among the substrates tested. Indeed, we observed that a concentration of oxalate in the range found in renal tubular fluid and urine (100 μM) can significantly convert the function of SLC26A6 from Cl^--HCO_3^- exchange to Cl^--oxalate exchange (Jiang et al. 2002).

Accordingly, it is possible that in cells physiologically exposed to appreciable intracellular oxalate concentrations, as in the kidney, SLC26A6 may predominantly function as a Cl^--oxalate exchanger, whereas in the absence of such high oxalate concentrations, SLC26A6 may principally mediate Cl^--HCO_3^- exchange. SLC26A6 is expressed in a wide range of epithelial and non-epithelial tissues (Lohi et al. 2000, Knauf et al. 2001, Waldegger et al. 2001, Wang et al. 2002) in most of which it is likely to function as a Cl^--HCO_3^- exchanger. In fact, Muallem and coworkers have demonstrated activation of SLC26A6-mediated Cl^--HCO_3^- exchange by CFTR, and proposed that SLC26A6 may play a major role in mediating CFTR activated HCO_3^- secretion in epithelia (Ko et al. 2002). Future studies involving generation of SLC26A6 null mice will be essential for determining the role of this anion exchanger in mediating ion transport in the proximal tubule and in other epithelia.

Finally, it should be noted that the array of apical membrane ion exchangers illustrated in Figure 1 provides the proximal tubule with the ability to regulate $NaHCO_3$ and NaCl reabsorption independently despite a major role for Na^+-H^+ exchange in both processes. In particular, regulation of CFEX (SLC26A6) would determine the comparative rates of $NaHCO_3$ and NaCl reabsorption resulting from a given level of NHE3 activity. Differential regulation of NHE3 and SLC26A6 in the proximal tubule may thereby control the relative rates of $NaHCO_3$ and NaCl reabsorption in response to physiologic and pathophysiologic stimuli.

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Peter S. Aronson, M.D.
Section of Nephrology
Department of Medicine
Yale School of Medicine
P.O. Box 208029
New Haven, CT 06520-8029
USA
Phone: +1 203 7854902
Fax: +1 203 7854904
E-Mail: peter.aronson@yale.edu

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Epithelial Ion Transport in Inner Ear and Kidney

Thomas Böttger (Halle/Saale)

With 2 Figures

Abstract

Epithelial transport of ions is of great importance in kidney and the inner ear. I discuss two proteins involved in epithelial transport, the loss of function of which gives exciting new insights into the physiology of kidney and inner ear. Mutations in Barttin, an essential β-subunit of ClC-K chloride channels, reveal important functions in salt reabsorption of the kidney and in potassium secretion of the stria vascularis of the inner ear. The potassium/chloride cotransporter KCC4 is important for potassium recycling in the inner ear as well as for acid secretion of the kidney and hence for the acid-base balance of the organism.

Zusammenfassung

Epithelialer Transport ist von großer Bedeutung für die Funktion von Niere und Innenohr. In diesem Artikel werden zwei Proteine diskutiert, deren Funktionsverlustmutationen neue, interessante Einblicke in die Physiologie von Niere und Innenohr geben. Mutationen in Barttin, einer essentiellen β-Untereinheit von ClC-K-Chloridkanälen, zeigen die Funktion dieser Ionenkanäle bei der Salzabsorption der Niere und bei der Kaliumsekretion durch die Stria vascularis des Innenohrs. Der Kalium/Chlorid-Kotransporter KCC4 ist wichtig für das Kalium-Recycling im Innenohr ebenso wie für die Säuresekretion der Niere und damit für den Säure-Base-Haushalt des Organismus.

1. Introduction

Epithelial transport is of great importance for the function of the kidney as well as for the function of the inner ear. Therefore it is not surprising, that we find similar mechanisms, even the same molecules that are involved in both, hearing and kidney function. Among them are molecules involved in acid-base balance (Karet et al. 1999) or molecules involved in ion transport such as the Na/K/Cl Cotransporters NKCC1 involved in inner ear function (Delpire et al. 1999) and NKCC2 essential for renal function (Simon et al. 1997). Here, I concentrate on two recently characterized molecules that both have a role in hearing and kidney function.

2. K/Cl Cotransporter KCC4 and the Chloride Channel β-subunit Barttin Are Crucial for Inner Ear Function

Hearing critically depends on an electrochemical potential between the endolymph-filled scala media and the surrounding tissues of the inner ear. This electrochemical potential is the result

of a high potassium (K^+) concentration in the endolymph and an electrical potential of approx. +100 mV between the scala media and the surrounding tissues. The electrochemical gradient, that is generated in the highly vascularised stria vascularis, is the driving force for the processes in the organ of Corti required for hearing. Thus, there is a morphological segregation of the site where the electrochemical gradient is generated, and of the site where the gradient is used by excitable cells. This is a unique property of the inner ear (Fig. 1*A*). After sound stimuli K^+ from endolymph enters the apical membrane of the sensory hair cells in the organ of Corti through mechanosensitive channels. The K^+ influx depolarizes these cells. The depolarization is the trigger for synaptic transmission from hair cells and for motility of the outer hair cells (OHCs) as the basis of the sensitivity and frequency selectivity of mammalian hearing. K^+ is released from the hair cells into the organ of Corti following its electrochemical gradient *via* potassium channels, in the OHCs especially including the KCNQ4 potassium channel located in the basolateral membrane (Kharkovets et al. 2000) (Fig. 1*C*). The K^+ liberated within the organ of Corti is taken up by epithelial cells namely the Deiters' cells (DC) and is transported *via* a gap junction system (Cohen-Salmon et al. 2002) back to the stria vascularis in a potassium recycling pathway (Wangemann 2002). In addition there is a potential supply of potassium to the stria vascularis by the blood.

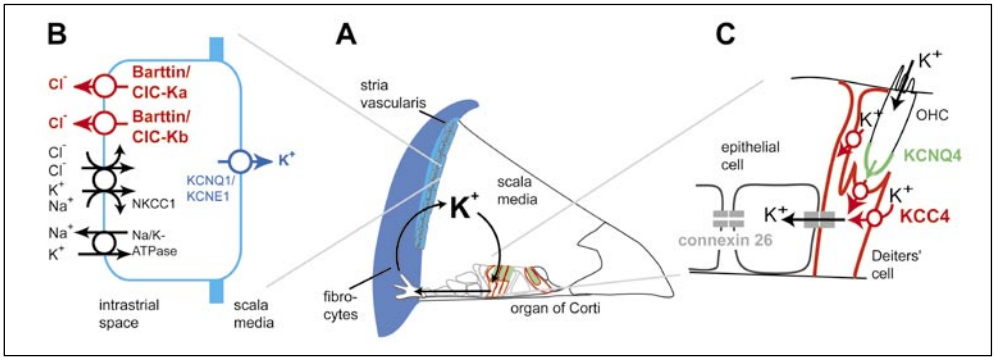

Fig. 1 Ion transport in the inner ear. (*A*) Scheme of a cochlear turn. Note the spatial segregation of generation (stria vascularis) and use (organ of Corti) of the K^+-electrochemical gradient. The scala media is filled with the K^+-rich endolymph (approx.: $[K^+]$ 150 mM). (*B*) marginal cell of the stria vascularis takes up K^+ from intrastrial space und secretes K^+ into the endolymph by active uptake through the action of basolaterally located Na/K ATPase and Na/K/Cl cotransporter and apical KCNQ1/KCNE1 potassium channels. Cl^- is recycled through basolateral Barttin/CLC-K channels. (*C*) Potassium enters OHCs from the endolymph through apical mechanosensitive channels and leaves the cells basolaterally through K^+ channels (KCNQ4). K^+ is then taken up by the KCC4 K/Cl cotransporter, transported *via* gap junctions, that connect epithelial cells and fibrocytes, back to the stria vascularis for re-secretion to endolymph.

KCC4 appears to be a main component for the uptake of K^+ leaving the sensory hair cells into the epithelial cells of the organ of Corti. Knock-out mice lacking KCC4 protein develop deafness due to degeneration of the OHCs within a week after onset of hearing. A complete loss of the Corti organ is seen during the following weeks. Surprisingly, KCC4 protein is not expressed in the OHCs, which degenerate first, but KCC4 protein is found at the supporting cells of the OHCs, the Deiters' cells (Fig. 1*C*) (Boettger et al. 2002). Loss of the OHCs and death of the respective supporting cells has also been observed in mice with a blocked K^+ flux

between the epithelial cells further downstream in the K^+-recycling pathway (Cohen-Salmon et al. 2002). It is suggested, that the rising K^+ concentration in the extracellular space surrounding the OHCs, due to the failure of K^+ removal, causes the death of the hair cells by membrane depolarization or osmotic stress. The K/Cl cotransporter KCC4 is especially suited to remove the K^+ from the extracellular space of the Corti organ. Because cotransporters like KCC4 are driven by the transmembrane gradients of the transported ions, this transport works without an additional energy source. This is compatible with the special morphology and function of the inner ear with a localization of the energy demanding processes to the stria vascularis and the withdrawal of metabolic stress from the sensory, highly specialized organ of Corti. The rising K^+ concentration in the extracellular space of the Corti organ during sound exposure (Johnstone et al. 1989) favors the influx of K^+ into the Deiters' cells through the cotransporter. Moreover, no other molecules are known that could mediate the uptake of K^+ into the Deiters' cells; where is no marked expression of Na/K ATPases (Spicer and Schulte 1998), nor of Na/K/Cl cotransporters (NKCCs), nor of suitable K^+ channels in these cells (Nenov et al. 1998).

K^+ is transported back to the stria vascularis, the highly vascularized, stratified tissue where the electrochemical potential is generated (Fig. 1*A*). The electrical potential is generated by the exit of potassium ions out of the intermediate cells into an intrastrial space *via* a K^+ channel. K^+ is then taken up by the marginal cells *via* the basolateral membrane through the combined action of a Na/K ATPase and NKCC1. Potassium leaves the marginal cells *via* the heteromeric K^+ channel KCNQ1/KCNE1 at the apical membrane into the endolymph (Fig. 1*B*). Mutation of NKCC1 (Delpire et al. 1999), KCNQ1 (Neyroud et al. 1997) or KCNE1 (Vetter et al. 1996) leads to deafness due to loss of potassium secretion. The combined action of Na/K ATPase, NKCC1 and the apical K^+ channel result in a net accumulation of Cl^- in the marginal cells. We suggest the heteromeric Cl^- channels ClC-Ka/Barttin and ClC-Kb/Barttin as the molecular basis for a Cl^- conductance that is needed for the function of the marginal cell (Fig. 1*B*). Mutation of barttin in the human Bartter syndrome type IV (BSND) results in deafness and renal failure (Birkenhäger et al. 2001). It has been shown that Barttin is an essential β-subunit for the ClC-Ka and ClC-Kb chloride channels (Estévez et al. 2001, Waldegger et al. 2002). Barttin is essential for the expression of ClC-K Cl^- channels at the plasmamembrane. Barttin and ClC-K proteins are co-expressed at the basolateral membrane of the marginal cells of the stria vascularis (Estévez et al. 2001). Both ClC-K1 and ClC-K2 are found in the inner ear. Probably this is the cause that no hearing loss is associated with mutations in ClC-Kb encoded by ClCNKB in Bartter syndrome type III. Here, in principle ClC-Ka/barttin could rescue the functional consequences of ClC-Kb/barttin loss. However, if the common subunit of both ClC-K chloride channels is not functional, a loss of function of both ClC-K channels is predicted. Hence, loss of barttin function would impair K^+ secretion due to accumulation of intracellular chloride in the marginal cells of the stria vascularis in accordance with the deafness observed in the human BSND syndrome.

