Department of Physiology
Faculty 3 (Theoretical Medicine)

The University of Saarland, Homburg/Saar

The Role of Tyrosine Phosphorylation in the regulation of TRPV6

Dissertation to obtainment the grade of doctor in medicine

Faculty of Medicine
UNIVERSITY OF SAARLAND
2005

Submitted by: Hajaj Alshaldi
born on 8. Mai 1974 in Hama

Table of Contents

List of Tables

List of Figures

Abbreviations

VOCCs	Voltage-operated calcium channel
ROCCs	Receptor-operated calcium channel
SOCCs	Store-operated calcium channel
TRP	Transient receptor potential
PM	Plasma Membrane
ER	Endoplasmic Reticulum
SR	Sarcoplasmic Reticulum
TRPC	Transient receptor potential for canonical or Classical
TRPV	Transient receptor potential for vanilloid
TRPM	Transient receptor potential for melastatin
TRPP	Transient receptor potential for polycystins
PKD	Polycystic kidney disease
TRPA	Transient receptor potential for Ankyrin
TRPML	Transient receptor potential for mucolipin
TRPN	Transient receptor potential for no no mechano-receptor potential
CaT2	Calcium Transporter 2
CaT1	Calcium Transporter 1
ECaC1	Epithelial Calcium channel 1
ECaC2	Epithelial Calcium channel 2
HEK	Human Embryonic Kidney
2-APB	2-aminoethoxydiphenylborate
CRAC	calcium release activated calcium current
RBL	rat basophilic leukemia
RT-PCR	reverse transcription-polymerase chain reaction
mRNA	Messenger RNA
$1,25\text{-}(OH)_2D_3$	1,25-dihydroxycholecalciferol
1α-OHase	1α hydroxylase
ATP	adenosine triphosphate
PTKs	protein tyrosine kinase
PTPs	protein tyrosine phosphatase
DMEM	Dimethylsulfoxide
CBS	Calf bovine serum
DMSO	Dimethylsulfoxide
GFP	green fluorescence protein
YFP	yellow fluorescence protein
TG	Thapsigargin
DMHV	bis-(*N,N*-dimethyl-hydroxamido)-hydrooxovanadate
BTP2	3,5-bistrifluoromethyl pyrazole Derivative

Abstract

The transient receptor potential (TRP) channel family member, TRPV6 (formerly known as CaT1 or ECaC2) have been implicated in maintaining the body Ca^{2+} balance by facilitating Ca^{2+} (re)absorption in kidneys and small intestines. In this study, we examined the role of the nonreceptor cellular tyrosine phosphtase PTP1B in the modulation of TRPV6 channel. In HEK-293 cells, TRPV6-mediated Ca^{2+} influx was increased in the presence of a tyrosine phosphatase inhibitor (bis-(*N*,*N*-dimethyl-hydroxamido) hydroxo-vanadate; DMHV). Also cotransfection of TRPV6 with PTP1B resulted in twofold increase in TRPV6-mediated Ca^{2+} influx. At the same time, Co-expression of the inactive substrate-trapping PTP1B (D181A) and TRPV6 in HEK-293 resulted in the same increase in TRPV6-mediated Ca^{2+} influx in the presence and absence of DMHV.

These data indicate that tyrosine phosphorylation is involved in the regulation of the TRPV6 channel protein. Importantly, point mutation of Tyr(161,162) abolished the increase in TRPV6-mediated Ca^{2+} influx. In aggregate, these data indicate that tyrosine dephosphorylation is involved in the regulation of the TRPV6 channel protein.

Chapter1

Introduction

Calcium is the most abundant cation in the human body and serves a number of important physiological functions, including fertilization, proliferation, development, learning and memory, contraction and secretion. Exceeding its normal spatial and temporal boundaries can result in cell death through both necrosis and apoptosis (Michael J. Berridge et al. 2000). Our understanding of calcium signaling is at two levels. First, the elementary calcium signals themselves have been shown to provide local control of many physiological functions like the release of synaptic and secretory vesicles, the activation of ion channels and the generation of nuclear specific calcium signals (Lipp P et al. 1997). Second, in addition to control the local functions of cells, elementary calcium signals are responsible for generation of global calcium signals such as waves and oscillations. A feature that makes calcium such a versatile ion in signaling is the ability of the cell to precisely regulate the cellular concentrations of free and sequestered calcium. These concentrations are regulated by the simultaneous interplay of multiple counteracting processes, which can be divided into calcium 'on' and 'off' mechanisms depending on whether they serve to increase or decrease cytosolic calcium. The calcium 'on' mechanisms include channels located in the plasma membrane (PM) which regulate the supply of calcium from extracellular space, and channels in the endoplasmic reticulum and sarcoplasmic reticulum (ER and SR respectively) which regulate the supply of calcium from intracellular Ca^{2+} stores (Berridge M.J. 2000). An equal diverse set of 'off' mechanisms is employed by cells to remove calcium from the cytoplasm. These include Ca^{2+}-ATPases in the PM and ER/SR, and exchangers that utilize gradients of other ions to provide the energy to transport calcium out of the cell like the Na^{+}/Ca^{2+} exchanger. Mitochondria also play an important part in regulating cytosolic calcium levels. These organelles have a low affinity, but high-capacity rapid Ca^{2+} uniporter that can significantly reduce cytosolic calcium transients and diminish cellular responses (Montero M et al. 2000).

1.1 Calcium entry channels

Calcium utilize several different types of calcium influx channel, which can be grouped on the basis of their activation mechanisms. Voltage-operated calcium Channels (VOCCs) are employed largely by excitable cell types such as muscle and neuronal cells (William A. Catterall. 2000), where they are activated by depolarization of the PM. Receptor-operated calcium channels (ROCCs) that are particularly prevalent on secretory cells and at nerve terminals. ROCCs are activated by the binding of an agonist to the extracellular domain of the channel. Mechanically activated calcium channels are present on many cell types and respond to cell deformation. Store-operated calcium channels (SOCCs) are activated in response to depletion of the intracellular calcium store, either by physiological calcium-mobilising messengers or pharmacological agents. Many different types of cells have been shown to have an enhanced calcium entry following calcium pool depletion, SOCCs may be one of the most ubiquitous PM calcium channels. At present, the best candidates for the molecular identity of SOCCs are homologous of a protein named transient-receptor-potential protein (TRP) that functions in Drosophila photoreception (Boulay G. et al. 1999).

1.2 TRP Channels

TRP channels constitute a large and diverse family of channel proteins that are expressed in many tissues and cell types in both vertebrates and invertebrates (J Vriens et al. 2004). The large functional diversity of TRPs is reflected in their diverse permeability to ions, activation mechanisms, and involvement in biological processes ranging from pain perception to male aggression. The first member of the family to be identified, *Drosophila melanogaster* TRP, was discovered in the analysis of a mutant fly whose photoreceptors failed to maintain a sustained response to a prolonged stimulus of light (Hardie and Minke 1992).Mammalian homologs of the *Drosophila* TRP gene encode a family of at least 20 ion channel proteins which can be divided by sequence homology into at least six subfamilies, designated TRPC (for canonical or classical), TRPV (for vanilloid), TRPM (for melastatin), TRPP (for polycystins, PKD-type), TRPA (for ankyrin), TRPML (for mucolipin), and the more distant subfamily TRPN (N for “nomp” no mechano-receptor potential) (Figure1) . The TRP channels are made of subunits with six membrane-spanning domains. By analogy to the relatively well-understood K_v class, TRP channels probably form tetramers in which the

amino acids that link the fifth and sixth transmembrane domains line the pore. But these channels are less refined in their selectivity for ions, as most of them allow any cation, including Ca^{2+}, to move into the cell (David E. Clapham, et al. 2001).An intriguing subfamily within the TRP superfamily is the TRPV subfamily consisting of six members. This group of channel includes TRPV1– 4, which respond to heat, osmolarity, odorants, and mechanical stimuli, whereas epithelial Ca^{2+} channels TRPV5-6 have been implicated in maintaining the body Ca^{2+} balance by facilitating Ca^{2+} (re)absorption in kidney and small intestine (Peng JB et al. 2003. Hoenderop JG et al. 2002).

1.3 Human Calcium Transport protein TRPV6

The epithelial calcium channel family is restricted to two members, TRPV5 and TRPV6, which in former literature were called CaT2 and CaT1, or ECaC1 and ECaC2, respectively. Both genes were identified nearly simultaneously by two groups (Hoenderop JG et al. 2001).The structure of TRPV6 shows typical topology features shared by all members of the TRP family, including six transmembrane regions, a short hydrophobic stretch between transmembrane segments 5 and 6, which is predicted to form the Ca^{2+} pore, and large intracellular N- and C-terminal domains. These intracellular regions contain several conserved putative regulatory sites that might be involved in regulation of channel activity and trafficking, like phosphorylation sites, PDZ motifs , and ankyrin repeat domains (Hoenderop JG et al. 2002) (Figure.2).

1.3.1 Functional Properties

When expressed in human embryonic kidney (HEK) 293 cells, TRPV6 mediates a saturable Ca^{2+} uptake and manifests distinct electro-physiological features, including hyperpolarization-dependent Ca^{2+} entry and Ca^{2+} -dependent inactivation (Hoenderop JG et al. 2003). TRPV6 displays large inward currents that are strongly dependent on extracellular Ca^{2+} and reverse at high positive membrane potentials. Studies in HEK-293 cells expressing TRPV6 have shown that this channel exhibits an ion permeation sequence for divalent cations (Ca^{2+} $>Sr^{2+}\approx Ba^{2+}>Mn^{2+}$) (Vennekens R et al. 2000). The characteristic pore region of TRPV6 is unique for its high Ca^{2+} selectivity with a Ca^{2+}-to-Na^{+} permeability ratio (P_{Ca}/P_{Na}) being over 100. A single aspartic residue in the pore region at position 542 (D542) is crucial for Ca^{2+}

permeation. Mutation of D542 to alanine abolishes Ca^{2+} permeation, but does not affect the permeation of monovalent cations (Nilius B et al. 2001). Like voltage-gated Ca^{2+} channels, this epithelial calcium channel is permeable for monovalent cations in the absence of divalent cations with the permeation sequence $Na^{+} > Li^{+} > K^{+} > Cs^{+}$.

1.3.2 Modulation of Channel Activity

1.3.2.1 Intracellular Calcium

Sustained increases in intracellular Ca^{2+} may cause cell death. Ca^{2+}-transporting cells express calbindins to buffer the increases in intracellular Ca^{2+} resulting from calcium entry through these channels. To avoid elevations in intracellular Ca^{2+} to toxic levels that are beyond the buffering capacity of the calbindins, TRPV6 exhibits Ca^{2+}-dependent inactivation (Niemeyer BA et al. 2001). TRPV6 shows both a fast phase (within 50 ms) and a slow phase of inactivation (over a period of ~1s). The fast phase is determined by the first intracellular loop of TRPV6 and H587, a positively charged amino acid residue downstream of the last transmembrane domain of TRPV6 (Suzuki M et al. 2002). The slow phase involves direct binding of calmodulin to the TRPV6 C-terminal region (Niemeyer *et al*. 2001). Ca^{2+}-dependent binding of calmodulin to TRPV6 inactivates the TRPV6 channel. A protein kinase C site is present within the calmodulin binding site in human TRPV6, and the phosphorylation of this site prevents calmodulin binding, thereby maintaining the activity of TRPV6 in order to allow more Ca^{2+} to enter the cell (Niemeyer *et al.* 2001).

1.3.2.2 Pharmacology

Little is known about effective pharmacological tools to modulate TRPV6. La^{3+}, Gd^{3+},Ruthenium red and econazole appeared to be the most effective inhibitors. Blockers of store-operated Ca^{2+} entry channels (SOC), e.g., SKF96365 and 2-aminoethoxydiphenylborate (2-APB), are nearly ineffective for TRPV6 (Nilius B et al. 2001). Xestospongin, a noncompetitive inositol 1,4,5-triphosphate receptor antagonist seems, however, to block TRPV6 (Vassilev PM et al. 2001).

1.3.3 CRAC and TRPV6

One of the unsolved problems in channel physiology is the nature and mechanism of the activation of SOC. Putney showed that the depleted calcium stores generate a signal which opens plasma membrane calcium channels (Putney. Capacitative calcium entry. 1997). The first electrical measurement of SOC was achieved in mast cells, and this current was referred to as "calcium release activated calcium current" (CRAC) (Hoth M, and Penner R.1992). CRAC is still the best-characterized SOC. It is a highly Ca^{2+}-selective channel (P_{Ca} /P_{Na} >100); permeable to Ca^{2+}, Sr^{2+}, Ba^{2+}, but not Mg^{2+}; exhibits anomalous mole fraction behavior, which means that it becomes permeable for monovalent cations in the absence of extracellular divalent cations and is inactivated by an increase in the intracellular Ca^{2+} concentration (Parekh AB and Penner R. 1997). These properties are reminiscent to the last described biophysical properties of TRPV6 except the activation by depletion of intracellular Ca^{2+} stores. Recently, Yue et al, reported that when TRPV6 is expressed at low levels, it can be activated by depleting calcium stores. Thus TRPV6 was proposed to constitute part or all of the CRAC channel pore, with the assumption that other cellular components are necessary for controlling TRPV6 via calcium stores (Yue L et al. 2001). In several other studies, a correlation between CRAC and TRPV6 also has been reported (Schindl R et al. 2002), (Vanden Abeele F et al. 2003), (Cui J et al. 2002), (Sternfeld et al. 2004). Despite the similarity of TRPV6 and CRAC channel currents, a comparison of TRPV6 expressed in HEK-293 cells and CRAC in RBL cells revealed certain differences between the TRPV6 and CRAC channel properties (Votes et al. 2001). These include the following: (1) CRAC (but not TRPV6) can be activated by ionomycin-mediated store depletion and can be blocked by 2-aminoethoxydiphenyl borate (2-APB); (2) TRPV6 (but not CRAC) can be blocked in a voltage-dependent manner by increasing intracellular Mg^{2+}; and (3) the channels display a difference in relative permeability to Na^{+} and Cs^{+} (Votes *et al* .2001). However, other differences exist and all these differences and similarities call into question whether and/or to what extent TRPV6 contributes to the CRAC channel.