3. Kidney Function of Barttin and KCC4

Bartter syndrome is characterized by a severe salt loss in the kidney. In the distal loop of Henle sodium chloride is taken up by the action of apical NKCC2 cotransporters and ROMK K^+ channels. Chloride leaves the cells basolaterally *via* ClC-K Cl^- channels (Fig. 2*A*, *B*). Mutation of NKCC2 (Simon et al. 1996a) and ROMK (Simon et al. 1996b) leads to Bartter syndrome

type I and II. Mutations of ClC-Kb (Simon et al. 1997) lead to Bartter syndrome type III. Loss of ClC-K1 (corresponding to human ClC-Ka) results in a diabetes insipidus-like phenotype in mice (Matsumura et al. 1999). Mutation of Barttin leads, besides of deafness, to a severe form of Bartter Syndrome (Birkenhäger et al. 2001). Barttin protein is found in several regions of the distal tubule of the kidney in mouse. It overlaps completely with the expression of ClC-K1 (Ka in humans) in the thin ascending limb of Henle's loop, and with ClC-K2 (Kb) in the thick ascending limb (TAL), the connecting tubule and the intercalated cells of the cortical collecting duct (CCD) (Estévez et al. 2001). With barttin being essential for the function of ClC-Ka and ClC-Kb, patients with BSND have a more severe renal phenotype than patients with ClCNKB mutations alone due to the additional loss of the ClC-Ka Cl^- channel function.

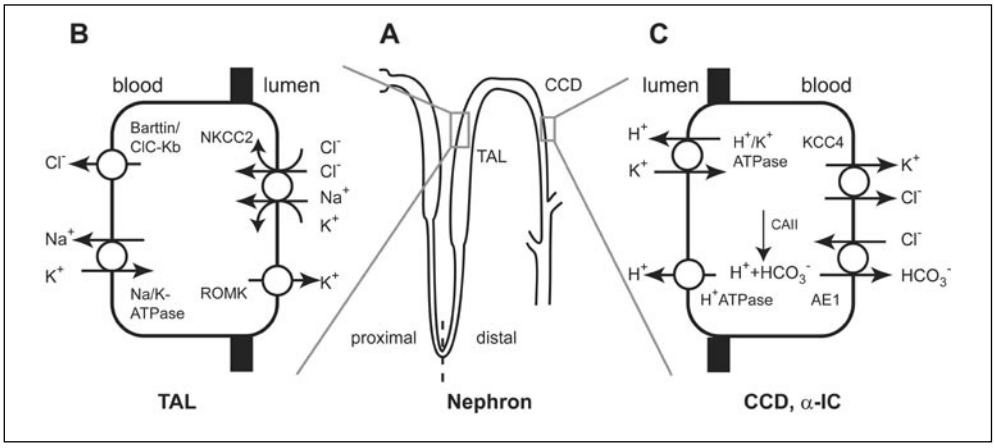

Fig. 2 Kidney function of Barttin and KCC4. (*A*) Scheme of nephron illustrates the position of the thick ascending limb (TAL) and of the cortical collecting duct (CCD). (*B*) TAL cells transport NaCl from lumen (urine) to blood. NKCC2 mediates the uptake of Na^+, K^+ and Cl^- into the cell. K^+ is recycled back to the lumen through ROMK. Na^+ leaves the cell *via* the basolaterally located Na/K ATPase, Cl^- *via* the basolateral ClC-Kb/Barttin channels. (*C*) In α-IC cells of the CCD, H^+ is released together with HCO_3^- in a reaction catalyzed by carbonic anhydrase II (CAII). H^+ is secreted *via* proton ATPase and H/K ATPase into the urine; HCO_3^- is exchanged for Cl^- by AE1, and Cl^- leaves the cell *via* KCC4. Loss of KCC4 leads to increased intracellular chloride concentration, increased pH of the urine and reduced base excess in the blood.

Mice lacking KCC4 protein suffer from renal metabolic acidosis, with increased urinary pH and reduced base excess in blood. KCC4 protein is found in the basolateral membrane of the H^+-secreting α-intercalating cells (α-ICs) of the CCD (Boettger et al. 2002), here overlapping with Barttin/ClC-K2 expression. The α-ICs determine the pH of the urine by secreting H^+ into the lumen of the CCD (Fig. 2*C*). H^+ is liberated within the α-ICs together with bicarbonate by carbonic anhydrase II (CAII) (Sly et al. 1985), secreted *via* the apical proton ATPase (Karet et al. 1998, Smith et al. 2000) or alternatively *via* H/K ATPase activity (Silver and Soleimani 1999). The bicarbonate released by CAII leaves the cell at the basolateral membrane *via* the anion exchanger I (AE1) in exchange for Cl^- (Bruce et al. 1997, Rodríguez-Soriano 2000) (Fig. 2*C*). AE1 action thus results a rise of the intracellular Cl^- concentration $[Cl^-]_i$ of the α-ICs. An increasing $[Cl^-]_i$ favors the outward transport of Cl^- *via* the KCC4 cotransporter at the

basolateral membrane. Loss of KCC4 should impair the recycling of Cl^- over the basolateral membrane, thus should lead to reduced bicarbonate export and increasing pH in the α-ICs. This deteriorates the H^+ secretion of the α-ICs resulting in the observed renal metabolic acidosis. In support of this hypothesis we found an increased $[Cl]_i$ in the α-ICs of the KCC4 knock-out mice. Thus KCC4 is essential for chloride recycling at the α-ICs of the CCD of the kidney.

The role of the Barttin/ClC-KB Cl^- channel in the α-ICs remains unresolved; in principle the Cl^- channel could also provide an exit for Cl^- out of the α-ICs and thereby rescue the loss of KCC4 in these cells. This question cannot be solved in the context of the BSND syndrome, therefore targeted ablation of Barttin/ClC-KB Cl^- channel, comparison and crosses to KCC4 knock-out mice will give new insights in the physiological role of the two Cl^- exits of these cells.

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Dr. Thomas Böttger
Martin-Luther-Universität Halle-Wittenberg
Zentrum für medizinische Grundlagenforschung
Magdeburger Straße 18
06112 Halle/Saale
Germany
Phone: +49 345 5571616
Fax: +49 345 5571436
E-Mail: thomas.boettger@medizin.uni-halle.de

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Voltage-activated Ca^{2+} Channels ($Ca_v1.3$) in Cochlear Hair Cells

Jutta Engel[1], Marcus Michna[1], Martina Knirsch[1], Nobuhiko Waka[1,2], and Stefan Münkner[1]

With 3 Figures

Abstract

Cochlear inner (IHC) and outer hair cells (OHC) are secondary sensory cells of epithelial origin. IHCs receive 95% of all cochlear afferent nerve fibers; they are the genuine sensory cells that respond to a mechanical stimulus with a graded receptor potential that is transmitted to afferent fibers *via* glutamatergic synapses. In contrast, OHCs function as cochlear amplifiers by transforming mechanically induced voltage changes into phase-coupled length changes which feeds energy into the system and reduces the threshold for IHC activation. While OHCs receive the majority of all cochlear inhibitory efferent fibers it is not known at present whether the few afferent fibers that contact OHCs are functional. Voltage-activated Ca^{2+} channels play a crucial role for synaptic transmission in IHCs and perhaps also in OHCs as well as for hair cell development. We studied whole cell Ca^{2+} currents in neonatal mouse IHCs and OHCs between postnatal day 1 (P1) and P9 with the patch clamp technique using Ca^{2+} or Ba^{2+} as charge carriers. Rapidly activating and deactivating Ca^{2+} channel currents with little (Ba^{2+} currents, I_{Ba}) to prominent (Ca^{2+} currents, I_{Ca}) inactivation were recorded in both cell types. I_{Ba} activation parameters in IHCs and OHCs were similar but not identical. The main difference was the lower current density in OHCs that amounted to 38% of the IHC value (65 pA/pF). I_{Ba} could be partially blocked by the L-type Ca^{2+} channel blockers nifedipine and nimodipine and increased by the L-type activator Bay K 8644. The underlying Ca^{2+} channel α1-subunit was identified by recording I_{Ba} in $Ca_v1.3$ (or α1D)-deficient mice. In $Ca_v1.3^{-/-}$ hair cells, I_{Ba} density was dramatically reduced indicating that > 92% of the IHC and > 97% of the OHC I_{Ba} flows through $Ca_v1.3$ channels.

Zusammenfassung

Kochleäre innere (IHC) und äußere (OHC) Haarzellen sind sekundäre Sinneszellen epithelialen Ursprungs. IHC erhalten 95% aller afferenten kochleären Nervenfasern, sie sind die eigentlichen Sinneszellen, die auf einen mechanischen Reiz mit einem graduierten Rezeptorpotential antworten, das über glutamaterge Synapsen auf afferente Fasern übertragen wird. Hingegen wirken OHC als kochleäre Verstärkerelemente, indem sie mechanisch ausgelöste elektrische Spannungsänderungen in phasengekoppelte Längenänderungen umwandeln, die Energie in das System hineinpumpen und damit die Schwelle für die Anregung der IHC verringern. OHC werden von 95% aller inhibitorischen efferenten Fasern innerviert. Ob die wenigen afferenten Fasern, die die OHC innervieren, eine Funktion besitzen, ist noch nicht bekannt. Spannungsgesteuerte Ca^{2+}-Kanäle sind außer für die synaptische Übertragung in reifen IHC – und vielleicht auch in OHC – auch für die Haarzellentwicklung wichtig. Wir haben Ganzzell-Ca^{2+}-Ströme in neonatalen IHC und OHC der Maus zwischen postnatalem Tag 1 (P1) und P9 mit der *Patch-clamp*-Technik mit Ca^{2+} oder Ba^{2+} als Ladungsträger untersucht. In beiden Zelltypen konnten schnell aktivierende und deaktivierende Ca^{2+}-Kanal-Ströme mit sehr geringer (Ba^{2+}-Ströme, I_{Ba}) bis starker (Ca^{2+}-Ströme, I_{Ca}) Inaktivierung abgeleitet werden. Die Eigenschaften von I_{Ba} waren in IHC und OHC ähnlich, aber nicht identisch. Der Hauptunterschied war die geringere Stromdichte

1 Institute of Physiology II and Department of Otolaryngology – Tuebingen Hearing Research Centre (THRC), University of Tuebingen, Gmelinstr. 5, 72076 Tuebingen, Germany.

2 Department of Otolaryngology, Kansai Medical University, Osaka, Japan.

in OHCs; sie betrug nur 38 % des Wertes der IHC von 65 pA/pF. I_{Ba} konnte mit den L-Typ-Kanalblockern Nifedipin und Nimodipin teilweise gehemmt und mit dem L-Typ-Aktivator BayK8644 gesteigert werden. Die verantwortliche Ca^{2+}-Kanal-α1-Untereinheit wurde durch Ableitungen an $Ca_v1.3$ (oder α1D)-defizienten Mäusen identifiziert: in $Ca_v1.3^{-/-}$-Haarzellen war die Ba^{2+}-Stromdichte sehr stark reduziert, was darauf schließen läßt, daß > 92 % des Ba^{2+}-Stroms in IHC und > 97 % in OHC durch $Ca_v1.3$-Kanäle fließt.

1. Function of Inner and Outer Hair Cells

The cochlear sensory epithelium consists of inner (IHC) and outer (OHC) hair cells with different function and innervation (for review see Pujol et al. 1997, Geisler 1998). IHCs are the genuine sensory cells that transmit mechanical stimuli into graded receptor potentials and graded release of glutamate. The transmitter evokes EPSPs in afferent nerve fibers of the spiral ganglion cells, the axons of which form the auditory nerve. IHCs receive > 90 % of all cochlear afferent fibers. The OHC is a relatively late evolutionary development which by its unique electro-mechanical transduction mechanism provides an amplification mechanism for weak acoustical stimuli. When a sound wave exerts a force on the sterecilia of either IHC or OHC, transduction channels open at the stereocilia leading to influx of K^+ and Ca^{2+} ions and depolarization. In IHCs, depolarization triggers fast and sensitive opening of Ca^{2+} channels and transmitter release (Moser and Beutner 2000, Platzer et al. 2000) while in OHCs depolarization causes cell shortening (Brownell et al. 1985) by the depolarization-driven conformational change of the motor protein prestin (Zheng et al. 2000, Oliver et al. 2001). The periodic shortening of OHCs is believed to feed energy into the cochlear partition and thus amplifies weak stimuli that otherwise would not have been detectable for the IHCs. OHCs show pronounced efferent inhibition by the medial olivocochlear bundle. Although OHCs are connected to few afferent fibers (< 10 % of all afferents; 5 – 10 per OHC; Felix 2002) it is unknown whether they play a role in afferent signaling of acoustical stimuli.

Mice are born deaf and start hearing at P12 (Rybak et al. 1992). From P0 – P20, many maturational changes essential for normal hearing take place in the organ of Corti including hair cells. Ca^{2+} influx is necessary for hair cell development since $Ca_v1.3$-deficient mice show degeneration that starts in OHCs around P15 and later on in IHCs (Platzer et al. 2000, Glueckert et al. 2003).

2. Ca^{2+} Currents and Channels in Inner and Outer Hair Cells

Ca^{2+} currents were measured in the whole-cell configuration with Cs^+ ions in the pipette solution to block K^+ channels and with $Tris^+$ ions in the bath solution replacing Na^+ and K^+ to prevent K^+ and Na^+ currents (Platzer et al. 2000, Michna et al. 2003). Ca^{2+} (at the physiological concentration of 1.3 mM or at 10 mM to get larger currents) or Ba^{2+} (which generates 1.8 times larger currents than with equimolar Ca^{2+} concentration) were used as charge carriers for voltage-activated Ca^{2+} channels. Figure 1 shows the rapid activation of an IHC inward current in response to a depolarization from −89 mV to −24 mV and rapid de-activation upon repolarization (control). When Ca^{2+} was omitted from the bath solution, the inward current was abolished. Re-admission of Ca^{2+} restored the inward current (wash). IHC Ca^{2+} currents (I_{Ca}) activated with time constants of about 0.3 ms at V_{max} at 22 °C and started activating at voltages < −60 mV (Fig. 1*B*) (Platzer et al. 2000). IHC Ca^{2+} channel currents showed little

voltage-dependent inactivation of I_{Ba} (11.1 ± 4.8 %, n = 18) in 10 mM Ba^{2+} after 300 ms and moderate Ca^{2+}-dependent inactivation of I_{Ca} in 10 mM Ca^{2+} (21.1 ± 9.3 %, n = 5) (Platzer et al. 2000, Koschak et al. 2001). These figures contrast to a reduced I_{Ba} inactivation of 3.6 ± 1.5 % (n = 10) and an enhanced Ca^{2+}-dependent inactivation of 50.7 ± 23.7 % (n = 10) in OHCs after 300 ms (Fig. 2*A*, *B*). The IHC I_{Ba} had activation parameters of V_{max} = −5.0 ± 4.1 mV, $V_{0.5}$ = −19.6 ± 4.6 mV, and k = 7.6 ± 0.7 mV (n = 13). Current properties in OHCs measured under identical conditions were similar (V_{max} = −1.9 ± 6.9 mV, $V_{0.5}$ = −15.0 ± 7.1 mV, and k = 8.2 ± 1.1 mV, n = 34) (Michna et al. 2003). The most prominent difference was that IHC had a threefold larger mean current density (64.7 ± 29 pA/pF, n = 5) compared to OHCs (24.4 ± 10.8 pA/pF, n = 105).

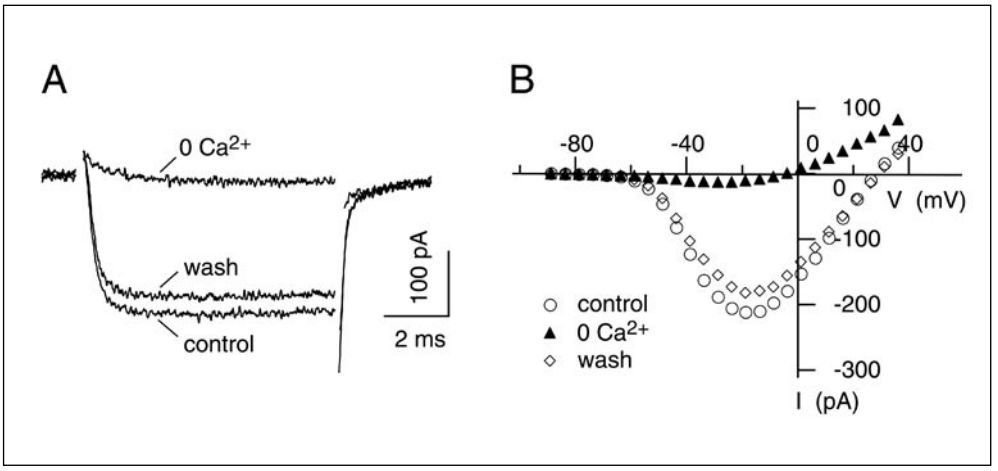

Fig. 1 Rapidly activating Ca^{2+} currents in a neonatal IHC (P3). (*A*) Peak inward currents (responses to depolarizations to −24 mV from a holding potential of −89 mV) in 1.3 mM Ca^{2+} (control, wash) that disappear in 0 Ca^{2+} solution. (*B*) Current-voltage relations reveal that I_{Ca} activates <−60 mV and peaks around −25 mV.

I_{Ba} was increased by 5 μM BayK8644 (by 292 % in IHCs and by 178 % in OHCs) and was partially blocked by dihydropyridines. Nifedipine (10 μM) inhibited 45 % of the total I_{Ba} in IHCs and 34 % of that in OHCs with an IC_{50} of 2.7 μM (Fig. 2) suggesting that at least part of I_{Ba} was L-type. Recording I_{Ba} in IHCs and OHCs from neonatal $Ca_v1.3^{-/-}$ mice revealed a dramatic decrease of the current density compared to wildtype HCs indicating that about 92 % of I_{Ba} in IHCs (Platzer et al. 2000) and 97 % in OHCs (Michna et al. 2003) flow through $Ca_v1.3$ channels. Despite the fact that the IHC I_{Ca} density declines from a peak at P6 and that the coupling between I_{Ca} and exocytosis becomes more effective (Beutner and Moser 2001, Marcotti et al. 2003) I_{Ba}/I_{Ca} properties seem to be unchanged during further maturation of IHCs > P9 (Engel, unpublished). OHC I_{Ba} peaks at P2 (Michna et al. 2003) and declines to 20 % at P19 (Knirsch and Engel, unpublished).

The subcellular distribution of $Ca_v1.3$ channels in IHCs and OHCs was studied using the immunofluorescence method with cochlear cryosections (Waka et al. 2003). Anti-$Ca_v1.3$ staining (courtesy of M. McEnery) revealed a punctate membrane staining in the lateral and basal parts of the IHC and a concentrated staining at the base of the innermost OHC at P4 (Fig. 3).

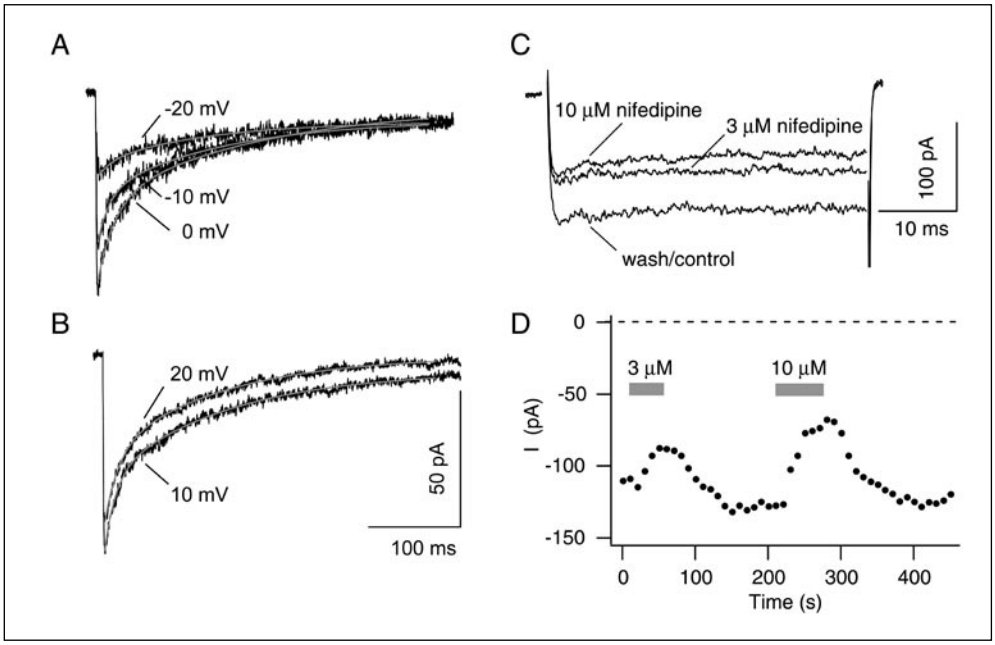

Fig. 2 Properties of Ca^{2+} channel currents in neonatal OHCs. (*A*), (*B*) I_{Ca} in a P6 OHC (10 mM Ca^{2+}) evoked by depolarizations to the potentials indicated showed Ca^{2+}-dependent inactivation the kinetics of which could be fitted with double-exponential functions. Fast inactivation time constants ranged from 6 to 28 ms; slow time constants from 94 to 253 ms. (*C*) Nifedipine partially blocked OHC I_{Ba} (P4) as shown by peak current traces with 3 μM, after washout, and with 10 μM nifedipine. (*D*) Time course of the peak current amplitude during application of 3 and 10 μM nifedipine.