1.3.4 Tissue Distribution of TRPV6

The tissues that express TRPV6 at high levels, as assessed by Northern analysis or *in situ* hybridization are (1) gastrointestinal tract: TRPV6 expression in the intestine was found in duodenum, jejunum, and cecum (Hoenderop et al. 2001). But Peng demonstrated expression of TRPV6 throughout the entire digestive tract from esophagus to colon (Peng et al. 2000). (2) Placenta: During pregnancy Ca^{2+} absorption in the placenta is solely responsible for the nutrient supply to the developing fetus. The Ca^{2+} needs of the fetus increase progressively throughout the pregnancy. This Ca^{2+} is transported by the syncytiotrophoblasts, where the cells that line the chorionic villi tissue and correspond to the epithelial layer separate the maternal and fetal circulation. Moreau demonstrated the expression of TRPV6 by RT-PCR in cytotrophoblasts freshly isolated from human term placenta (Moreau R et al. 2002). (3) Bone: the major Ca^{2+} store of the body, is an important tissue involved in Ca^{2+} homeostasis. Interestingly, TRPV6 mRNA have been detected in bone cells isolated from mouse femurs (Nijenhuis T et al. 2003). (4) Exocrine tissues, such as pancreas, testis, prostate, mammary gland, sweat gland, and salivary gland: TRPV6 mRNA is found in the exocrine acinar cell of the pancreas but not in pancreatic duct and β-cells (Zhuang L et al. 2002). In the human pancreas, acinar cells showed a granular and apical membrane staining of TRPV6. If TRPV6 is present in the membrane of the zymogen granules, it would have to be in an inactive state to prevent the release of calcium from the zymogen granules. Once it is incorporated into the plasma membrane, it would then become active to take up Ca^{2+} released from the granule (Peng JB et al. 2003). As TRPV6 is a Ca^{2+}-selective Ca^{2+} channel, its involvement in Ca^{2+} signaling in pancreatic acinar cells is likely. However, the role of TRPV6 in the process has not been shown. TRPV6 may play a role in the clearance of Ca^{2+} released from the zymogen granules by re-uptake of Ca^{2+} into the cells, thereby replenishing the Ca^{2+} pool equivalent to store operated Ca^{2+} entry.

1.3.5. Regulation of TRPV6

1.3.5.1 Regulation by 1,25-$(OH)_2D_3$

Vitamin D_3 is one of the main hormones in controlling Ca^{2+} balance (Reichel H et al. 1989). There are two sources of vitamin D_3 in the body. It is either ingested from the diet or

synthesized in the skin form its precursore 7-dehydrocholesterol in the presence of sunlight (Neer RM. 1975). Since vitamin D_3 itself is physiologically inactive, it is converted by an activation process, involving 25-hydroxylation in the liver followed by 1α-hydroxylation in mitochondria of the renal proximal tubulet to synthesize the biologically active 1,25-$(OH)_2D_3$. (Fraser DR and Kodicek E. 1970). The formation of 1,25-$(OH)_2D_3$ depends on the Ca^{2+} status of the body. When that Ca^{2+} is sufficient with adequate dietary Ca^{2+} intake and normal plasma Ca^{2+} concentration, 1α-OHase activity is low because there is no need for additional Ca^{2+}. However, when Ca^{2+} is insufficient, with a low dietary Ca^{2+} intake and decreased plasma Ca^{2+} concentration, the activity of this enzyme increases to produce 1,25-$(OH)_2D_3$ to ensure that additional Ca^{2+} will be absorbed from the gastrointestinal tract. Recent studies consistently indicated that the expression of TRPV6 is tightly controlled by 1,25-$(OH)_2D_3$ (Bouillon R et al. 2003).

1.3.5.2 Regulation by Estrogens

Estrogen deficiency results in a negative Ca^{2+} balance and bone loss in postmenopausal women (Nordin BE et al. 1979), this estrogen deficiency-dependent-bone loss is associated with a rise in plasma and urinary calcium. In addition to bone, the intestine and kidney are also potential sites for estrogen action and involved in Ca^{2+} handling and regulation. Recent findings suggest that TRPV6 expression is regulated by estrogen, as duodenal expression of TRPV6 mRNA of 1α-OHase knockout mice and ovariectomized rats is upregulated after 17β-estradiol administration (Van Abel M. 2003). It is clear that the expression of TRPV6 is influenced by the estrogen status. Estrogens, hormonal changes during pregnancy, and lactation have distinct, vitamin D-independent effects at the genomic level on active duodenal Ca^{2+} absorption mechanisms, mainly through a major upregulation of TRPV6 (Van Cromphaut SJ et al. 2003). The mechanism of estrogen-controlled upregulation of epithelial Ca^{2+} channel mRNA remains to be elucidated. Interestingly, recently an estrogen-responsive element in the promoter sequence of the mouse TRPV6 gene was described (Weber K et al. 2001).

1.3.5.3 Regulation by Dietary Ca^{2+}

Dietary Ca^{2+} is one nutrient that has been the focus of multiple studies in an effort to discover its preventive actions. It has been implicated in the reduction of risk in osteoporosis, and a significant link between Ca^{2+} intake and bone mass has been reported. Although recommended daily allowance of calcium is 600 mg/day for adults, ~850mg/day or more is recommended later in life. 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 decrease Ca^{2+} reabsorption and expression of Ca^{2+} transport proteins (Van Abel M et al. 2003). Studies revealed that Ca^{2+} supplementation can upregulate gene transcription encoding for Ca^{2+} transporters in the absence of circulating 1,25-$(OH)_2D_3$, and the Ca^{2+}-responsive element in the promoter region of TRPV6 gene are present (Gallin WJ, and Greenberg ME. 1995).

1.4 Protein Tyrosine Phosphatase

Protein phosphorylation is a major molecular mechanism through which protein function is regulated. Although proteins are known to be covalently modified in many other ways, including ADP-ribosylation, acylation, carboxymethylation, tyrosine sulfation and glycosylation, none of these mechanisms is nearly as widespread and readily subject to regulation by physiological stimuli as is phosphorylation. Regulation of protein phosphorylation involves a protein kinase, a protein phosphatase and a substrate protein. In all cases, the kinases catalyze the transfer of the terminal (γ) phosphate group of ATP to the hydroxyl moiety in the respective amino acid residue. Because phosphate groups are highly negatively charged, phosphorylation of a protein alters its charge, which can then alter the conformation of the protein and ultimately its functional activity. Protein phosphatases catalyze the cleavage of this phosphoester bond through hydrolysis (Eric. J. Nestler and Paul Greengard. Book. Basic Neurochemistry. Sixth Edition). Protein tyrosine phosphorylation is controlled through the coordinated actions of protein tyrosine kinases (PTKs) and phosphatases (PTPs). Protein tyrosine phosphatases (PTPs) are represented by a structurally diverse family, which in the human genome is encoded by ~ 100 genes (Andersen, J.N. et al.2001). The prototypic member of the PTP family is the enzyme PTP1B. This enzyme was originally purified from human placenta as a 37 KDa catalytic domain (Tonks. N.K., Diltz,

C.D. and Fischerl, E.H. 1988). A variety of structural studies have provided important insights into the mechanism of substrate recognition and catalysis. The catalytic domain of PTPs is approximately 200 to 300 amino acids in length and is composed of a central β sheet enclosed by α helices. The highly conserved motif, (I/V)HCXXGXGRS/TG, forms the base of the active-site cleft and contains the cysteinyl residue (Cys 215 in PTP1B) that is essential for catalysis. In the first step, there is nucleophilic attack by the sulfur atom of the thiolate side chain of the Cys on the substrate phosphate, coupled with protonation of the tyrosol-leaving group of the substrate by the side chain of a conversed acidic residue (Asp 181 in PTP1B) acting as a general acid. This leads to formation of a cysteinyl-phosphate catalytic intermediate. In the second step, mediated by Gln 262, which coordinates a water molecule, and Asp 181, which functions as a general base, there is hydrolysis of the catalytic intermediate and a release of phosphate (Denu, J.M. and Dixon,J.E.1998). The phosphotyrosine-recognition subdomain, confers substrate specifity of PTPs by creating a deep pocket (9 Aº) so that only the phosphtyrosine moiety is long enough to reach the cysteine nucleophile located at the base of this pocket. Through these properties PTPs can modulate the cellular level of tyrosine phosphorylation, and an appropriate level of tyrosine phosphorylation is essential for regulating cell growth, differentiation, metabolism, progression through the cell cycle, cell-cell communication, cell migration, gene transcription, ion channel activity, the immune response, and apoptosis/survival decisions.

1.5 Statement of purpose

The purpose of this study is to investigate the role which PTP1B may play in TRPV6-mediated calcium influx in HEK-293 cells. By inhibiting PTP1B, we can determine if PTP1B contributes to the regulation of TRPV6-mediated Ca^{2+} influx through dephosphrylation the tyrosine residues in TRPV6 and it is important to determine which residue is implicated in this dephosphorylation. If this role of PTP1B exists, it calls into question whether and/or to what extent Ca^{2+} store depletion affects the dephosphorylation of TRPV6 by PTP1B.

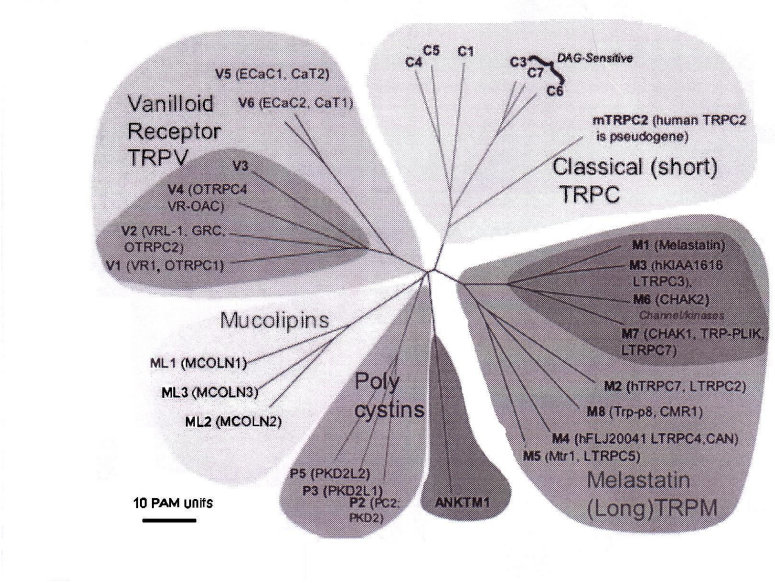

FIG.1. Mammalian TRP family tree.

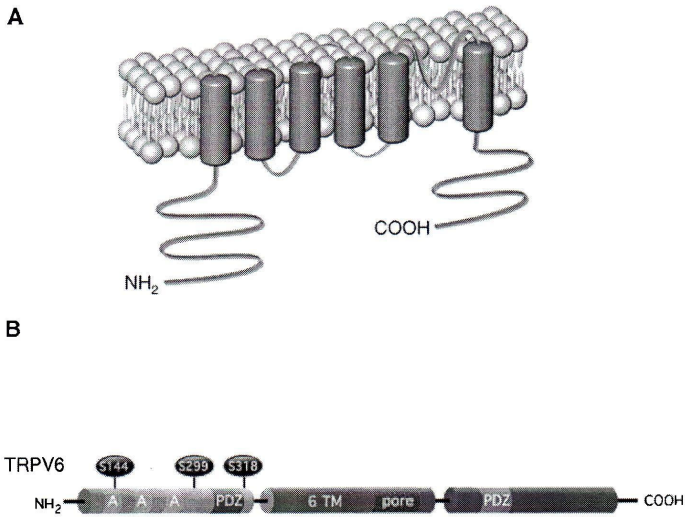

FIG.2. Structural organization of TRPV6. ***A*** : TRPV6 are 730 amino acids long with a predicted molecular mass of 83 KDa. TRPV6 contains a core domain consisting of 6 transmembrane (TM) segments. In addition, a large cytosolic amino and carboxy terminus are present containing ankyrin repeats. Between TM5 and TM6 there is a short hydrophobic stretch predicted to be the pore-forming region of this channel. ***B*** : Potential regulatory sites in the amino and carboxy tail of TRPV6 include ankyrin repeats, PDZ motifs and conserved PKC phosphorylation sites.

Chapter 2

Materials and Method

2.1 Cell Culture

HEK cells (human embryonic kidney), cell line 293 from (American Type Culture Collection, ATCC) were used in the experiments. Cells were cultivated on coverslips in culture dishes containing 2 ml of DMEM, a medium supplemented with L-Glutamine, 4500 mg Glucose, sodiumpyruvat (Invitrogen), 10% CBS (Calf bovine serum) (PAA Laboratories), Penicillin 100 U/ml, Streptomycin 100µg/ml (Invitrogen). Cells were kept in an incubator at a temperature of 37 °C, 95 % O_2, 8,5% CO_2 and 100% humidity for 24 hours.

2.2 Loading Fura-2-AM

For measurement of cytosolic calcium concentrations we used the fluorescence indicator Fura-2 (Molecular Probes, Eugene OR, USA). To load the cells, Fura-2-AM (1 µM) solved in Dimethylsulfoxide (DMSO, Sigma) was added to the cells and cells were incubated for another 15 minutes. Fura-2 is a dual-wavelength ratiometric dye, which is a polycarboxylate anion that cannot cross lipid bilayer membranes. The Fura-2-AM, however, is a polar ester that can cross lipid membranes. Because the AM group is labile to enzymatic hydrolysis by esterases present in the cell, AM esters of the Fura-2 are processed intracellularly to liberate the Ca^{2+}-sensitive polycarboxylate form which, being multiply charged, is trapped in the cell.

2.3 Transfection

Transfection of cells was performed with FUGENE 6 transfection reagent (Roche, Germany), a multi-component lipid-based transfection reagent that complexes with and transports DNA into the cell.
Benefits of FUGENE 6 reagent like high transfection efficiency and no cytotoxicity support its use in transfection; the transfection protocol was as follows.

1. Dilution of FuGENE 6 reagent with serum-free medium (without antibiotics and fungicides): The 100 µl of Opti-MEM serum–free medium (Invitrogen) per 35mm culture

dish was mixed with 6 µl FuGENE 6 reagent by vortexing for 1 second. The mixture was incubated for 5 minutes at room temperature. Subsequently, 2 µg of DNA was added and the transfection reagent: DNA complex was incubated for a minimum of 15 minutes at room temperature.

2. Adding the FuGENE/DNA complex to the cells**:** Culture dishes were removed from the incubator and the transfection reagent/ DNA complex was added to the cells in a drop-wise manner. The dishes were swirled to ensure distribution of the added solution over the entire plate surface.