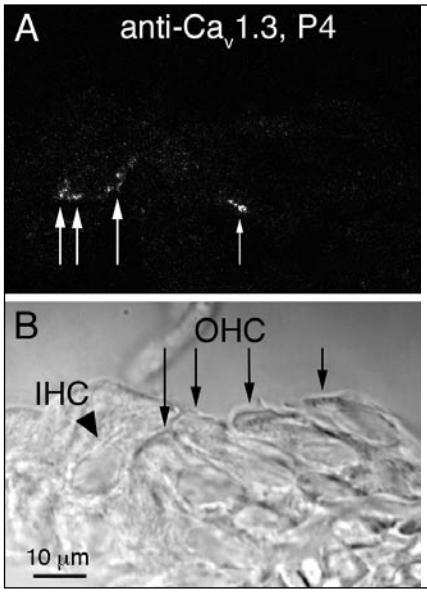

Fig. 3. Immunohistochemical staining of $Ca_v1.3$ in the mouse organ of Corti (P4). (*A*) Anti-$Ca_v1.3$ immunofluorescence is present at the basolateral membrane of the IHC (large arrows) and at the base of the innermost OHC (small arrow). (*B*) The corresponding differential interference contrast image is shown for comparison.

3. Conclusions

IHC I_{Ca} shows properties that are quite unusual for L-type Ca^{2+} currents – very negative activation and extremely fast activation as well as very little inactivation. These properties meet the demands put on the IHC: Around the IHC resting potential of −65 mV (Kros et al. 1998, Oliver et al. 2003) some of the Ca^{2+} channels should already be open so that tiny depolarizations cause a change in the rate of exocytosis. The fast activation and deactivation of the $Ca_v1.3$ channels is one prerequisite for the high temporal resolution of acoustical stimuli, especially for phase-locking up to several kHz. Weak inactivation of $Ca_v1.3$ currents guarantees continuous presynaptic Ca^{2+} influx even upon long-lasting acoustical stimulation.

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Dr. Jutta Engel
Eberhard-Karls-Universität Tübingen
Physiologisches Institut, Abt. II und
Universitäts-HNO-Klinik
Gmelinstraße 5
72076 Tübingen
Germany
Phone: +49 7071 2988194
Fax: +49 7071 294950
E-Mail: jutta.engel@uni-tuebingen.de

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The Role of Protein Kinase C in the Regulation of the Activity of the Cystic Fibrosis Chloride Channel

Guillermo A. Altenberg, Yongyue Chen, Brian Button, and Luis Reuss
(Galveston)

Abstract

CFTR is a Cl^- channel that is activated by phosphorylation of its regulatory (R) domain by PKA. The effects of phosphorylation of CFTR by PKC have not been studied in detail. We have identified three separate responses to PKC stimulation: (*i*) *Permissive effect*: requirement of phosphorylation by PKC for complete activation by PKA agonists. (*ii*) *Activation*: increase in CFTR activity upon stimulation of PKC. (*iii*) *Potentiation effect*: stimulation of PKC after stimulation by PKA agonists produces an activation that is several folds that of the individual kinase stimulation. We studied voltage-clamped *Xenopus laevis* oocytes, injected with cRNA for expression of wild-type CFTR, CFTR mutants or chimeras of CFTR. Stimulation of PKA and PKC was accomplished by exposure of the cells to supramaximal concentrations of pharmacological agonists. *Permissive effect*: a basal level of phosphorylation by PKC is required for its activation by PKA agonists. Our mutagenesis studies of human (hCFTR) and *Xenopus* (*X*CFTR) CFTR showed that Ser605 is essential, but not sufficient because it has to be present in combination with Ser686 or Ser790. We conclude that phosphorylation of Ser605 and either Ser686 or Ser790 allows for normal activation by PKA agonists. *Activation*: experiments in oocytes have shown that stimulation of PKC produces a significant activation of *X*CFTR, but not hCFTR. Therefore, the differential response is not due to the host cell, but to differences in the amino-acid sequences between the CFTR orthologs. Studies with *X*CFTR/hCFTR chimeras and phosphorylation-defective mutants revealed that a single residue, Thr665, is required for the activation by PKC agonists. The PKC consensus sequence that includes Thr665 is conserved from dogfish to chimpanzee, but is not present in humans, where the basic residue at position 667 (Arg667 in *X*CFTR) is His. The observation that the substitution His667Arg in hCFTR yields a molecule that can be activated by PMA strongly supports the conclusion that phosphorylation of Thr665 by PKC activates CFTR. *Potentiation effect of dual kinase stimulation*: potentiation effect was observed in *X*CFTR, but not in hCFTR when these proteins were expressed in *Xenopus* oocytes, suggesting that this phenomenon is also CFTR-molecule specific. Studies with *X*CFTR/hCFTR chimeras showed that differences between sequences of the *X*CFTR and hCFTR R domains are not responsible for the lack potentiation effect in hCFTR. Differences between the NBD2 of XCFTR and hCFTR are essential for the potentiation effect. In summary, phosphorylation of CFTR by PKC has three separate and independent effects. The permissive effect is due to phosphorylation of R domain residues that are conserved in hCFTR and *X*CFTR. The activation of CFTR by stimulation of PKC is due to phosphorylation of a single residue, Thr665, in a PKC consensus site that is absent in hCFTR. The potentiation effect of dual-kinase stimulation is complex and involves at least two regions of *X*CFTR that are far away in the primary sequence, but differences between the R domains of *X*CFTR and hCFTR are not involved.

Zusammenfassung

CFTR ist ein Cl^--Kanal, der durch Phosphorylierung seiner regulatorischen (R) Domäne durch Proteinkinase A (PKA) aktiviert wird. Die Effekte der Phosphorylierung des CFTR durch PKC sind noch nicht im Einzelnen untersucht worden. Wir haben drei verschiedene Antworten auf die PKC-Stimulation identifiziert: (*i*) *Permissiver Effekt*: Notwendigkeit der Phosphorylierung durch PKC für eine vollständige Aktivierung durch PKA-Agonisten. (*ii*) *Aktivierung*: Anstieg der CFTR Aktivierung nach Stimulation der PKC. (*iii*) *Kooperativer Effekt*: Stimulation der PKC nach Stimulation durch PKA-Agonisten bewirkt eine Aktivierung um das Mehrfache der Stimulation der einzelnen Kinase. Wir haben „spannungs-geklemmte“ *Xenopus-laevis*-Oozyten nach Injektion von cRNA zur Expression des Wild-Typ-CFTR, CFTR-Mutanten und Chimären von CFTR untersucht. Stimulation der PKA und PKC wurde durch Behandlung der Zellen mit supra-maximalen Konzentrationen der pharmakologischen Agonisten erreicht.

Permissiver Effekt: Ein basales Niveau der Phosphorylierung durch PKC ist für die Aktivierung durch PKA Agonisten notwendig. Unsere Untersuchungen der Mutagenese der humanen (hCFTR) und *Xenopus* (*X*CFTR) CFTR zeigten, daß Ser605 essentiell, aber nicht ausreichend ist, da es in Kombination mit Ser686 oder Ser790 vorliegen muß. Wir schlußfolgern, daß Phosphorylierung von Ser605 und entweder Ser686 oder Ser790 für normale Aktivierung durch PKA-Agonisten ausreicht. *Aktivierung*: Untersuchungen an Oozyten zeigten, daß Stimulation der PKC zu einer signifikanten Aktivierung von *X*CFTR, aber nicht von hCFTR führt. Deshalb liegt die unterschiedlich abgestufte Aktivierung nicht an der exprimierenden Zelle, sondern an den Unterschieden in der Aminosäuresequenz zwischen den orthologen CFTR-Genen. Untersuchungen mit *X*CFTR/hCFTR-Chimären und Phosphorylierungs-defekten Mutanten zeigten, daß ein einziger Rest, Thr665, für die Aktivierung durch PKC-Agonisten nötig ist. Die übereinstimmende PKC-Sequenz, die Thr665 einschließt, ist im Katzenhai und Schimpansen konserviert, aber bei Menschen nicht vorhanden, wo der basische Rest in Position 667 (Arg667 in *X*CFTR) ein Histidin ist. Die Beobachtung, daß Substitution von His667Arg im hCFTR ein Molekül ergibt, das durch PMA aktiviert werden kann, unterstützt die Schlußfolgerung, daß Phosphorylierung von Thr665 durch PKC den CFTR aktiviert. *Kooperativer Effekt durch zweifache Kinase-Stimulation*: Der Kinase-kooperative Effekt wurde in *X*CFTR beobachtet, aber nicht in hCFTR, wenn diese Proteine in *Xenopus*-Oozyten exprimiert waren, was nahe legt, daß dieses Phänomen auch spezifisch für das CFTR-Molekül war. Untersuchungen mit *X*CFTR/hCFTR-Chimären zeigten, daß Unterschiede zwischen Sequenzen von *X*CFTR- und hCFTR-Domänen nicht für das Fehlen des Kinase-kooperativen Effekts verantwortlich sind. Es sind mindestens 2 *X*CTR-Domänen, die in der Primärsequenz voneinander entfernt sind, für den kooperativen Effekt nötig (eine beinhaltet NBP und die andere die C-terminale zytoplasmatische Region).

Zusammenfassend hat die Phosphorylierung von CFTR durch PKC drei separate und unabhängige Effekte. Der „permissive Effekt" erfolgt durch Phosphorylierung von Resten der R-Domäne, die im hCFTR und *X*CFTR konserviert sind. Die Aktivierung von CFTR durch Stimulation der PKC erfolgt durch Phosphorylierung eines einzigen Restes, Thr665, in einer übereinstimmenden PKC-Stelle, die im hCFTR nicht vorhanden ist. Der kooperative Effekt der Zweifach-Kinase-Stimulation ist komplex und involviert mindestens zwei Regionen des *X*CFTR, die in der Primärsequenz weit entfernt liegen, aber Unterschiede zwischen R-Domänen von *X*CFTR und hCFTR sind nicht involviert.

1. Introduction

Mutations of the cystic fibrosis transmembrane conductance regulator (CFTR) cause cystic fibrosis, the most common lethal genetic disease among Caucasians. CFTR is a Cl^- channel that mediates conductive Cl^- transport across apical membranes of secretory epithelia. This channel is a member of the ATP-binding cassette (ABC) superfamily and consists of five domains: two membrane-spanning domains of six transmembrane-spanning helices each, two nucleotide-binding domains (NBD1 and NBD2) that gate the channel by binding and hydrolyzing ATP, and a regulatory domain (R domain). The R domain contains numerous consensus phosphorylation sites for protein kinase A (PKA) and protein kinase C (PKC).

PKA phosphorylates the R domain and increases the mean open time of the channel. The number of CFTR channels in the membrane can increase by exposure to PKA agonists. However, this phenomenon is cell-type dependent (Loffing et al. 1998). The mechanism by which phosphorylation regulates CFTR gating is controversial (reviewed by Gadsby and Nairn 1999, Sheppard and Welsh 1999, Ma 2000). The regulation of CFTR by its R domain cannot be explained by a simple pore-plugging mechanism. It involves complex interactions between consensus sites that are not functionally equivalent to each other.

The effects of phosphorylation of CFTR by PKC have been less studied. We have identified three separate responses to PKC stimulation:

(*i*) Permissive effect: requirement of phosphorylation by PKC for complete activation by PKA agonists.
(*ii*) Activation: increase in CFTR activity upon stimulation of PKC.
(*iii*) Potentiation of the response to PKA agonists by prior PKC stimulation: stimulation of

PKC increases the CFTR activation in response to PKA agonists several fold. Here, we discuss these three effects.

We studied the regulation of CFTR by PKC-mediated phosphorylation in voltage-clamped *Xenopus laevis* oocytes injected with cRNA for expression of wild-type CFTR, CFTR mutants or chimeras of CFTR. The oocytes were bathed in HEPES-buffered solution and their conductance was determined. Stimulation of PKA and PKC was accomplished by exposure of the cells to supra-maximal concentrations of pharmacological agonists.

2. Permissive Effect

Although phosphorylation by PKC produces only a minor activation of human CFTR (hCFTR), a basal level of phosphorylation by PKC is required for its activation by PKA agonists. A recent study showed that the residues responsible for this permissive effect of PKC are located in NBD1 and/or the R domain (Chappe et al. 2003). Substitution of 9 Ser/Thr residues in these domains with Ala reduced by > 90 % the activation of hCFTR by PKA. A similar effect was observed in cells expressing wild-type hCFTR that were exposed to the PKC inhibitor chelerythrine. We also observed the permissive effect in *Xenopus* CFTR (*X*CFTR). Exposure of oocytes expressing wild-type *X*CFTR to the PKC blocker calphostin C essentially abolished the activation by PKA agonists. In addition, substitution of 8 Ser/Thr residues of the R domain and NBD1 with Ala produced a similar decrease in the response of *X*CFTR to PKA stimulation. We identified 4 PKC consensus sequences common to hCFTR and *X*CFTR and performed additional mutagenesis studies in hCFTR to identify the residues responsible for the permissive effect. Our results showed that Thr604 is essential, but not sufficient because it has to be present in combination with Ser686 or Ser790. We conclude that the permissive effect requires phosphorylation of two residues conserved in the hCFTR and *X*CFTR R domains. Recent results by Chappe et al. (2004) are in general agreement with our findings, although they found a decreased response to PKA stimulation in the Ser686Ala mutant. The reason for this difference is not clear.

3. Activation

Previous studies from our laboratory demonstrated that the apical membrane of the gallbladder of *Necturus maculosus* (Heming et al. 1994) and *Xenopus laevis* express CFTR. In contrast with hCFTR where PKA agonists activate the current, but PKC agonists have a much smaller effect, stimulation of PKC activates *Necturus* CFTR and *X*CFTR at least as much as phosphorylation by PKA. The differential responses to stimulation of PKA and PKC are not due to the host cell, but to differences in the amino-acid sequences between the CFTR orthologs. We took advantage of this to identify the domains and residues involved in the activation of *X*CFTR by PKC stimulation. First, we made chimeras in which the R domain was exchanged and demonstrated that differences between the hCFTR and *X*CFTR R domains were responsible for the differential activation by PKC agonists in *X*CFTR. Substitution of Ser/Thr residues of the *X*CFTR R domain with Ala revealed that a single residue, Thr665, is required for the activation by the phorbol ester PMA. Interestingly, the PKC consensus sequence that includes

Thr665 is conserved from dogfish to chimpanzee, but is not present in humans, where the basic residue at position 667 (Arg667 in *X*CFTR) is His. A human-like sequence for this site was also reported in zebrafish, but the effects of modulation of PKC activity on zebrafish CFTR have not been studied. The observation that the substitution His667Arg in hCFTR yields a molecule that can be activated by PKC stimulation strongly supports the conclusion that phosphorylation of Thr665 by PKC activates CFTR (Button et al. 2001).

In principle, the increase in conductance by PKC stimulation could result from increases in number of active channels in the membrane (N), open probability of single channels (P_o) and/or single-channel conductance (γ). We used the response to thiol reagents of CFTR mutants to address the possibility that exocytic insertion of new channels from an intracellular pool is responsible for the activation of *X*CFTR by PKC stimulation. This methodology was recently used by Liu et al. (2001) for studies in hCFTR. Our results indicate that the activation of *X*CFTR by PMA occurs without insertion of new CFTR molecules in the plasma membrane. Patch-clamp studies showed that an increase in open probability is the dominant mechanism of CFTR activation.

4. Potentiation of the Response to PKA Agonists by Prior PKC Stimulation

The potentiation of the response to PKA stimulation by PKC agonists was observed in *X*CFTR, but not in hCFTR when these proteins were expressed in *Xenopus* oocytes, suggesting that this phenomenon is also CFTR molecule specific. Results with cell-impermeable thiol reagents showed that the potentiation occurs without insertion of new CFTR channels in the plasma membrane. This effect is independent of the activation of *X*CFTR by PKC stimulation. This is supported by the observation that *X*CFTR mutants that are not activated by PKC (e.g., Thr665Ala) still have the potentiation effect. Although not definitive, our results suggest that the potentiation effect results from an enhanced response to PKA. Mutations of PKA consensus sites that greatly reduce the response to PKA stimulation produced a proportional decrease in the response to the dual-kinase stimulation. As in the case of activation, the main biophysical mechanism of the potentiation effect is an increase in single channel open probability.

We decided to exploit the differences between hCFTR and *X*CFTR to identify the domains responsible for the potentiation effect. Since the R domain is critical for the activation of CFTR by stimulation of PKA or PKC, as well as for the permissive effect described above, we first studied R domain chimeras. An *X*CFTR chimera with the human R domain displayed the potentiation effect, while an hCFTR chimera with the *Xenopus* R domain did not. Therefore, differences between sequences of the *X*CFTR and hCFTR R domains are not responsible for the lack of potentiation in hCFTR. This is a surprising result, given the dominant role of the R-domain in the regulation of CFTR by phosphorylation (see Gadsby and Nairn 1999 and Sheppard and Welsh 1999). We next tested the possible participation of N- and C-terminal sequences, as well as NBD1 and NBD2. The N- and C-terminal regions have been shown to interact either with other domains of the molecule (Naren et al. 1999, Wang et al. 2002, Neville et al. 1998) or with accessory proteins (Naren et al. 1997, Wang et al. 2000) to modify channel function. We engineered chimeras based on the *X*CFTR in which the cytoplasmic N- and/or C-terminal sequences or the NBDs were replaced by the corresponding sequences of hCFTR. The results showed that differences between *X*CFTR and hCFTR NBD2 are necessary for the potentiation effect, whereas differences in the other regions studied are not. hCFTR with the

NBD2 of *X*CFTR did not display potentiation, suggesting that the phenomenon of potentiation is complex and involves several domains.

In summary, phosphorylation of CFTR by PKC has three separate and independent effects. The permissive effect is due to phosphorylation of R domain residues that are conserved in hCFTR and *X*CFTR. The activation of CFTR by stimulation of PKC is due to phosphorylation of a single residue, Thr665, in a PKC consensus site that is absent in hCFTR. The potentiation effect of dual-kinase stimulation is complex and involves differences between the NBD2 of hCFTR and *X*CFTR, but differences between the R domains of *X*CFTR and hCFTR are not involved.

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Dr. Guillermo A. Altenberg
Department of Neuroscience and Cell Biology
and Sealy Center for Structural Biology
University of Texas Medical Branch
Galveston, Texas 77555-0437
USA
Phone: +1 409 7723026
Fax: +1 409 7723381
E-Mail: galtenbe@utmb.edu

Christian Gottfried Nees von Esenbeck: Briefwechsel mit Johann Wolfgang von Goethe nebst ergänzenden Schreiben

Acta Historica Leopoldina Nr. *40*
Bearbeitet von Kai Torsten Kanz (Lübeck)
(2003, 470 Seiten, 12 Abbildungen, 34,80 Euro, ISBN 3-8047-2001-3)

Der Band enthält die zwischen dem Botaniker und Präsidenten der heutigen Deutschen Akademie der Naturforscher Leopoldina, Christian Gottfried Nees von Esenbeck (1776–1858), und dem Dichter und Naturforscher Johann Wolfgang von Goethe (1749–1832) in den Jahren 1816 bis 1831 gewechselten mehr als 150 Schreiben. Damit wird eine der bedeutendsten naturwissenschaftlichen Korrespondenzen des alten Goethe erstmals vollständig und in kommentierter Form zugänglich gemacht. Ergänzend werden die Korrespondenz zwischen Nees von Esenbeck und Goethes Sekretär Eckermann sowie weitere Briefe ediert. Neben einer Einführung in die Ausgabe sind die Texte vollständig durch Register (Personen, Sachen, Orte) erschlossen.

Die historische Bedeutung Christian Gottfried Daniel Nees von Esenbecks liegt neben seiner vierzigjährigen Amtszeit als XI. Präsident der Leopoldina (1818–1858) in seinem wissenschaftlichen Lebenswerk als Botaniker, Naturphilosoph und Wissenschaftsorganisator, in seiner Tätigkeit als Hochschullehrer an den Universitäten Erlangen, Bonn und Breslau sowie in seinem engagierten sozialpolitischen Wirken. Sein Leben und Werk ist in vielfacher Weise ein Spiegel der Wissenschafts- wie auch der allgemeinen Geschichte der ersten Hälfte des 19. Jahrhunderts.

Seine umfangreiche Korrespondenz, die sich über weit mehr als 4000 Briefe ausdehnt, stellt einen Schlüssel zum Verständnis seines Wirkens dar. Darüber hinaus sind die Briefe für eine Vielzahl weiterer Fragestellungen aus der Wissenschafts-, Literatur- und Kulturgeschichte sowie der politischen Geschichte (Arbeiterbewegung, Christkatholizismus, Revolution von 1848) von herausragendem Quellenwert. Der vorliegende Band eröffnet die schwerpunktmäßig ausgewählten Editionen dieser Korrespondenzen in der Reihe *Acta Historica Leopoldina*.