3. Cells were then returned to the incubator for about 48 hours.

The DNA, which we used, was TRPV6-pcDNA3 wt (wild type), which encodes the calcium channel TRPV6, and the hPTP1B-pcDNA3 wt (wild type), which encodes the enzyme protein tyrosine phosphatase (PTP). Other DNAs used in this study were hPTP1B-pEGFP-C1 and TRPV6-pEYFP. The GFP DNA-coding sequence can be inserted at the beginning or end of the gene, yielding a chimeric product consisting of the protein with a GFP domain attached. This GFP-fusion protein reveals the location of the protein and can give us an idea about the efficiency of the transfection (Figure 3). Another DNA, hPTP1B-(D181A)-pcDNA3 mutant, is a mutant of PTP1B, in which the invariant aspartate Asp-181 functioning as a general acid in protonating the tyrosol leaving group of the substrate, is changed to Ala. This PTP1B mutant retains the ability to bind substrates in a cellular context but is markedly attenuated in its catalytic function.(Andrew J.Flint et al. 1997). Additional DNA like hPTP1B-(D181A)pEGFP-C1 mut which encode GFP-PTP mutant protein, and the DNA for TRPV6-(Y161F,Y162F)-N1-pEYFP mut were also used.

2.4 DNA Constructs

In our experiments, DNAs are used in different vectors. Some details about these vectors and proteins are in the following table:

DNA-Name	Vector	Protein
TRPV6-pcDNA3	pcDNA3	TRPV6
TRPV6-pEYFP	pEYFP-N1	TRPV6-EYFP
TRPV6(Y161F,Y162F)-N1-pEYFP	pEYFB-N1	TRPV6-(Y161F,Y162F)-EYFP
hPTP1B-pEGFP-C1	pEGFP-C1	EGFP-hPTP1B
hPTP1B-pcDNA3	pcDNA3	hPTP1B
hPTP1B-(D181A)-pEGFP-C1	pEGFP-C1	EGFP-hPTP1B-(D181A)
hPTP1B-(D181A)-pEGFP-C1	pEGFP-C1	EGFP-hPTP1B-(D181A)
YFP-C1-TRPV6 1-698AA	YFP-C1	YFP-TRPV6 AA 1-698

Table1: The DNAs are used, the vectors and the transcripted proteins.

2.5 Video-Imaging and Measurement of The Intracellular FreeCalcium Concentration in HEK-293 cells

For measuring transient changes in the concentrations of intracellular calcium that allowcells to respond to extracellular signals the fluorescent calcium indicator, Fura-2, has been used. Calcium binds to Fura-2, this binding will cause a shift in the excitation fluorescence spectrum from 380 nm to 340 nm, (Grynkiewicz et al.1985). Thus, by measuring and comparing the ratio of fluorescence emission at the two excitation wavelengths, one can

determine the ratio of calcium-bound indicator to calcium-free indicator, providing a good approximation of the concentration of free calcium. (Figure 4).

3.5.1. *Calibration of the intracellular calcium concentration*

For quantitative evaluation of intracellular calcium concentration from fluorescenceintensity measurements a fluorescence microscope with a video imaging system (T.I.L.L.-Photonics, München) was used. This microscope (Axiovert 135, Zeiss), with high-quality objective lenses (Fluar 10x, NA=1,3), both dry objectives, and oil immersion objective (Fluar 40x, NA=1,3). In the experiments a dry objective (Fluar 10x) is used despite the low light power of this lens, but it gives us the possibility to measure the changes of intracellular calcium in a big number of cells. Simultaneously the 40x oil immersion objective is used to gather more detailed information but from a limited number of cells. The fluorescence microscope is connected to an video- imaging system (T.I.L.L.- PHOTONICS, Germany), and the main device in this system is the polychrome II, a monochromatising device with integrated light source. The monochromatic light is linked to the microscope via light guide and an epifluorescence condenser. The fluorescence image is detected from a CCD Camera IMAGO with a 640*480 Pixel (CCD ship 4095 Graustufen). The raw fluorescent signals from intracellulary trapped Fura-2 can be informative in a qualitative way. A semi-quantitative estimate of intracellular calcium can be made, depending on the Grynkiewicz- equation for a dual-wavelenght ratiometric fluorescent indicator (Fura-2):

$$[Ca^{2+}] = K_d \left(\frac{R - R_{min}}{R_{max} - R}\right) \left(\frac{s_{f,2}}{s_{b,2}}\right)$$

In this equation, we suppose that:

$R = F1 / F2$

$F1$ = Emission λ 380 nm

$F2$ = Emission λ 340 nm

R_{min} = Ratio minimal

R_{max} = Ratio maximal

R_{min} is the limiting value of the ratio R when all of the indicator is in the calcium free form. R_{max} is the limiting value of R when the indicator is saturated with calcium. Experimentally, the factor $S_{f,2}/S_{b,2}$ is simply the ratio of the intensity measured when all of the indicator is calcium bound; both measurements are taken at λ 2.

3.6. Experimental Procedures

After loading the cells with Fura-2-AM, glass coverslips with transfected HEK-293 cells were fixed on a thick slide making a small simple chamber, in which the cells could be superfused and observed by the objectives. The cells were superfused with control and calcium free solutions depending on the gravity flow method. During the experiment, intracellular calcium changes are detected by the microscope and are saved to a computer connected to the microscope. The analysis of the data and statistic are made by SiGMA PLOT and Excel XP programs.

3.7. Solutions and chemicals

3.7.1. Solutions

We used two solutions; control-, and Ca^{2+}-free solutions.

	Control solution	Ca^{2+}- free solution
NaCl	140 mM	140 mM
KCl	4.7 mM	4.7 mM
$CaCl_2$	1.3 mM	-
$MgCl_2$	1 mM	1 mM
HEPES	10 mM	10 mM
Glucose	10 mM	10 mM
PH (NaOH)	7.4	7.4

Table2: solutions are used n the experiments.

3.7.2. CHEMICALS

PAA-Laboratories :

10% FBS (fetal bovine serum).
Trypsin.
EDTA.

INVITROGEN :

D-MEM Medium.
Penicillin.
Streptomycin.
Opti-MEM.

Molecular Probes, Eugene OR (USA):

Fura-2/AM.

ROTH, Karlesruhe , Germany :

Nacl

Merck, Darmstadt, Germany :

Kcl
MgCl2
CaCl2
NaOH

Sigma , Steinheim, Germany :

DMSO.
HEPES.

Roche, Germany :

FuGENE 6 Reagent.

CALBIOCHEM,Germany:

2-Aminoethoxy-diphenylborate (2APB).
Thapsigargin.
bis(N,N-Dimethylhydroxamido)hydrooxovanadate (DMHV).

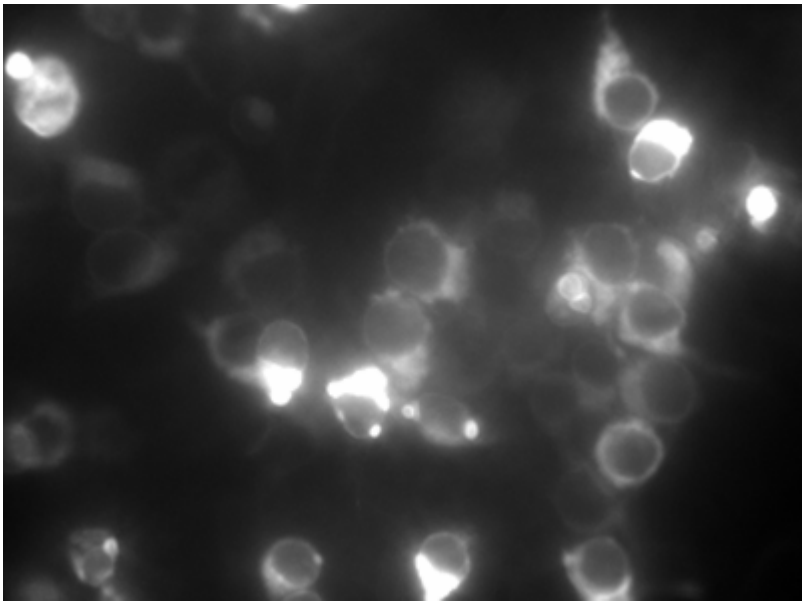

Figure 3: HEK-293 cells transfected with TRPV6 and GFP-PTP Protein Showing location of PTP protein and efficiency of the transfection (magnified x40).

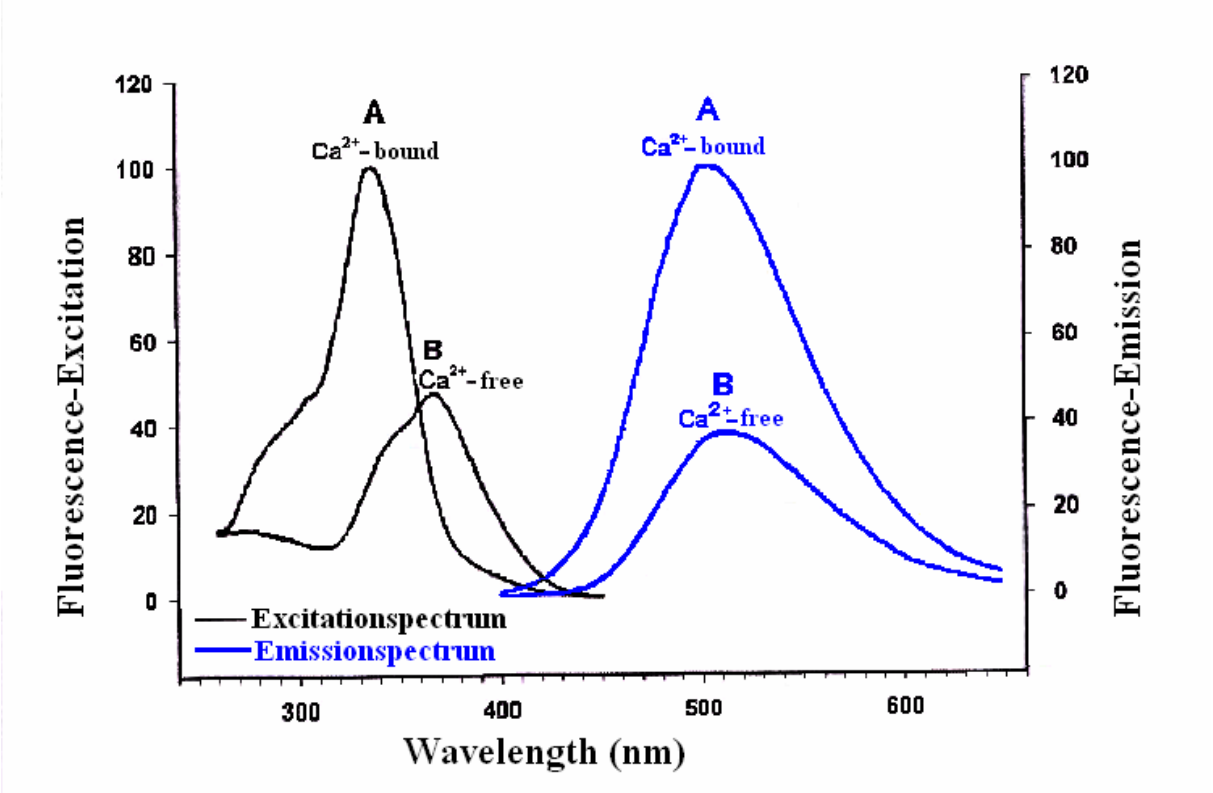

Figure 4: Excitation and Emission spectrum of Fura-2. The left part of the figure (black line) shows the excitation spectrum for Ca^{2+}-bound form of the indicators (A) and the Ca^{2+}-free form (B), by increasing calcium concentration shifts the excitation maximum to short lengths. In right part (blue line) we show the emission spectrum for the Ca^{2+}-bound form (A), and the Ca^{2+}-free form (B) of the indicator.

Chapter 3

Results

3.1 Examination the effect of DMHV on TRPV6-mediated Ca^{2+} influx

TRPV6 has been proposed to manifest pore properties of CRAC channel, particularly due to its high selectivity for Ca^{2+} (Yue *et al*. 2001). In addition, Hsu et al. (Shyuefang Hsu *et al*. 2003) has suggested that CRAC influx is modulated by tyrosine phosphorylation and dephosphorylation which involves the tyrosine phosphatase PTP1B. Therefore in our work, we analyzed the possibility that TRPV6-mediated Ca^{2+} influx is modulated by tyrosine dephosphorylation through PTP1B.

TRPV6 was expressed in HEK-293 cells. To ensure efficient expression of TRPV6, these cells were transiently transfected with the clone TRPV6-pEYFP, which encodes our channel attached to the yellow fluorescent protein (YFP). This YFP-part of the protein, gave a directly visible display of TRPV6 expression. In the same way, the expression of other clones were detected by using the green fluorescent protein (GFP) or YFP. For all used clones, cells expressed transfection efficiency at the rate of 70-80% after 48 hours.

3.1.1 Store-operated influx in HEK-293 cells

In the first set of experiments we characterized store-operated Ca^{2+} influx in untransfected HEK-293 cells. Depletion of Ca^{2+} from the endoplasmic reticulum was induced by 500 nM TG which inhibits the SERCA pump. In the absence of extracellular Ca^{2+}, TG causes a very small, transient Ca^{2+} rise in HEK-293 cells resulting from the leakage of Ca^{2+} from internal stores followed by Ca^{2+} extrusion across the plasma membrane. As illustrated in Fig. 5, after approximately 10 minutes, exchange of the external solution to a 1.3 mM Ca^{2+}-solution (control solution) allows Ca^{2+} influx across the plasma membrane resulting in long lasting elevations of $[Ca^{2+}]_{cyt}$.

This retrograde process by which plasma membrane calcium channels are opened through depletion of intracellular Ca^{2+} stores has been called "capacitative calcium entry" (Putney,J.W.,Jr. 1997).

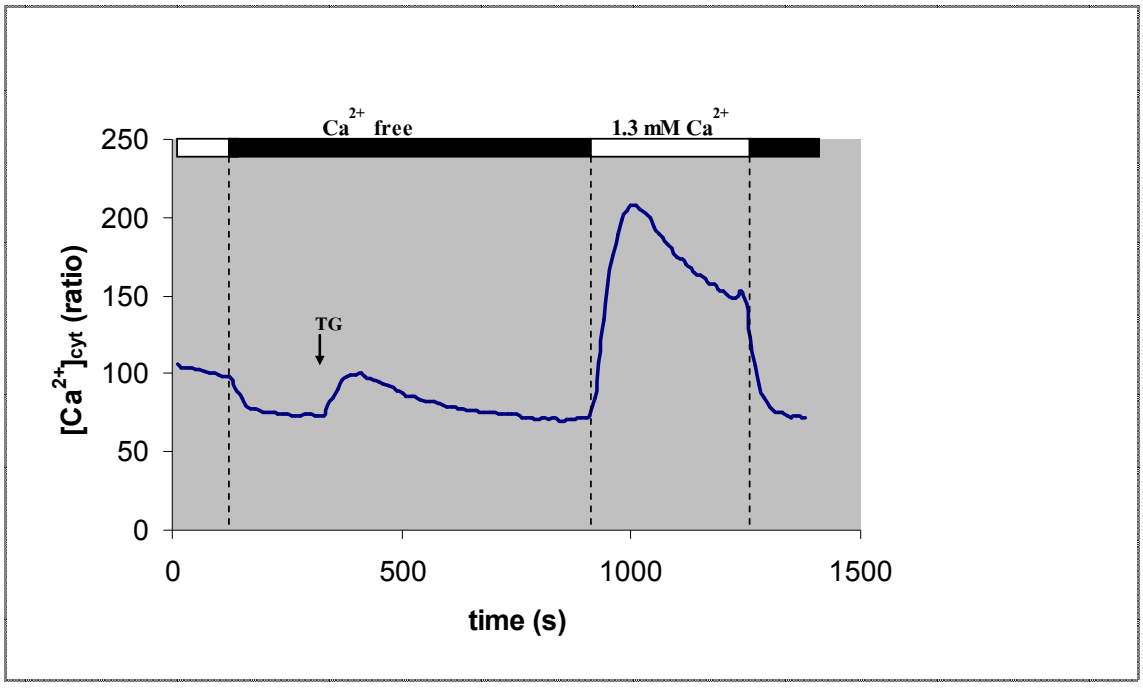

Figure 5: Ca^{2+} influx through endogenous SOC Channels. HEK-293 cells were loaded with Fura-2 and were incubated in the presence or absence of extracellular Ca^{2+}. Thapsigargin (500 nM) was applied in Ca^{2+}-free external solution to deplete ER Ca^{2+} stores. Addition of 1.3 mM Ca^{2+} led to a rapid increase in $[Ca^{2+}]_{cyt}$ through store- operated Ca^{2+} channels.

3.1.2 2-APB effects on store-operated and TRPV6 mediated Ca^{2+} influx in HEK-293 cells

Since the presented study investigates the role of PTP1B in regulating TRPV6-mediated Ca^{2+} entry, we had to discriminate between Ca^{2+} entry through TRPV6 channels and endougenous CRAC channels. Therefore, the effects of 2-Aminoethoxydiphenyl borate (2-APB), which had been described to block store-operated Ca^{2+} entry (Bootman *et al*. 2002, M. Prakriya, R.S. Lewis. 2001) but not to inhibit Ca^{2+} currents through heterologously expressed TRPV6 (T.

Voets. et al. 2001) were studied in both wild type (Figure 6) and TRPV6 transfected cells (Figure 7).

3.1.2.1 Effect of 2-APB on store-operated Ca^{2+} influx in HEK-293 cells

First, we tested whether 2-APB blocks store-operated Ca^{2+} channels in wt HEK-293 cells. As described before, the Ca^{2+} content of the ER was depleted by TG in the absence of Ca^{2+}. Readdition of Ca^{2+} (1.3 mM) to the superfusion buffer allowed Ca^{2+} influx into the cell, indicated by a rapid increase in $[Ca^{2+}]_{cyt}$ to a peak level. Subsequently, $[Ca^{2+}]_{cyt}$ decreased and came to a new level called "Ca^{2+} plateau". As shown in figure 6, the addition of 2-APB (50 µM) to the superfusion buffer in the presence of extracellular Ca^{2+} caused a decrease in $[Ca^{2+}]_{cyt}$ to the basic level indicating complete block of Ca^{2+} entry.

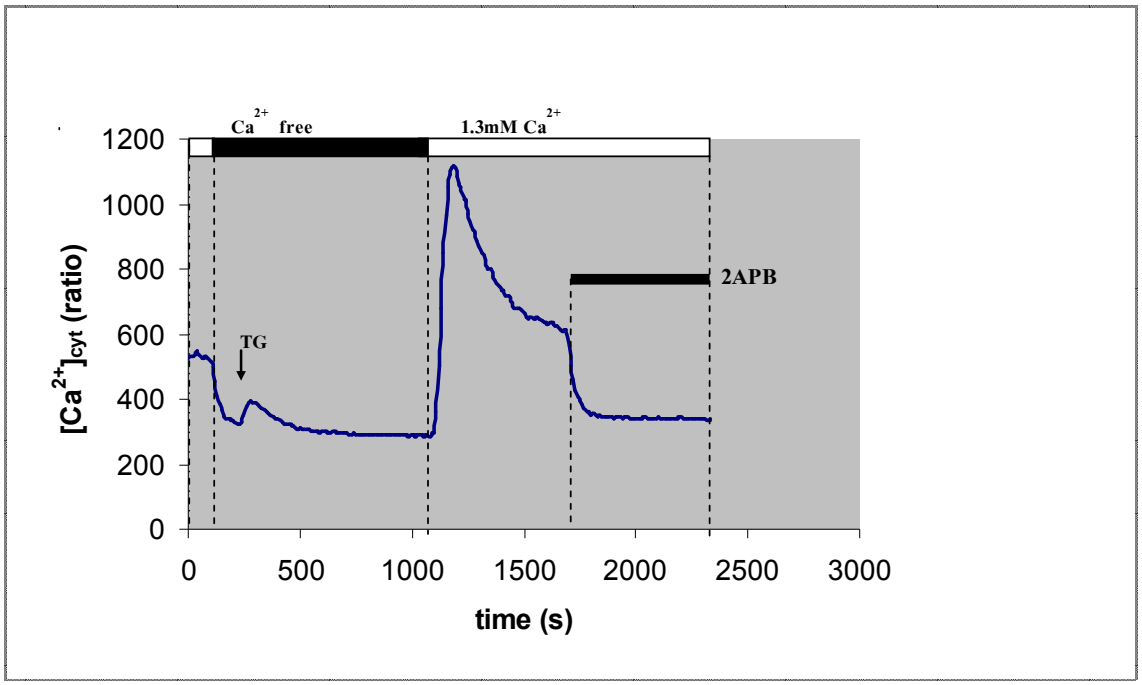

Figure 6: The inhibitory effect of 2-APB. HEK293 cells were loaded with Fura-2. Calcium (1.3 mM) was added to the extracellular buffer after depletion of ER by TG. Decrease $[Ca^{2+}]_{cyt}$ after adding 2-APB (50 µM) indicated its effect in blocking Ca^{2+} entry.

3.1.2.2 Effect of 2-APB on Ca^{2+} influx in TRPV6-transfected HEK-293 cells

3.1.2.2.1 Effect of 2-APB on Ca^{2+} plateau

As shown in fig 7, 2-APB could be used successfully to distinguish between transfected and untransfected cells. Whereas 2-APB (50 µM) produced total inhibition of Ca^{2+} influx in untransfected HEK-293 cells, it did not cause the same reduction in the Ca^{2+} plateau in TRPV6-transfected HEK-293 cells. Proportional to the transfection rate, some cells showed a complete inhibition of the plateau after the application of 2-APB. These cells were designated as "untransfected". The rest of the cells only showed either no effect or a small suppression in the plateau (Figure 7). We interpret this to mean that TRPV6 is unaffected by 2-APB. Since the Ca^{2+} influx is reduced after inhibition of the endogenous SOCs, a lower $[Ca^{2+}]_{cyt}$ could be reached.

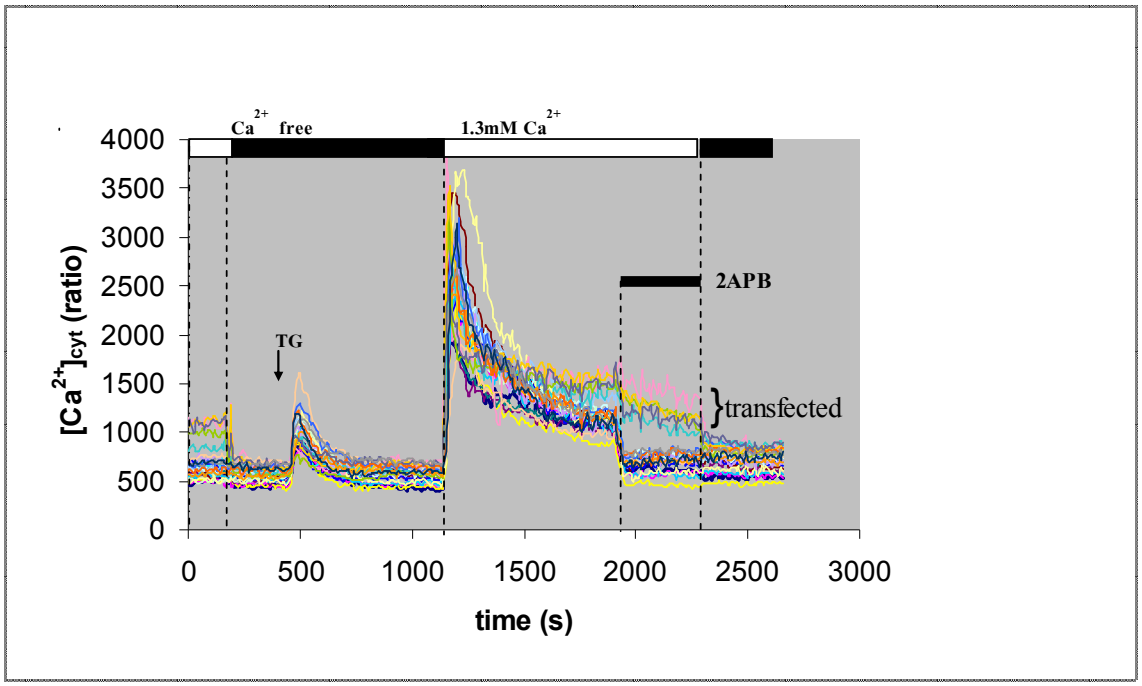

Figure 7: 2-APB effect in TRPV6-transfected HEK293 cells. After adding 2-APB (50 µM) to the extracellular 1.3 mM Ca^{2+}-solution, untransfected cells show a marked decrease in $[Ca^{2+}]_{cyt}$ to a level similar to the level of $[Ca^{2+}]_{cyt}$ in the absence of extracellular Ca^{2+}. TRPV6-transfected HEK293 cells show a small decrease in $[Ca^{2+}]_{cyt}$

3.1.2.2.2 Discrimination TRPV6-transfected HEK-293 cells by using 2-APB

In most experiments, 2-APB was applied from the beginning of the experiment (Figure 8). Under these conditions, untransfected cells only responded to TG but did not show any other change in $[Ca^{2+}]_{cyt}$.

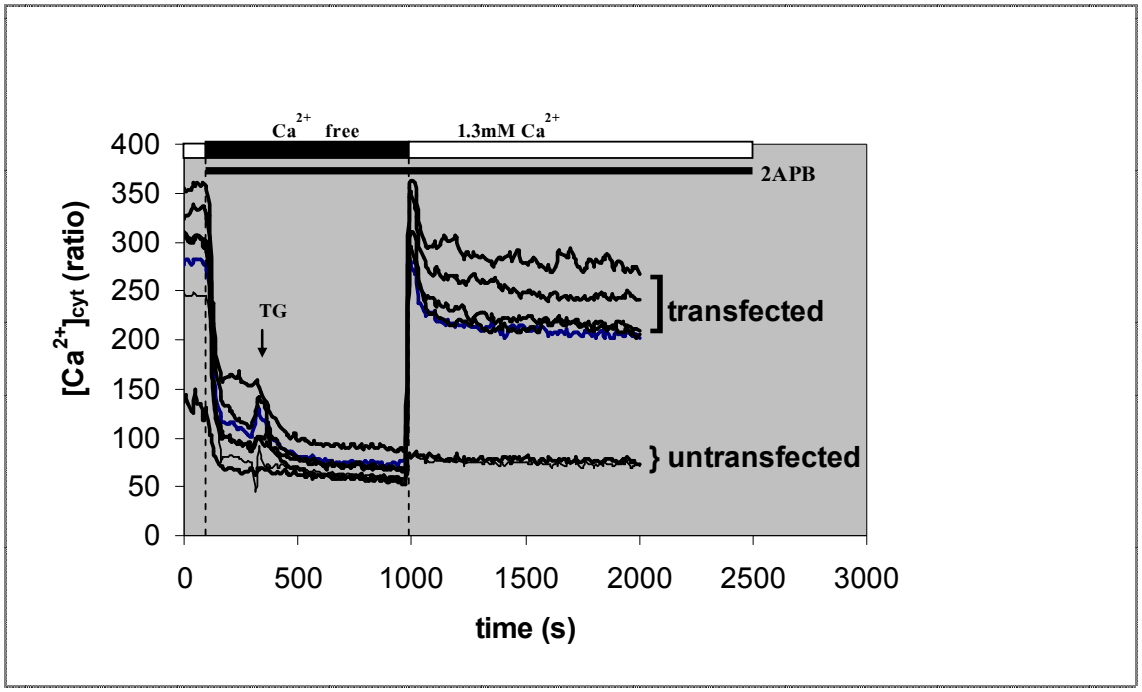

Figure 8: 2-APB effect in TRPV6-transfected HEK293 cells. In these experiments, Tg 500 nM depleted intracellular Ca^{2+} store in the presence of 50µM 2-APB in the absence of extracellular Ca^{2+}. Exchanging the extracellular solution to 1.3 mM Ca^{2+}-solution in the presence of 2-APB (50 µM) allowed Ca^{2+} influx through TRPV6 channels. Untransfected cells did not show any Ca^{2+} influx.