In Kommission bei Wissenschaftliche Verlagsgesellschaft mbH Stuttgart

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Guanylin as a Luminal Activator of Epithelial CFTR-dependent Chloride Secretion

Matthias Hönscheid[1], Hasan Kulaksiz[2], Yalcin Cetin[2], and Andreas Schmid[1]

With 1 Figure

Abstract

The bioactive peptide guanylin induces epithelial chloride secretion in various tissues by activation of a cGMP-dependent signal cascade. Guanylin is produced by certain epithelial cells. It is stored in intracellular vesicles and can be secreted by exocytosis across the luminal membrane. Therefore, guanylin mainly exerts its effect on epithelial cells that are located downstream to guanylin-releasing cells. We found that the guanylin-signaling system, which initially was found in the intestine, also plays a role in human exocrine pancreas and in human lung. In these organs, guanylin acts as a paracrine activator of chloride secretion which locally tunes and co-ordinates epithelial electrolyte transport within pancreatic ducts and small distal airways.

Zusammenfassung

Guanylin ist ein bioaktives Peptid, das in verschiedenen Epithelien durch die Aktivierung eines cGMP-abhängigen Signalweges zur Steigerung der Chloridsekretion führt. Die entscheidende Rolle spielt dabei nicht das im Blut zirkulierende Guanylin, sondern Guanylin, das von Epithelzellen über die luminale Membran sezerniert wurde. Guanylin wirkt damit vorwiegend auf die Epithelzellen, die sich im gleichen Organ distal zum jeweiligen Sekretionsort befinden. Wir konnten zeigen, daß das Guanylin-Signalsystem, das ursprünglich für Darmepithelzellen beschrieben wurde, auch in distalen Luftwegen und in Pankreasgängen eine Rolle spielt. In diesen Gangsystemen kann über Guanylin der epitheliale Elektrolyt-Transport koordiniert und damit die Sekretionsleistung von verschiedenen Gangabschnitten aufeinander abgestimmt werden.

1. Introduction

Epithelial chloride transport is important for the electrolyte homeostasis of the body and is closely coupled to fluid secretion and fluid reabsorption in many organs. Inadequate balance of chloride transport is the reason of several diseases such as diarrhea or the hereditary disease cystic fibrosis. The precise regulation of chloride transport is therefore important for maintenance of normal epithelial function. The regulation is primarily accomplished by the cooperation of hormones which are circulating in the blood, and neurotransmitters which are released from nerve varicosities within the respective tissue. Together they regulate the activity of epithelial chloride channels by changing the cytosolic calcium concentration and/or the activity of protein kinases and phosphatases in the target cells.

1 Department of Physiology, University of the Saarland, 66421 Homburg/Saar, Germany.
2 Department of Anatomy and Cell Biology, Philipps University, 35033 Marburg, Germany.

Studies from the last years revealed that in several epithelia chloride transport is also regulated by paracrine acting substances. One of these locally acting substances is guanylin. Guanylin is a small peptide which is synthesized in certain epithelial cells. It can be released by exocytosis into the lumen were it acts from the luminal surface of the epithelium. The guanylin signal cascade involves a membrane-spanning receptor with guanylate cyclase activity, the activation of a cGMP-dependent protein kinase and the phosphorylation of chloride channel proteins in the luminal plasma membrane.

2. Guanylin

Human guanylin is a small peptide consisting of 15 amino acids. It is produced in a 94-amino-acid precursor form, the prohormone proguanylin. From proguanylin it is released by cleavage between Asp-79 and Pro-80. Guanylin contains 4 cysteine residues which form two intramolecular disulfide bonds. For the correct folding of guanylin and for the correct formation of the two disulfide bonds, the N-terminus of proguanylin is essential. Furthermore, the N-terminus of proguanylin interacts with its guanylin sequence at the C-terminus and thereby largely suppresses a biologic activity of the prohormone (Lauber et al. 2003). The process which under physiological conditions leads to release of guanylin from proguanylin is not known so far. A proteinase which cleaves proguanylin between Asp-79 and Pro-80 has not been identified. Whether the acidic cleavage of the Asp-79-Pro-80 bond that can be observed *in vitro* is also important for the situation *in vivo* is still an open question.

3. Receptor-Guanylyl Cyclase GC-C

In mammalian tissues two classes of guanylyl cyclase (GC) has been identified. The soluble GC (sGC) has been isolated from the cytosolic fraction of cells. It is a protein heterodimer formed by two subunits, and it contains a heme group and is activated by binding of NO. In contrast, the receptor guanylyl cyclases (pGC) are membrane proteins with an extracellular and a cytosolic domain. The first pGCs cloned from mammalian tissues were GC-A and GC-B (Chang et al. 1989), the receptors for the natriuretic peptides ANP, BNP and CNP. The sequence of the catalytic domain of pGCs showed high homology to the catalytic domain of sGCs but only little homology to adenylyl cyclases and other proteins. Primers against this region could therefore be used for polymerase chain reactions and further members of the GC family were identified (GC-C to GC-G) (Yuen et al. 1990). It is still unclear whether the enzymatic activity of GC-D, GC-E, GC-F and GC-G can be modulated by binding of any ligand to the extracellular domain of these proteins. However, it could be shown that the pGC isoform GC-C serves as a receptor for the heat-stable enterotoxin STa which is produced by pathogenic strain of *E. coli* (Schulz et al. 1990). The endogenous ligands which bind to GC-C are guanylin (Currie et al. 1992) and uroguanylin (Kita et al. 1994). Both agonists induce activation of GC-C and production of cGMP. The elevation in the cytosolic cGMP concentration leads to an activation of chloride channels *via* a cGMP-dependent protein kinase.

4. cGMP-dependent Protein Kinase cGK-II

There are two types of cGMP-dependent protein kinases (cGKs) in mammalian tissues. The soluble cGK-I is more ubiquitously expressed and plays an important role in the regulation of cardiovascular function and calcium homeostasis in platelets (Feil et al. 2003). In contrast, the membrane-bound isoform cGK-II is mainly expressed in epithelial cells in the intestine, kidney, pancreas and lung, where it is involved in regulation of epithelial transport. Furthermore, both cGKs are widespread in various areas of the brain, suggesting that cGKs are also involved in NO/cGMP-dependent signaling in the central nervous system (Arancio et al. 2001, El-Husseini et al. 1999). cGK-I and cGK-II share similarities in the amino acid sequence and in the structural organization. Both isoforms possess two distinct cGMP-binding domains, autophosphorylation modulates the enzymatic activity, and there are no hydrophobic trans-membrane domains. While cGK-I is a soluble protein found in the cytosol, cGK-II is a membrane-associated protein. Binding of cGK-II to the plasma membrane is necessary for interaction with the membrane-inserted target proteins, such as CFTR chloride channels (Gardner et al. 1995), and requires N-terminal myristoylation (Vaandrager et al. 1998).

5. Guanylin as a Luminal Activator of Chloride Secretion in Distal Airways and Pancreatic Ducts

CFTR chloride channels, which are the main target of the guanylin signal cascade in the intestine, are also crucially involved in transepithelial ion transport in pancreatic ducts and in airway epithelia. We therefore tested the hypothesis that guanylin is also an activator of chloride secretion in these epithelia. As cell model we used the permanently growing human pancreatic duct cell line Capan-1 and the Clara cell line H441 which are known to express functional CFTR chloride channels (Kulaksiz et al. 2001, 2002). Using the patch-clamp technique we could demonstrate that in both cell lines the stimulation with either guanylin or the heat stable enterotoxin STa from *E. coli* causes a rise in the whole-cell chloride membrane conductance. The effect of guanylin and STa could be mimicked by addition of the membrane permeant cGMP analogue dibutyryl-cGMP, indicating that cGMP is the intracellular messenger mediating chloride current activation. Enzymatic tests confirmed that incubation of the cells with either guanylin or STa led to an elevation in the cytosolic cGMP but not cAMP concentration.

RT-PCR and Western blot analysis as well as immunocytochemical methods demonstrated that both, Capan-1 and H441 cells, express all proteins necessary for guanylin-dependent signal-transduction. Immunostaining of human pancreatic tissue and tissue sections of human lung with specific antibodies demonstrated that all members of the guanylin signal cascade, the guanylin receptor GC-C, the cGMP-dependent protein kinase cGK-II as well as the cGK-II target CFTR are confined to the apical plasma membrane of Clara cells and pancreatic duct cells. Furthermore it could be shown that guanylin is produced and stored in distal pancreatic duct cells and Clara cells. Therefore, our data strongly suggest that in human lung and exocrine pancreas guanylin regulates chloride secretion as a paracrine activator which locally co-ordinates epithelial electrolyte transport within single pancreatic ducts and small distal airways (Fig. 1).

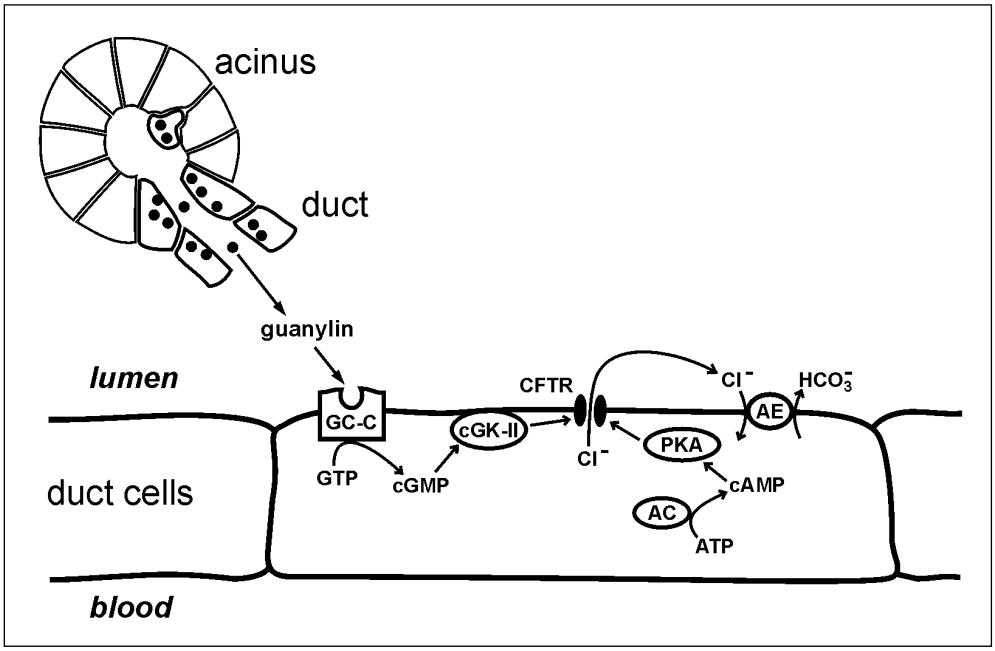

Fig. 1 In the exocrine pancreas guanylin is produced in centroacinar and distal duct cells. From these cells guanylin is released into the pancreatic juice. Guanylin binds to the receptor guanylyl cyclase C and the activated receptor produces cGMP. This leads to activation of the cGMP-dependent protein kinase cGK-II which phosphorylates and thereby opens the CFTR chloride channel in the luminal plasma membrane. The chloride-rich fluid is then modified by chloride-bicarbonate exchange through an anion exchanger (AE) which is also located in the luminal membrane of duct cells. CFTR activation by hormones and neurotransmitters acting from the basolateral membrane involves activation of a adenylyl cyclase (AC), production of cAMP and activation of the cAMP-dependent protein kinase A (PKA).