3.1.3 TRPV6-mediated Ca^{2+} influx in HEK-293 cells modulated by PTP1B inhibitor "DMHV"

As various ion channel activities are regulated by direct tyrosine phosphorylation and dephosphorylation, we set out to examine whether TRPV6–mediated Ca^{2+} influx is regulated by tyrosine dephosphorylation by PTP1B. For this matter, HEK-293 cells were transfected with the TRPV6 gene cloned into a pTracer-CMV2-vector (Invitrogen) and we examined the effect of PTP1B inhibitor on TRPV6 induced Ca^{2+} entry. After store depletion by TG (500 nM), readdition of Ca^{2+} buffer caused Ca^{2+} influx through TRPV6 channels in the presence of

2APB (50 µM). DMHV [bis-(*N,N*-dimethyl-hydroxamido) hydrooxovanadate, 20 µM], an inhibitor of protein tyrosine phosphatase PTP1B, was added to the Ca^{2+} buffer at the Ca^{2+} plateau. As illustrated in fig.9, DMHV (20 µM) induced a slow increase in the Ca^{2+} plateau within 45 minutes. To quantify the DMHV effect we defined the difference between the lowest Ca^{2+}-ratio in nom. Ca^{2+} free conditions before Ca^{2+} addition and the highest ratio after the readdition of Ca^{2+} (peak) as 100%. The DMHV induced increase in $[Ca^{2+}]_{cyt}$ between the $[Ca^{2+}]_{cyt}$ after the addition of DMHV and the end of DMHV administration was set in relation to this 100% value. Using this method we found that DMHV induced a 0,71 ±3.88 % increase. However, the increase, which was induced by DMHV as compared to the control (without DMHV), was not given by this value. To get an estimate for it we had to compare the DMHV value with cells which were not treated with DMHV.

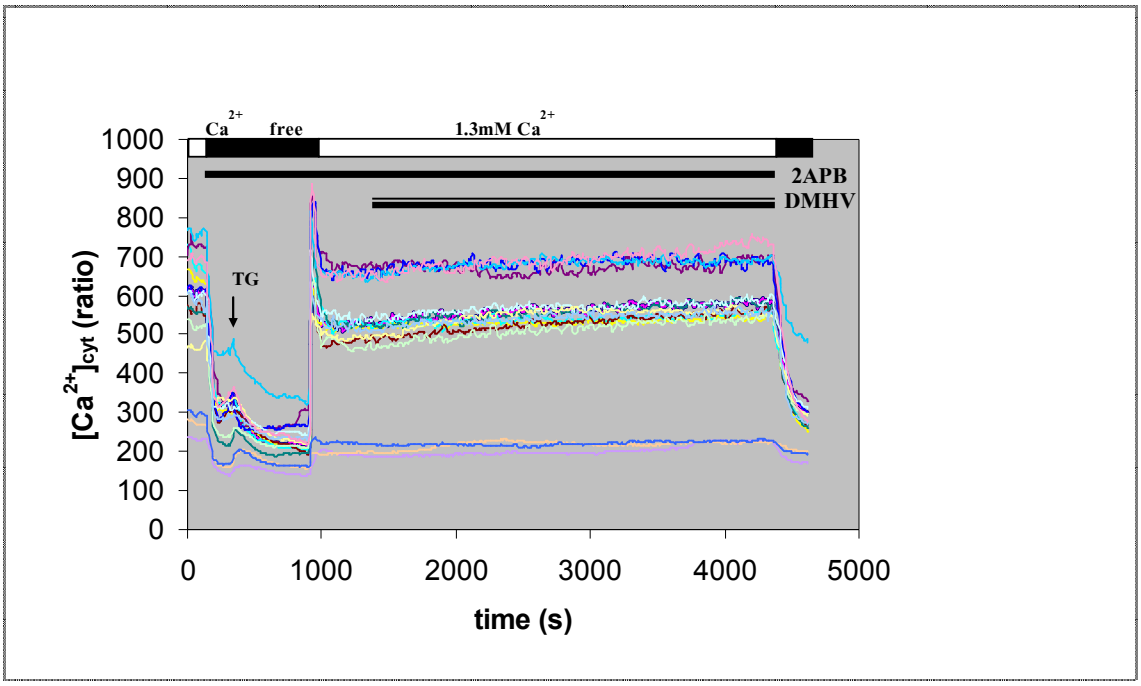

Figure 9: DMHV effect in store depleted TRPV6-transfected HEK-293 cells. In five experiments, these curves show $[Ca^{2+}]_{cyt}$ differences in HEK-293 cells under the effect of DMHV (20µM) over 45 minutes.

Fig. 10 presents such controls. Using the same quantification method as before, it could be shown that without DMHV the plateau decreased spontaneously by -6.9±5.2 %, n=80 cells (Figure 12). Hence, the DMHV effect was 7.61±2,61 %, $P < 0.05$. (Figure 11).

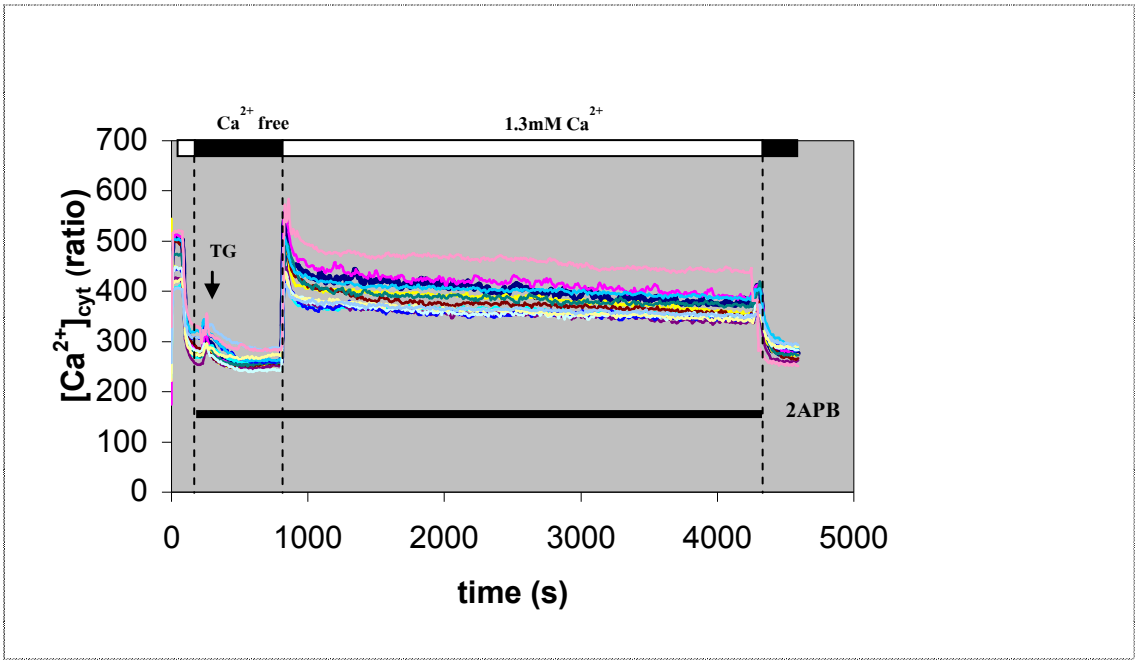

Figure 10: TRPV6-mediated Calcium influx in HEK-293 cells. These curves show $[Ca^{2+}]_{cyt}$ differences in HEK-293 in the absence of DMHV, 2APB (50µM) was presented in the superfusion buffer.

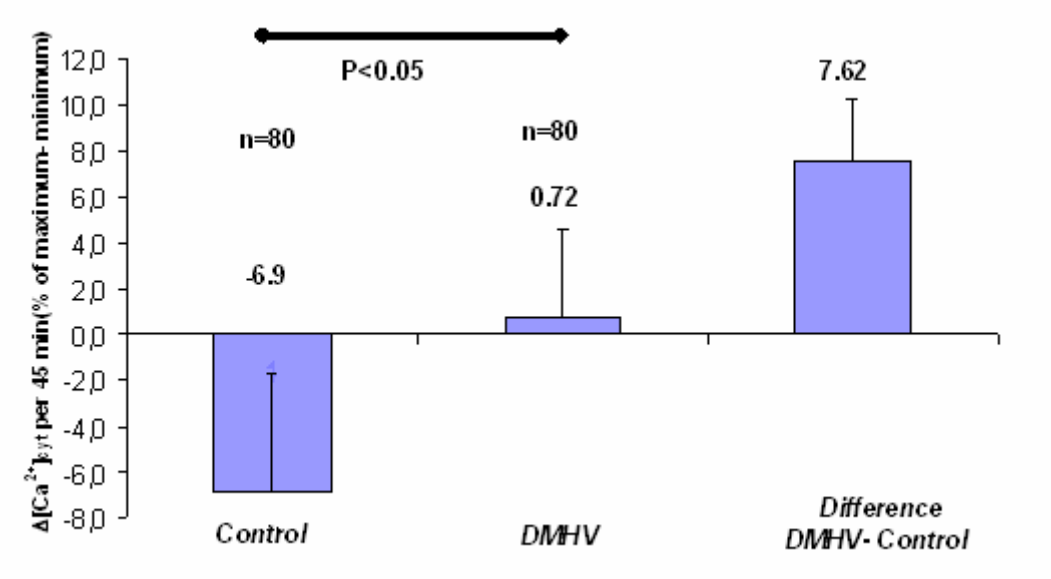

Figure 11: DMHV effect in TRPV6-transfected HEK-293 cells. Column **1** shows $[Ca^{2+}]_{cyt}$ change, which was -6.9±5.2% during the 45 minutes of the $[Ca^{2+}]_{cyt}$ plateau in TRPV6-transfected HEK-293 cells in the absence of DMHV. Column **2** shows $[Ca^{2+}]_{cyt}$ change in the presence of DMHV which was 0.7 ±3.8 %. Therefore the effect (column **3**) is 7.6±2.6 %.

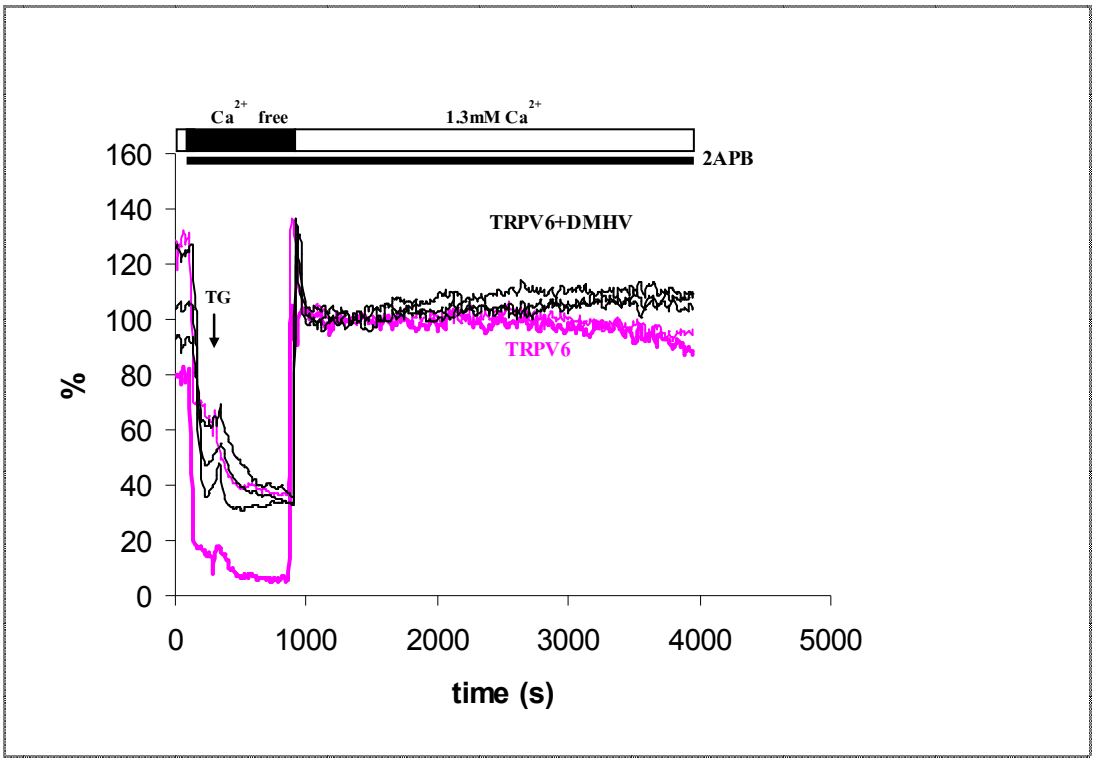

Figure 12: Ca^{2+} plateau in TRPV6-transfected HEK-293 cells in the presence and absence of DMHV. A comparison shows that the presence of DMHV induces an increase in $[Ca^{2+}]_{cyt}$ plateau in HEK-293 transfected with TRPV6.

3.1.4 Time-dependent increase in $[Ca^{2+}]_{cyt}$ by DMHV

As mentioned above, changes in $[Ca^{2+}]_{cyt}$ were measured during 45 minutes. This time was elected because DMHV displayed a time-dependent inhibition of PTP1B which reflected itself in a time-dependent increase in $[Ca^{2+}]_{cyt}$ .It was clear from measuring the DMHV effect on the $[Ca^{2+}]_{cyt}$ through different periods of time (0 min, 15 min, 30 min, 45 min) that the highest increase in $[Ca^{2+}]_{cyt}$ was measured after 45 minutes (Figure 13).

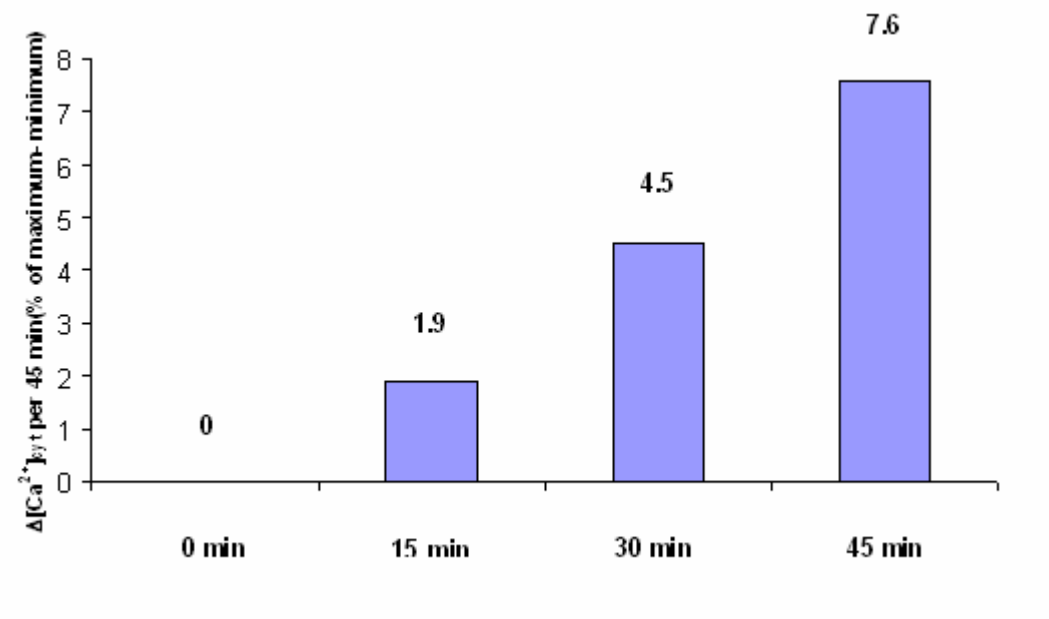

Figure 13: Increasing DMHV effect with time. In TRPV6-transfected HEK-293 cells, DMHV increased $[Ca^{2+}]_{cyt}$ with time. 0 minute it was 0 %, after 15 minutes it was 1.9%, after 30 minutes it was 4.5 % and after 45 minutes it was 7.6 %.

The cytosolic $[Ca^{2+}]_{cyt}$ could depend on the activity of the following transport mechanisms: TRPV6 channels which are responsible for the Ca^{2+} influx, Ca^{2+}- and potential- dependent K^+ channels which control the membrane potential and therefore the electrical driving force for Ca^{2+} entry, Ca^{2+}-ATPase in the plasma membrane which transports Ca^{2+} out of the cell, and mitochondrial Ca^{2+} transport.

3.1.5 DMHV effect on $[Ca^{2+}]_{cyt}$ in untransfected HEK-293 cells

Since TRPV6-transfected HEK-293 cells displayed an increase in $[Ca^{2+}]_{cyt}$ by inhibiting PTP1B, we tested if the inhibtition of endogenous PTP1B in untransfected HEK-293 cells showed any increase in $[Ca^{2+}]_{cyt}$. Fig. 14 shows examples of Ca^{2+} measurements in HEK-293 cells following store depletion by TG and subsequent activation of Ca^{2+} entry. In the presence of 2APB (50µM) which inhibited SOC channels, DMHV induced an increase in $[Ca^{2+}]_{cyt}$ 2.6± 0.75%, n=97 cells during the 45 minutes period.

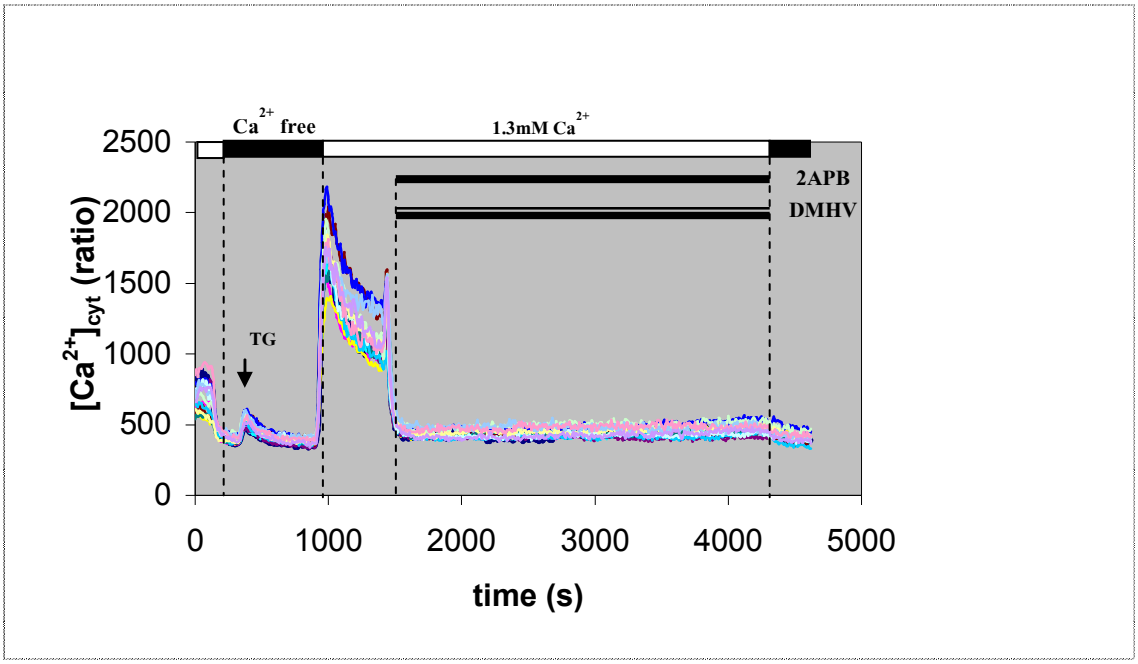

Figure 14: DMHV effect in HEK-293 cells. In untransfected cells, inhibition of PTP1B by DMHV (20µM) made an increase in $[Ca^{2+}]_{cyt}$ over 45 minutes in the presence of 2APB (50µM). Data summarized in Fig. 15 show that inhibition of endogenous PTP1B in untransfected HEK-293 cells induced an increase in $[Ca^{2+}]_{cyt}$ to 0±2.64 %, P > 0.4.

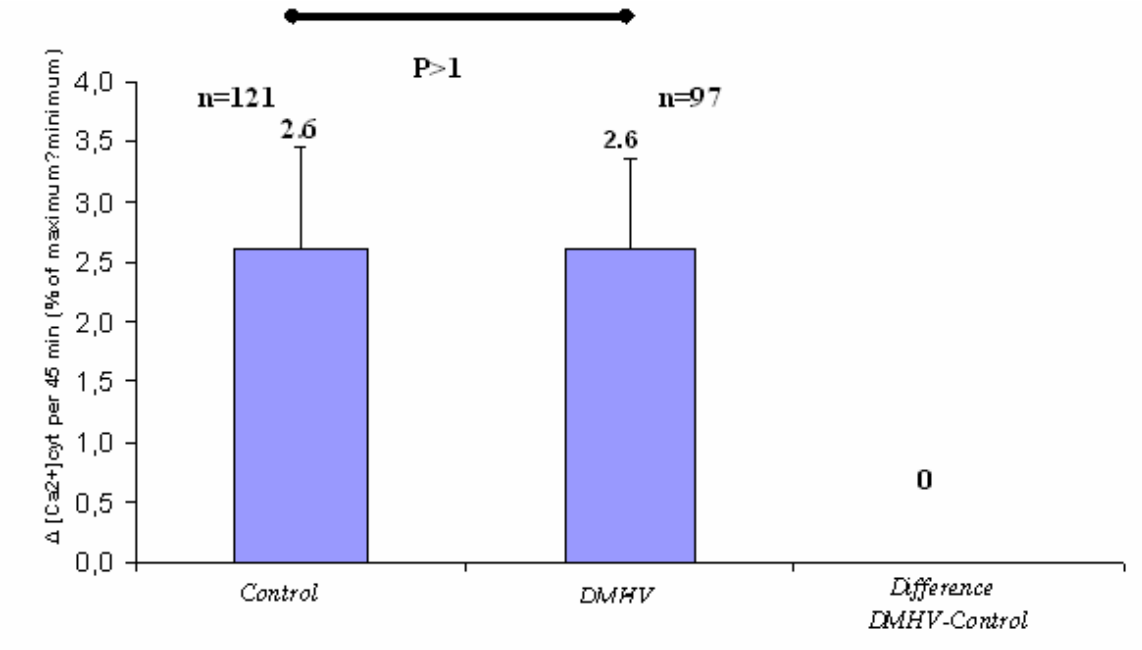

Figure 15: DMHV effect in HEK-293 cells. Column **1** shows the difference in $[Ca^{2+}]_{cyt}$ over 45 minutes in the absence of DMHV. But column **2** shows the difference in the presence of DMHV. Column **3** explains that DMHV effect is 0 in HEK-293 cells.

However, these experiments exhibit a good evidence that an increase in cytosloic Ca^{2+} is mediated by TRPV6 channel through a direct effect of DMHV on the channel or indirectly by inhibiting PTP1B.

3.1.6 DMHV effect on $[Ca^{2+}]_{cyt}$ in HEK-293 cells co-transfected with TRPV6 and PTP1B

In a more direct approach to determine whether the tyrosine phosphatase PTP1B is indeed involved in modulation of the TRPV6-mediated Ca^{2+} influx. TRPV6-transfected HEK-293 cells co-transfected with PTP1B were used in other groups of experiments. Interestingly, applying DMHV during 45 minutes in seven experiments induced an increase in the Ca^{2+} plateau by 6.41±7.91 %, n=87 cells (Figure 16).

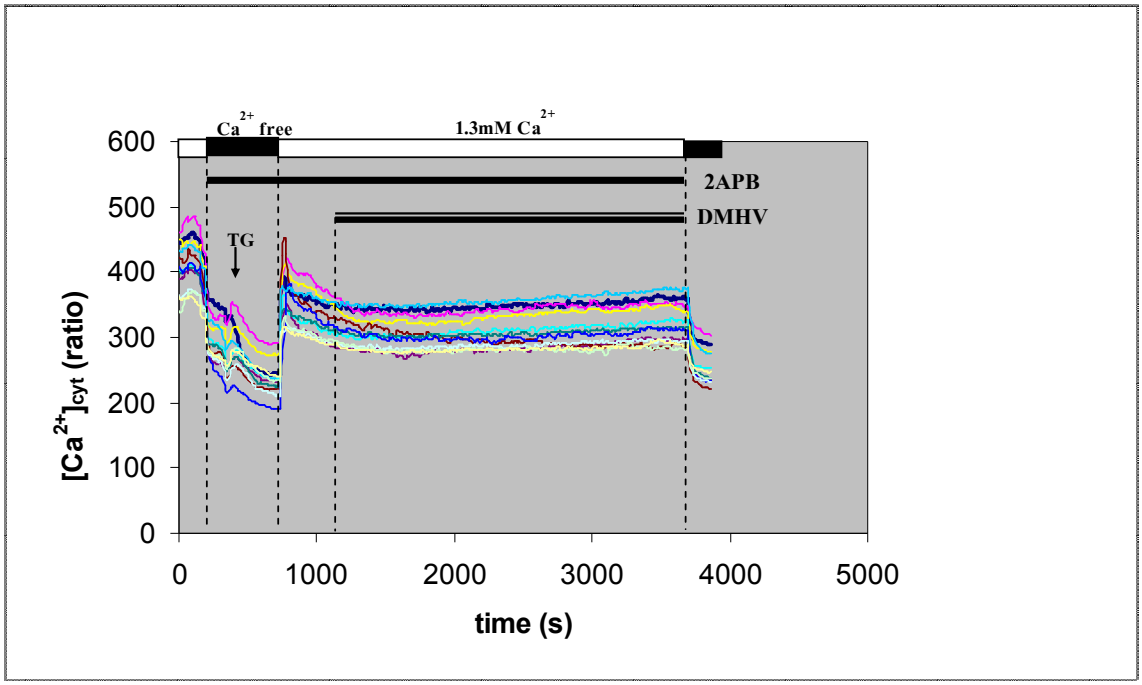

Figure 16: DMHV effect in TRPV6 transfected HEK-293 cells co-transfected with PTP1B.Transfected HEK-293 cells exhibited a higher increase in the presence of DMHV over 45 minutes.

In the absence of DMHV, cotransfected cells showed a decrease in the Ca^{2+} plateau which was even -5.8 ± 6.89 %, n=60 cells (Figure 17).

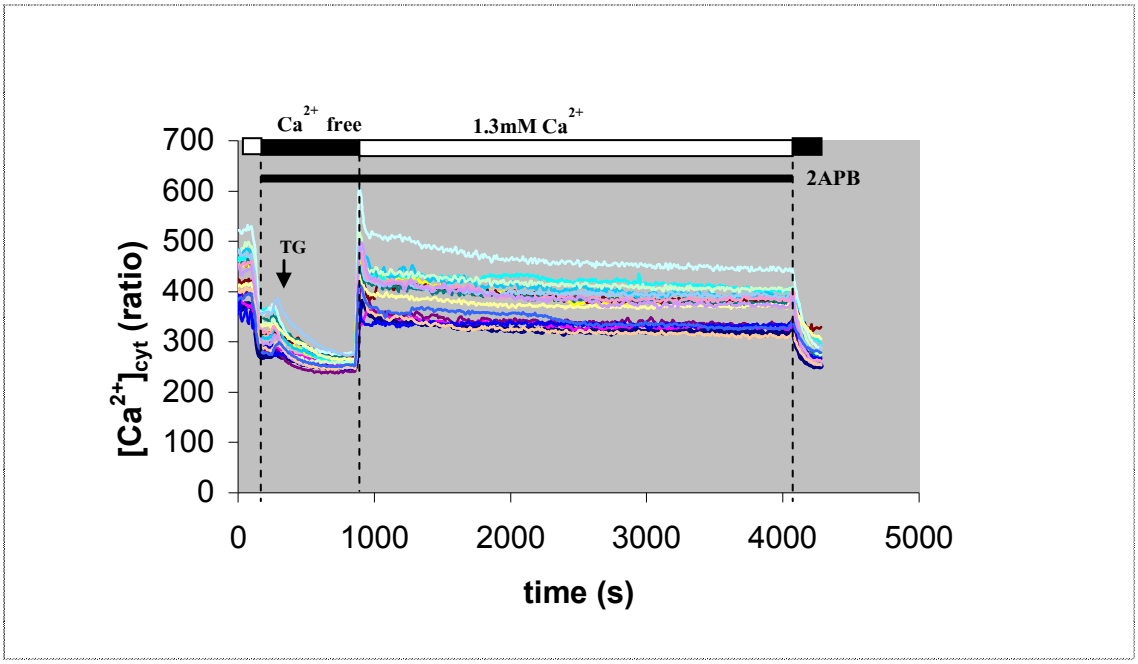

Figure 17: In the absence of DMHV, HEK-293 cells co-transfected with TRPV6 and PTP1B showed a decrease in $[Ca^{2+}]_{cyt}$ over 45 minutes.

In these experiments the DMHV effect on $[Ca^{2+}]_{cyt}$ was 12.17± 4.45 %, $P < 0.01$ as shown in Fig. 18.