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PD Dr. Andreas Schmid
Institut für Physiologie
Gebäude 58
Universität des Saarlandes
66421 Homburg/Saar
Germany
Phone: +49 6841 1626454
Fax: +49 6841 1626655
E-Mail: andreas.schmid@uniklinik-saarland.de

Bildgebung und Tumorheilung

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Verfahren der Bildgebung sind von großer Aktualität in der Medizin, vor allem in der Tumorbehandlung. Die Computertechnologie erlaubt heute diagnostische Einblicke, die noch vor wenigen Jahren undenkbar waren. Die Fortschritte in der Technik vollziehen sich mit so atemberaubendem Tempo, daß die Studien zum klinischen Nutzen der verschiedenen Verfahren, die zeitaufwendig und kostenintensiv sind, kaum mithalten können. Der Band versucht daher, Anwendungsmöglichkeiten und Einsatzgrenzen der modernen bildgebenden Verfahren für die medizinische Praxis auszuloten. Die »Bilderflut aus dem Körper« wird geordnet und die Aussagefähigkeit der einzelnen Methoden systematisiert. Behandelt werden u. a. Computertomographie (CT), Positronenemissionstomographie (PET), Magnetresonanztomographie (MRT) und Magnetresonanzspektroskopie (MRS). Ein Schwerpunkt ist den Anforderungen und Fragestellungen gewidmet, die von den tumorbehandelnden Fächern an die Bildgebung gestellt werden. Die Darstellung und Interpretation von Tumorstrukturen wird sowohl mit bildgebenden Verfahren aus Radiologie und Nuklearmedizin als auch mit Verfahren der Pathologie behandelt. Außerdem werden Zusammenhänge von Tumormasse und Tumorkontrolle bzw. von gewebespezifischer Bildgebung und Tumorheilung erörtert sowie Verfahren der interventionellen Radiologie dargestellt. Einen weiteren Schwerpunkt bilden Überlegungen zur Indikation der einzelnen Verfahren und zur Kosten-Nutzen-Problematik. Es wird diskutiert, welchen Gewinn die verbesserte Primärdiagnostik von Tumoren für den Patienten bringt und wie sich die verbesserte Diagnostik des Rezidivs auf dessen Therapie auswirkt. Dabei werden auch offene Fragen angesprochen, etwa bei der Erfassung postoperativ zurückbleibender Tumoranteile oder bei der Lokalisation von Lymphknotenmetastasen in verschiedenen Körperregionen bzw. bei Unterscheidung der Patienten in Responder oder Nicht-Responder in therapeutischen Maßnahmen. Der Band zeigt, daß man in der Tumorbehandlung von der Weiterentwicklung der bildgebenden Verfahren vor allem differenzierte Aussagen erwartet: über den Wert entsprechender therapeutischer Varianten für die Heilung oder ein palliativ symptomatisches Vorgehen, wenn eine belastende »kurative« Therapie überflüssig und letztendlich nicht erfolgreich sein wird.

In Kommission bei Wissenschaftliche Verlagsgesellschaft mbH Stuttgart

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Inhibition of Epithelial Na^+ Channels by CFTR and Purinergic Agonists: Mechanisms and Significance for CF

Karl Kunzelmann[1,2], Jane Sun[1], Daniel Markovich[1], Jens König[2], Bettina Mürle[2], Thilo Voelcker[2], Marcus Mall[3], and Rainer Schreiber[1]

Abstract

Epithelial Na^+ channels (ENaC)[4] are located in the luminal membrane of salt absorbing epithelia in kidney, airways, colon and glandular excretory ducts. CFTR Cl^- channels and other Cl^- channels inhibit ENaC and thus limit Na^+ absorption. Inhibition of ENaC during activation of CFTR has been demonstrated in numerous tissues and is probably mediated by an increase in the intracellular Cl^- concentration. ENaC is also inhibited by activation of purinergic receptors, as demonstrated in airways and renal cells. Both CFTR and purinergic inhibition of Na^+ absorption is Cl^--dependent. We may hypothesize a Cl^--sensitive site in one or several subunits of the epithelial Na^+ channel. Cystic Fibrosis is characterized by a loss of cAMP activated CFTR Cl^- conductance and enhanced Na^+ absorption in airways and colon. Apart from correcting the Cl^- channel defect, the primary goal of a novel pharmacotherapy is the inhibition of enhanced Na^+ conductance in CF airways. Purinergic stimulation with stabilized ATP or UTP derivatives such as INS365 (Inspire Pharmaceuticals, Durham, NC, USA) and thus downregulation of ENaC with parallel activation of Ca^{2+}-dependent Cl^- channels should restore Cl^- secretion and reduce excessive salt absorption. A better understanding of the Cl^--dependent mechanism of purinergic or CFTR-dependent inhibition of ENaC may lead to the identification of new therapeutic targets.

Zusammenfassung

Der epitheliale Na^+-Kanal (ENaC) ist exprimiert in der luminalen Membran von Kochsalz-resorbierenden Epithelien von Niere, Luftwegen, Kolon und exkretorischen Drüsen. In Coexpressionsexperimenten führt die Aktivierung von CFTR-Cl^--Kanälen oder anderer Cl^--Ionenkanäle zur Inhibition epithelialer Na^+-Kanäle, wodurch die Na^+-Resorption limitiert wird. Die Hemmung epithelialer Na^+-Kanäle durch CFTR wurde mittlerweile in verschiedenen Epithelien nachgewiesen und ist wahrscheinlich durch einen Anstieg der zytosolischen Cl^--Konzentration bedingt. ENaC wird ebenfalls durch Aktivierung purinerger Rezeptoren gehemmt, was bislang in Luftwegsepithelien und Nierenepithelzellen gezeigt wurde. Für CFTR-abhängige und purinerge Hemmung der Na^+-Absorption wurde eine Cl^--Abhängigkeit gefunden. Die vorliegenden Resultate weisen auf eine elektrostatische Interaktion von Cl^- mit dem Na^+-Kanal hin. Zystische Fibrose ist durch einen Verlust der cAMP-aktivierten Cl^--Leitfähigkeit auf Grund von Mutationen im CFTR-Protein und durch erhöhte Na^+-Absorption in Luftwegen und Darm charakterisiert. Die Pharmakotherapie der Erkrankung zielt darauf ab, den Cl^--Kanaldefekt zu kompensieren und die erhöhte Na^+-Resorption zu unterdrücken.

1 School of Biomedical Sciences, Department of Physiology and Pharmacology, University of Queensland, St. Lucia, QLD 4072, Brisbane, Australia.

2 Institut für Physiologie, Universität Regensburg, Universitätstraße 31, 93053 Regensburg, Germany.

3 CF/Pulmonary Research and Treatment Center, University of North Carolina at Chapel Hill; 7011 Thurston Bowles Building, Chapel Hill, NC 27599-7248, USA.

4 Abbreviations: CFTR: cystic fibrosis transmembrane conductance regulator; CF: cystic fibrosis; IBMX: 3-isobutyl-1-methylxanthine; cAMP: 5' cyclic adenosine monophosphate; ENaC: epithelial Na+ channel; UTP: uridine 5'-triphosphate, ATP: adenine 5'-triphosphate.

Purinerge Stimulation mit stabilisierten Derivaten von ATP oder UTP, wie z. B. INS365 (Inspire Pharmaceuticals, Durham, NC, USA), führt zur Hemmung des Na^+-Kanals und gleichzeitiger Aktivierung Ca^{2+}-abhängiger Cl^--Kanäle, was die Cl^--Sekretion wiederherstellt und die exzessive Salzresorption hemmt. Ein besseres Verständnis des Cl^--abhängigen Mechanismus der purinergen bzw. CFTR-abhängigen Hemmung von ENaC könnte zur Identifikation neuer therapeutischer Wege führen.

1. Introduction

Cystic fibrosis (CF) is a frequent common autosomal recessive disease in the Caucasian population with an incidence between 1/2500 and 1/3000. The pathophysiology and cellular pathology in CF has been examined in detail and is based on mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) (Riordan et al. 1989). The basic defects are enhanced Na^+ absorption in the surface epithelium along with reduced cAMP-dependent Cl^- secretion in the airway glands (Kunzelmann et al. 2000). Thus, normalizing pathological ion transport, particularly in the airways, has become a major goal for current approaches in CF therapy. Previous work has demonstrated the presence of purinergic receptors on both sides of the airway epithelium (Lazarowski and Boucher 2001). These receptors are activated by purine ligands like adenine 5'-triphosphate (ATP) or uridine 5'-triphosphate (UTP), which leads to a reduced net electrolyte absorption (Clarke and Boucher 1992). Because these mechanisms are preserved in CF, stimulation of the purinergic pathway is expected to have beneficial effects in CF airways by normalizing airway surface liquid homeostasis and increasing lung clearance.

2. Cystic Fibrosis

The progressive lung disease is the single most important and life limiting factor in CF. According to the so called isotonic volume hypothesis (Boucher 1999), at least two mechanisms seem to contribute to the pathophysiology of the CF lung disease:

(*i*) enhanced absorption of Na^+ and
(*ii*) impaired cAMP dependent Cl^- secretion.

The isotonic volume transport theory (Boucher 1999) predicts an isotonic contraction of the airway surface liquid (ASL), the thin watery layer that covers the ciliated airway epithelial cells. Isotonic dehydration of the ASL in CF is expected to lead to mucus impactions, airway obstruction, submucosal gland hypertrophy, and a decrease in mucociliary airway clearance (Boucher 1999).

3. CFTR-dependent Inhibition of ENaC

Evidence has grown over the past that in the intestinal epithelium CFTR regulates both electroneutral as well as electrogenic absorption of electrolytes (Kunzelmann and Mall 2002a). Regulation of the epithelial Na^+ channel ENaC by CFTR was first detected in an epithelial cell line and in fibroblasts transfected with ENaC and CFTR (Stutts et al. 1995). These initial results were confirmed by experiments in *Xenopus* oocytes coexpressing CFTR and ENaC

(Mall et al. 1996). ENaC currents were inhibited by activation of wtCFTR Cl^- currents, but not by mutant ΔF508-CFTR. CFTR's ability to downregulate ENaC relies on the proper direction and the magnitude of the Cl^- current through CFTR Cl^- channels (Briel et al. 1998). Thus, Cl^- flux through CFTR and/or changes in the intracellular Cl^- concentration could serve as the signal for inhibition of ENaC. The inhibitory effects of CFTR on ENaC are clearly suppressed in the presence of a low extracellular Cl^- concentration, suggesting that Cl^- influx and eventually accumulation in the cytosol is essential for the inhibition of ENaC (König et al. 2001, Briel et al. 1998). Such a mechanism exists in the mouse salivary duct epithelium (Dinudom et al. 1993). For this feedback regulation of ENaC, $G_{\alpha i2}$ subunits of Cl^--sensitive trimeric GTP-binding proteins play a central role. In addition, a Na^+ feedback mechanism exists, which has been well examined and which requires $G_{\alpha 0}$ subunits of trimeric G proteins (Dinudom et al. 1998, 1993). Nevertheless, Na^+ feedback inhibition of ENaC as observed in *Xenopus* oocytes is independent of G protein function (Hübner et al. 1999).