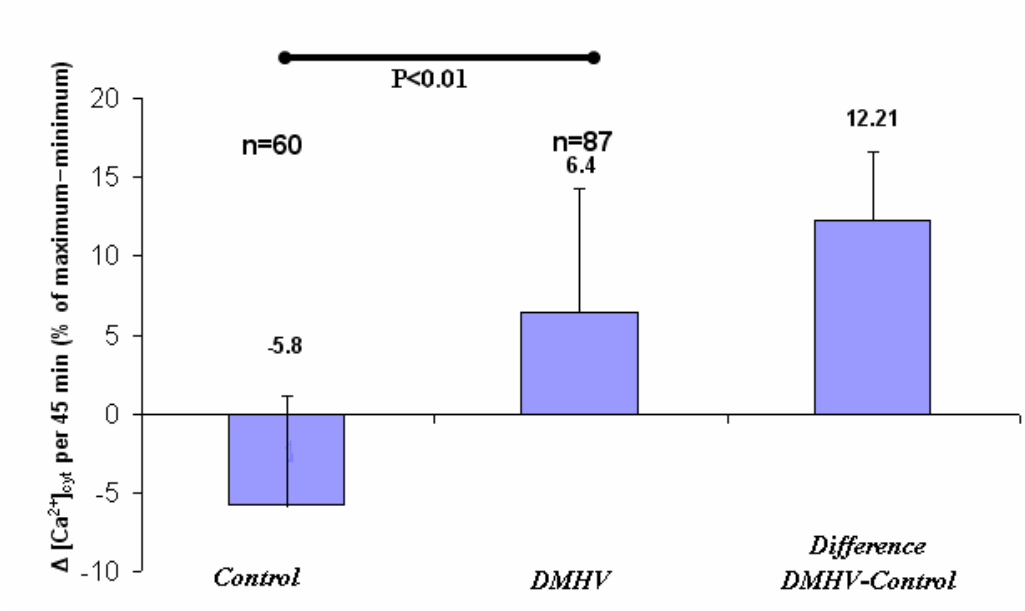

Figure 18: DMHV effect in HEK-293 cells co-transfected with TRPV6 and PTP1B.

In conclusion, the so far presented experiments show that the PTP1B inhibitor DMHV induce an increase in the TRPV6-mediated Ca^{2+} influx in HEK-293 cells through inhibition of endogenous PTP1B (Figure 19). In cells co-transfected with TRPV6 and PTP1B, the increase

in the Ca^{2+} influx was almost twofold. This suggests a significant role for PTP1B in the regulation of TRPV6 currents.

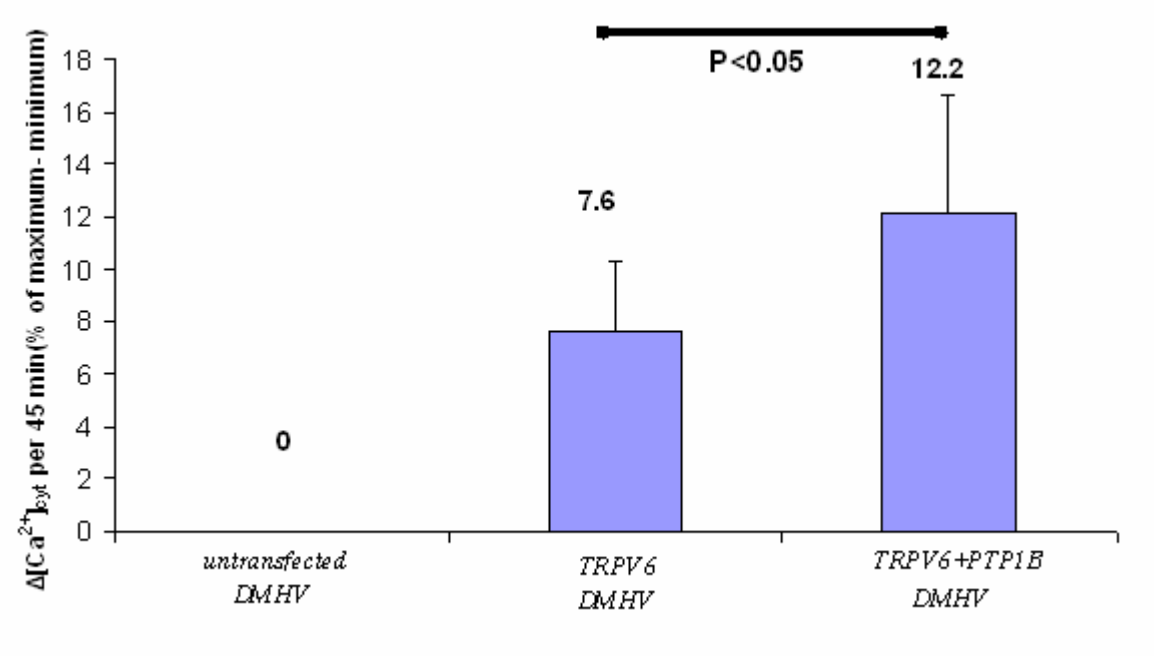

Figure 19: DMHV induced lang-lasting elevations in cytosolic calcium. In the second column, inhibition of endogenous PTP1B by DMHV in TRPV6-transfected HEK-293 cells induced an increase in $[Ca^{2+}]_{cyt}$ which disappeared in untransfected HEK-293 cells (First column); in cells co-transfected with PTP1B the increase in $[Ca^{2+}]_{cyt}$ was twofold.

3.2 DMHV effect on $[Ca^{2+}]_{cyt}$ in HEK-293 cells co-transfected with TRPV6 and PTP1B in the absence of 2-APB

Because DMHV-mediated Ca^{2+} increase was induced in the presence of other substances like 2APB and TG, we analyzed the increase in $[Ca^{2+}]_{cyt}$ in more detail to demonstrate that the measured effect only depended on the inhibition of PTP1B by DMHV, not on any potential side effects of the chemicals. Therefore, in the absence of 2-APB three experiments were made. DMHV (20 µM) induced an increase in $[Ca^{2+}]_{cyt}$ to 3.40±3.15%, n=32 cells (Figure 20).

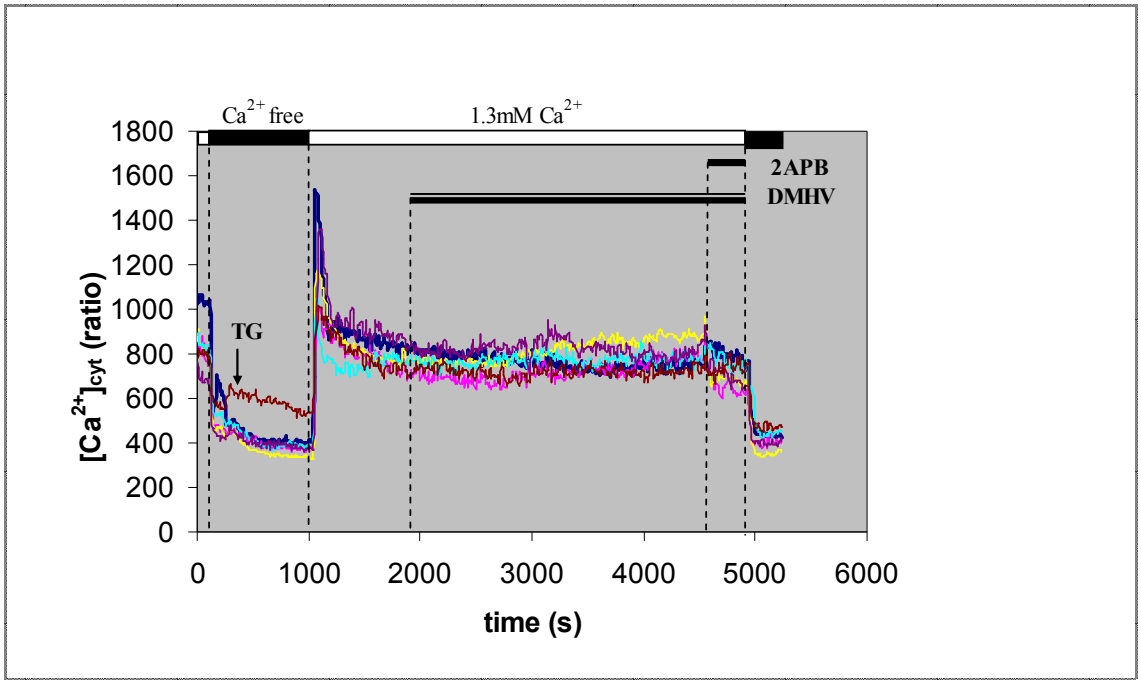

Figure 20: DMHV effect without 2APB. In the absence of 2APB, DMHV (20 µM) induced an increase in Ca^{2+} plateau over 45 minutes in HEK-293 cells transfected with TRPV6 and PTP1B. At the end of the experiment untransfected cells were discriminated by adding 2-APB (50 µM).

Control experiments showed a decrease in $[Ca^{2+}]_{cyt}$ to -7.3±2.5%, n=23 cells in the absence of DMHV. However, as shown in figure 21, we found that DMHV induced an increase in $[Ca^{2+}]_{cyt}$ to 10.70±4.47% .

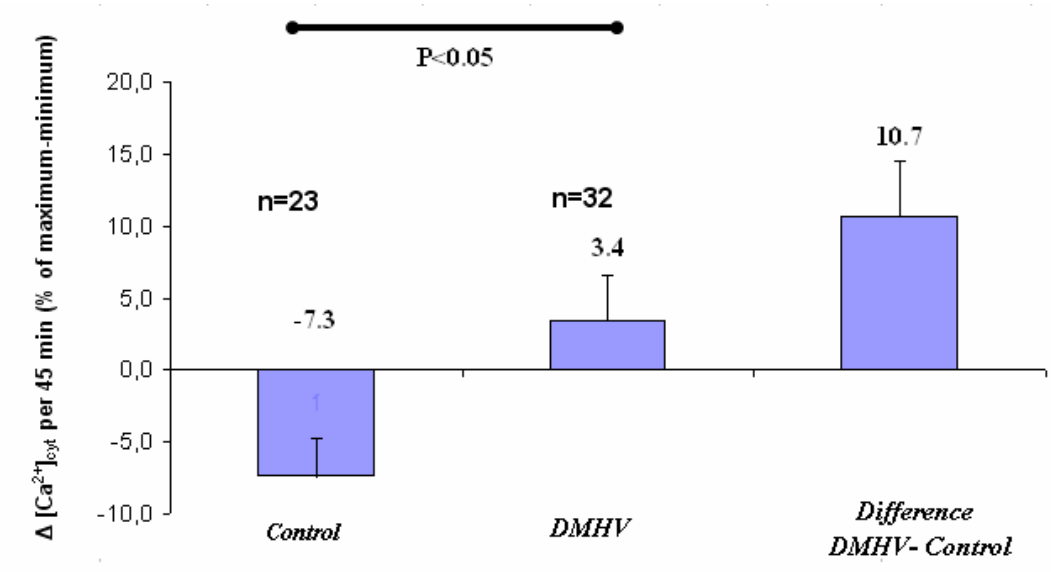

Figure 21: DMHV effect in HEK-293 cells co-transfected with TRPV6 and PTP1B in the absence of 2-APB. First column shows the decrease in $[Ca^{2+}]_{cyt}$ in control experiments and

second column shows the increase in $[Ca^{2+}]_{cyt}$ in the presence of DMHV the third column shows the increase in $[Ca^{2+}]_{cyt}$ which was induced by DMHV.

3.3 DMHV effect on $[Ca^{2+}]_{cyt}$ in HEK-293 cells co-transfected with TRPV6 and PTP1B in the absence of TG

As mentioned above, we chose 2-APB (50µM) to discriminate between TRPV6-mediated Ca^{2+} influx and endogenous Ca^{2+} influx. But previous evidence has suggested that 2-APB can deplete Ca^{2+} stores by inhibition of SERCA pumps (J.G. Bilmen et al. 2002). However, to investigate whether Ca^{2+} store depletion affects the dephosphorylation of TRPV6 by PTP1B, one of the pyrazole derivatives named BTP2 was selected. This 3,5-bistrifluoromethyl pyrazole derivative BTP2 was found to inhibit CRAC channels in T-cells (Zitt C et al.. 2004). TRPV6-transfected HEK-293 cells co-transfected with PTP1B were pre-incubated with BTP2 (100 nM) for 24 hours. In the presence of a Ca^{2+} free solution, $[Ca^{2+}]_{cyt}$ decreased which was followed by Ca^{2+} influx through TRPV6 channels and in the absence of TG resulting in long-lasting elevations of $[Ca^{2+}]_{cyt}$ when the external solution was changed to a 1.3 mM Ca^{2+}-solution (Figure 22). As illustrated in figure 22, in the presence of filled Ca^{2+} stores, DMHV (20µM) induced an increase in $[Ca^{2+}]_{cyt}$ 0.9±7.37%, n=40 cells (four experiments).

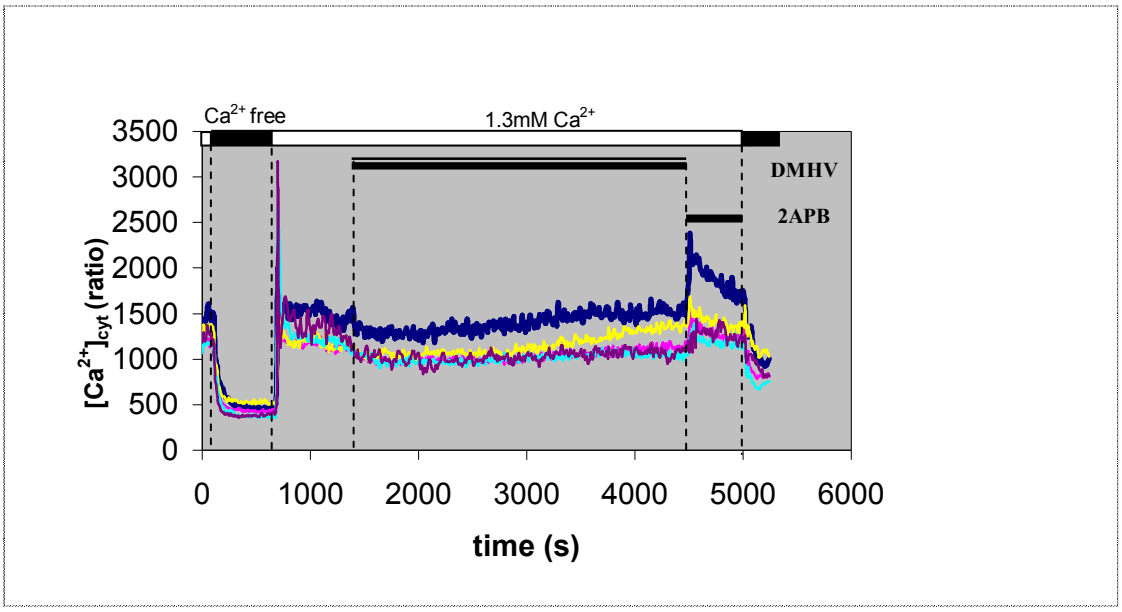

Figure 22: DMHV effect in the absence of TG. After pre-incubation of the HEK-293 cells transfected with TRPV6 and PTP1B with BTP2 (100nM) for 24 hours, DMHV induced a small increase in the Ca^{2+} plateau during 45 minutes in the absence of TG.