4. Purinergic Inhibition of ENaC

Activation of electrolyte secretion by extracellular nucleotides in both normal and CF airways activates Cl^- secretion indirectly, by activating K^+ channels, and directly by activation of Ca^{2+}-dependent Cl^- channels (Clarke and Boucher 1992). $P2Y_2$ receptors are co-localized with $P2Y_1$ receptors on the basolateral side, and with $P2Y_6$ and probably $P2Y_4$ receptors on the mucosal side of the epithelium (Communi et al. 1999, Hwang et al. 1996). Luminal $P2Y_2$ receptors are activated by ATP or UTP, while $P2Y_6$ receptors are stimulated through metabolic break down products of UTP, such as UDP (Lazarowski et al. 1997). Recent studies have identified that airway epithelial cells release nucleotides, which in turn regulate cellular functions in an autocrine/paracrine fashion. Nucleotides are released to the luminal compartment of the airways and reach concentrations that are sufficient to activate luminal purinergic receptors (Lazarowski and Harden 1999). It is very likely that a transient accumulation of nucleotides in the very thin film of airway surface liquid may be sufficient to activate purinergic receptors.

Activation of $P2Y_2$ and $P2Y_6$ (and eventually $P2Y_4$) receptors on the apical surface of airway epithelial cells have multiple effects on mucociliary clearance. They affect Ca^{2+} and protein kinase C dependent cellular functions, such as ion transport, ciliary beat frequency and mucin release (Lazarowski and Boucher 2001, Lethem et al. 1993). Ca^{2+}-dependent activation of luminal Cl^- channels in the airways has been known for some time (Welsh 1987, Koslowsky et al. 1994). Previous studies identified a family of Ca^{2+}/calmodulin-activated Cl^- channels (CaCC), expressed predominantly in the digestive and respiratory mucosa and which may be activated by ATP or UTP (Agnel et al. 1999, Mall et al. 2003). It should be noted that Ca^{2+} increase that is triggered by stimulation of purinergic receptors, also activates basolateral Ca^{2+}-dependent SK4 type K^+ channels. Co-activation of these K^+ channels does enhance the driving force for luminal Cl^- exit and therefore supports Cl^- secretion (Mall et al. 2003).

Activation of mucosal purinergic receptors does not only stimulate Cl^- secretion but also modulates Na^+ absorption. This has been demonstrated in kidney and airway epithelial cells (Mall et al. 2000, Cuffe et al. 2000). Importantly, purinergic inhibition of amiloride-sensitive Na^+ transport was observed in native nasal tissues and primary bronchial epithelial cell cultures from CF patients (Mall et al. 2000, Devor and Pilewski 1999). Although several transport proteins participate in transepithelial Na^+ absorption, such as luminal epithelial Na^+ channels

(ENaC), the basolateral Na^+/K^+ ATPase and basolateral K^+ channels, it is likely that purinergic stimulation inhibits ENaC directly. The inhibitory effect is independent of intracellular Ca^{2+} or Ca^{2+}-dependent protein kinase C (PKC) (Mall et al. 2000, Kunzelmann et al. 2002b). However, it requires the function of phospholipase C and involves Cl^- transport over the luminal membrane (Kunzelmann et al. 2002b). Two recent papers show activation of ENaC channels by phosphatidylinositols, probably by interaction with putative PIP_2-binding domains in the N termini of the β- and γ-subunits of ENaC. Hydrolysis of PIP_2 leads to deactivation of ENaC channels (Ma et al. 2002b, Yue et al. 2002). Thus cleavage of PIP_2 by ATP/UTP mediated activation of PLC is probably the mechanism how ENaC and thus amiloride sensitive Na^+ absorption is inhibited by purinergic stimulation (Ma et al. 2002a).

5. Pharmacotherapy in CF

Topical application of aerosolized ATP or UTP has a dual potential therapeutic effect in CF by counteracting Na^+ absorption and by promoting Ca^{2+}-dependent Cl^- secretion in airways (Olivier et al. 1996, Bennett et al. 1996). However, both UTP and ATP are rapidly degraded (< 1 min) from the airways by ecto-nucleotidases, limiting the therapeutic effects of both nucleotides. Thus, synthetic ligands of purinergic receptors have been developed, with a higher biological and chemical stability compared to UTP or ATP (Pendergast et al. 2001). Stabilized diuridine polyphosphates, like INS37217 and INS365 (Inspire Pharmaceuticals, Durham, NC, USA), show promising initial results and indicate safety and efficacy of this compound (Yerxa et al. 2002, Kellerman 2002). Apart from the treatment of CF, there is a large demand for these synthetic agonists for the treatment of chronic bronchitis and rhinosinusitis, diseases that affects large percentages of the population worldwide.

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Prof. Dr. Karl Kunzelmann
Universität Regensburg
Institut für Physiologie
Universitätsstraße 31
93053 Regensburg
Germany
Phone: +49 941 9434302
Fax: +49 941 9434315
E-Mail: uqkkunze@mailbox.uq.edu.au

»Bewahren und Verändern im Kontext biologischer und kultureller Evolution«
Gaterslebener Begegnung 2003

Gemeinsame Tagung des Institutes für Pflanzengenetik und Kulturpflanzenforschung (IPK) Gatersleben und der Deutschen Akademie der Naturforscher Leopoldina vom 22. bis 24. Mai 2003 in Gatersleben

Nova Acta Leopoldina N. F., Bd. *90*, Nr. 338
Herausgegeben von Anna M. Wobus (Gatersleben), Ulrich Wobus (Gatersleben) und Benno Parthier (Halle/Saale)
(2004, ca. 250 Seiten, ca. 48 Abbildungen, ca. 10 Tabellen, 29,95 Euro, ISBN 3-8047-2170-2)

Zum nunmehr zehnten Mal trafen sich in Gatersleben Natur- und Geisteswissenschaftler, Schriftsteller, Künstler, Journalisten und Politiker zum interdisziplinären Dialog über Fragen, die sich aus den Entwicklungen von Naturwissenschaft und Technik für die Gesellschaft ergeben. Naturwissenschaftler und Biologen sind dem Erhalt der in der Evolution entstandenen biologischen Vielfalt, aber auch dem Anspruch verpflichtet, dieses Potential verantwortungsvoll zum Wohle des Menschen einzusetzen und zu gestalten. Der Band enthält neben den *Anfragen an Wissenschaftler* (diesmal von der Schriftstellerin Helga Schütz, Potsdam) Beiträge zur Evolutionsbiologie (Konrad Bachmann, Gatersleben, Evolution und Information; Jörg Hacker, Würzburg, Evolutionäre Infektionsbiologie), Ethologie (Wulf Schiefenhövel, Andechs, Vom Instinkt zur Kultur: Zur Evolution geistiger Fähigkeiten Beispiele aus traditionalen Kulturen Melanesiens), Humangenetik (Peter Propping u. a., Bonn, Humane Reproduktionsbiologie: Eingriff in die natürliche Evolution des Menschen?), Kulturwissenschaft (Sigrid Weigel, Berlin, Evolution der Kultur oder Kulturgeschichte der Evolutionstheorie – Epistemische Probleme am Schnittpunkt der zwei Kulturen) und Ethik (Klaus Tanner, Halle/Saale, Zwischen »Heuristik der Furcht« und Hoffnung auf Veränderung). Er spannt damit den Bogen von naturwissenschaftlichen Problemen über die Reflexion wissenschaftshistorischer und -philosophischer Zusammenhänge bis hin zur Debatte ethischer Standpunkte. Verbunden mit Gedichten der beteiligten Schriftsteller und Abbildungen der Werke der in Gatersleben zur Begegnung ausstellenden Künstler vermittelt der Band einen unkonventionellen Blick auf ein nach wie vor brisantes Thema.

In Kommission bei Wissenschaftliche Verlagsgesellschaft mbH Stuttgart

Energie

Vorträge anläßlich der Jahresversammlung vom 17. bis 20. Oktober 2003 zu Halle (Saale)

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Während früher naturwissenschaftliche und technische Argumente in Diskussionen über Energiebedarf, -erzeugung und -nutzung bestimmend waren, werden diese heutzutage auf vielfältige Weise durch ökologische, soziale und kulturelle Aspekte verdrängt. Bürgerinitiativen, Umweltgruppen, Medien, Gewerkschaften, Parteien und auch Kirchen sind am Energiediskurs beteiligt. Daß Energie eine naturwissenschaftliche Basis hat, wird in der Öffentlichkeit häufig kaum noch wahrgenommen. Aus einem naturwissenschaftlich-technischen ist ein gesellschaftswissenschaftlich-kultureller Diskurs geworden. Angesichts der Vielzahl unterschiedlicher Prognosen zum Weltenergiebedarf und der Energienutzung, die Experten und Politik der Öffentlichkeit vorlegen, kann das nicht verwundern. Die Leopoldina nahm das zum Anlaß, um auf ihrer Jahresversammlung verschiedene Energieszenarien zu erörtern. Ausgehend von den naturwissenschaftlichen Grundlagen werden sowohl die Auswirkungen der verschiedenen Formen von Energieträgernutzung auf die Umwelt, aber auch Leitbilder, Zielvorstellungen und ethische Prinzipien der Energieversorgung hinterfragt. Im Hinblick auf Komplexität, Globalität und Langfristigkeit des Energiebereiches liefern die Beiträge des Bandes eine auf dem heutigen Erkenntnisstand gegründete wissenschaftliche Standortbestimmung zu Fragen der Energieerzeugung und Energienutzung. Dabei werden Windkraft und Biomasse ebenso ausführlich betrachtet wie Kernenergie und Kernfusion. Die Tendenzen in der Nutzung fossiler Energieträger unterliegen einer kritischen Analyse. Außerdem werden technische Fragen (Solarzellen, Brennstoffzellen, Aufwindkraftwerke, energieeffizientes Gebäudemanagement) und ökonomische Probleme (z. B. der Strommarktliberalisierung) umfassend dargestellt. Ein Positionspapier faßt die Sicht der Leopoldina auf die für zukünftige politische, ökonomische und wissenschaftliche Weichenstellungen aktuell bedeutsame Energieproblematik zusammen.

In Kommission bei Wissenschaftliche Verlagsgesellschaft mbH Stuttgart