On the other hand, similarly there was, however, a marked decrease in the $[Ca^{2+}]_{cyt}$ -10.2±3.31%, n=55 cells (four experiments) in the absence of DMHV over 45 minutes. Thus the DMHV-induced $[Ca^{2+}]_{cyt}$ elevation was 11.10±4.69% without depletion Ca^{2+} store by TG as depicted in figure 23.

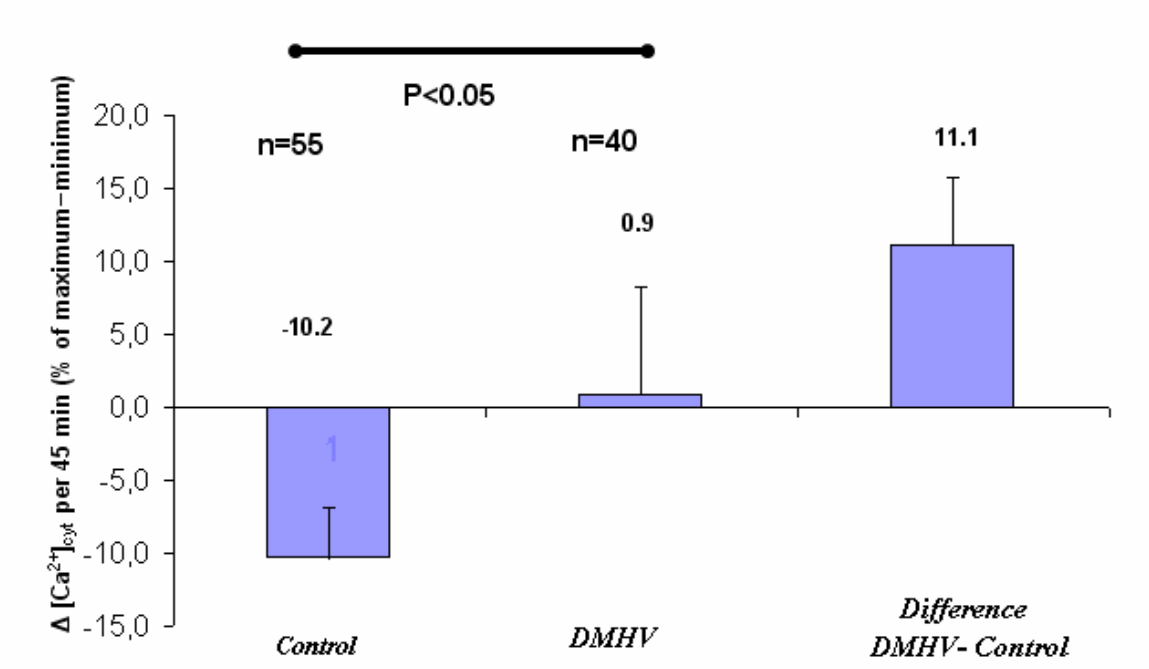

Figure 23: Measured DMHV effect in the in the presence of BTP2. The difference in $[Ca^{2+}]_{cyt}$ over 45 minutes in the two groups of experiments in BTP2-treated cells , shows the effect of DMHV on $[Ca^{2+}]_{cyt.}$

The effect of DMHV could be also observed in the absence of 2-APB and TG, indicating that 2-APB and TG did not induce this increase in $[Ca^{2+}]_{cyt}$ through a direct effect on the TRPV6 channels (Figure 24).

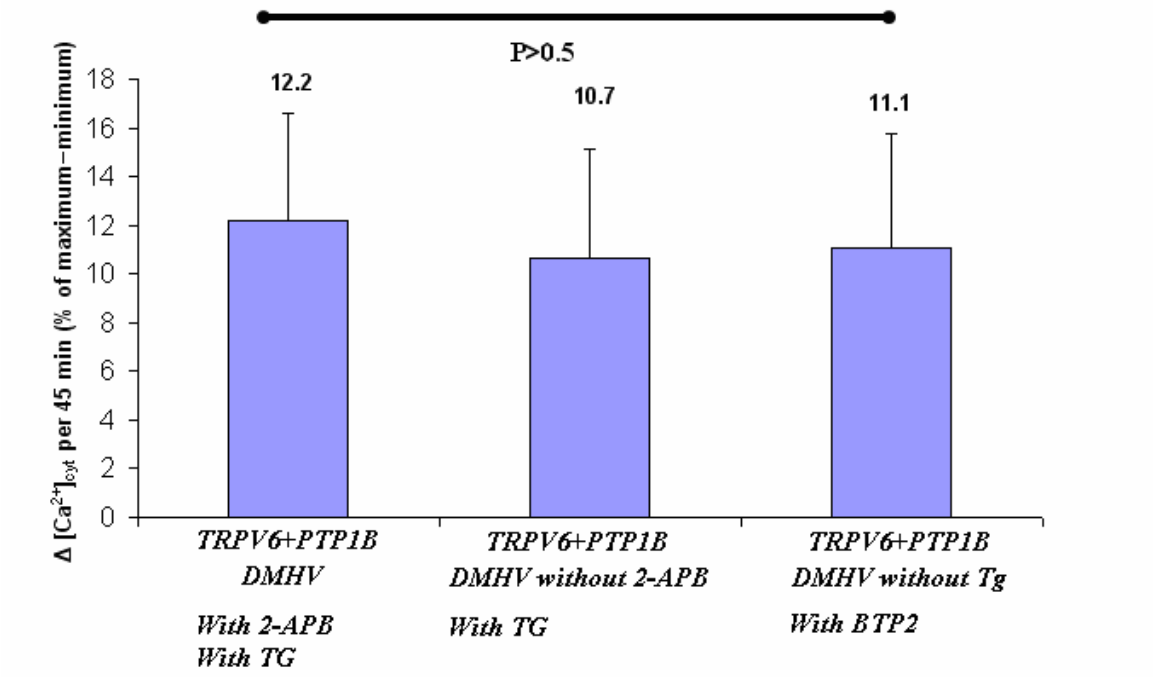

Figure 24: DMHV-mediated Ca^{2+} elevations in the absence of 2-APB and TG. First column shows DMHV-mediated Ca^{2+} elevation in cells cotransfected with TRPV6 and PTP1B in the presence of 2-APB and TG. In the absence of 2-APB or TG, cells showed similar elevations in $[Ca^{2+}]_{cyt}$ (see column 2 and 3 respectively).

3.4 DMHV effect on Ca^{2+} ATPase

The Ca^{2+} ATPase is one of the multiple processes which regulates the intracellular Ca^{2+} concentrations (Clapham, D.E. 1995). It plays its role in Ca^{2+} homeostasis by exporting Ca^{2+} to the outside of the cell. A potentially inhibitory effect of DMHV on Ca^{2+} ATPase was important to be detected. Figure 25 shows DMHV effect in untransfected HEK-293 cells following store depletion by TG and subsequent activation of Ca^{2+} entry, whereas at the end of the experiment Ca^{2+} free solution was added in the presence of DMHV (20μM) or in the absence of DMHV (control experiment). Data which are summarized in Fig. 26 reveal similar concentrations of cytosolic Ca^{2+} at different times in the presence or absence of DMHV.

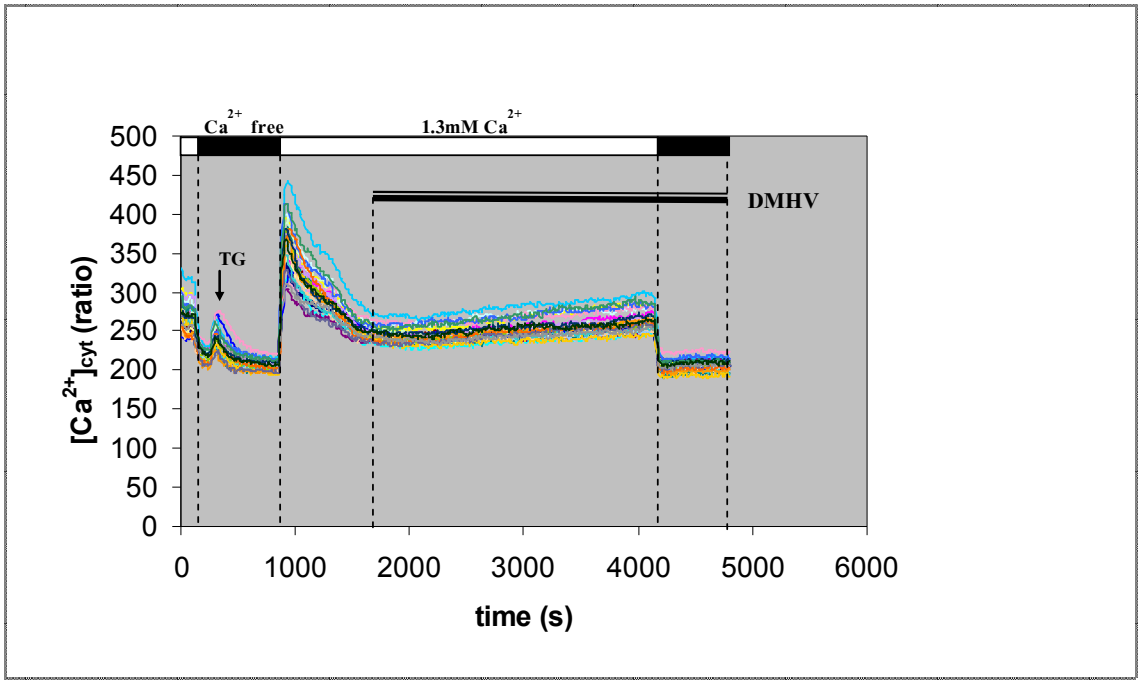

Figure 25: DMHV effect on Ca^{2+} ATPase. To test if there is an inhibitory effect of DMHV on the Ca^{2+} ATPase in untransfected HEK-293 cells, we added Ca^{2+} free solution in the presence of DMHV (20 µM) at the end of the experiment. For control we added the same solution in the absence of DMHV at the end of the experiment.

From these data we conclude that it is very unlikely that DMHV has any inhibitory effect on Ca^{2+}ATPase in the cellular membrane and therefore an increase in $[Ca^{2+}]_{cyt}$ did not result in inhibition of Ca^{2+}ATPase by DMHV.

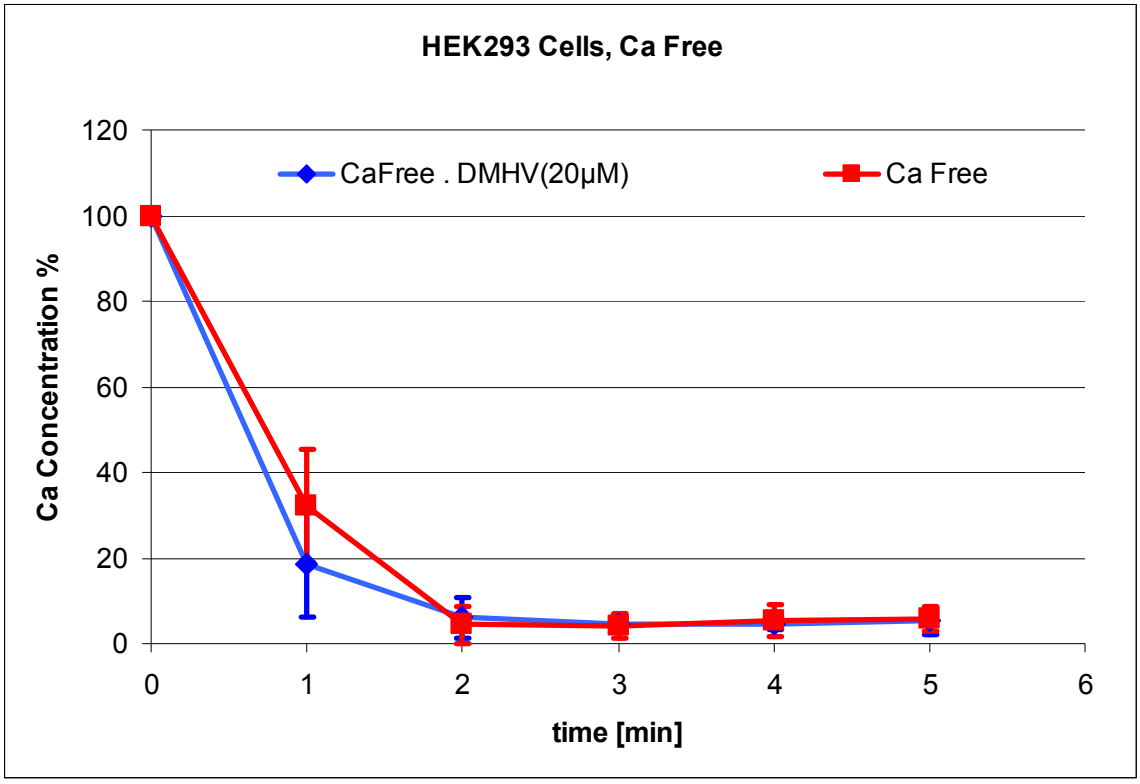

Figure 26: DMHV effect on Ca^{2+} ATPase in HEK-293 cells. **Red line** shows $[Ca^{2+}]_{cyt.}$ In HEK-293 cells in the presence of extracellular Ca^{2+} free solution at five different times and **blue line** shows similar $[Ca^{2+}]_{cyt.}$ in the same cells but in the presence of DMHV (20 µM).

3.5 Effect of the inactive PTP1B mutant PTPB(D181A) on TRPV6-mediated Ca^{2+} influx

DMHV has been described to be a potent inhibitor for tyrosine phosphatase PTP1B (Nxumalo F et al. 1998) by interacting with its highly conserved active site (Tracey AS. 2000). Co-expression of the inactive substrate-trapping PTP1B (D181A) and TRPV6 in HEK-293 cells resulted in an increase in the Ca^{2+} plateau (Figure 27),

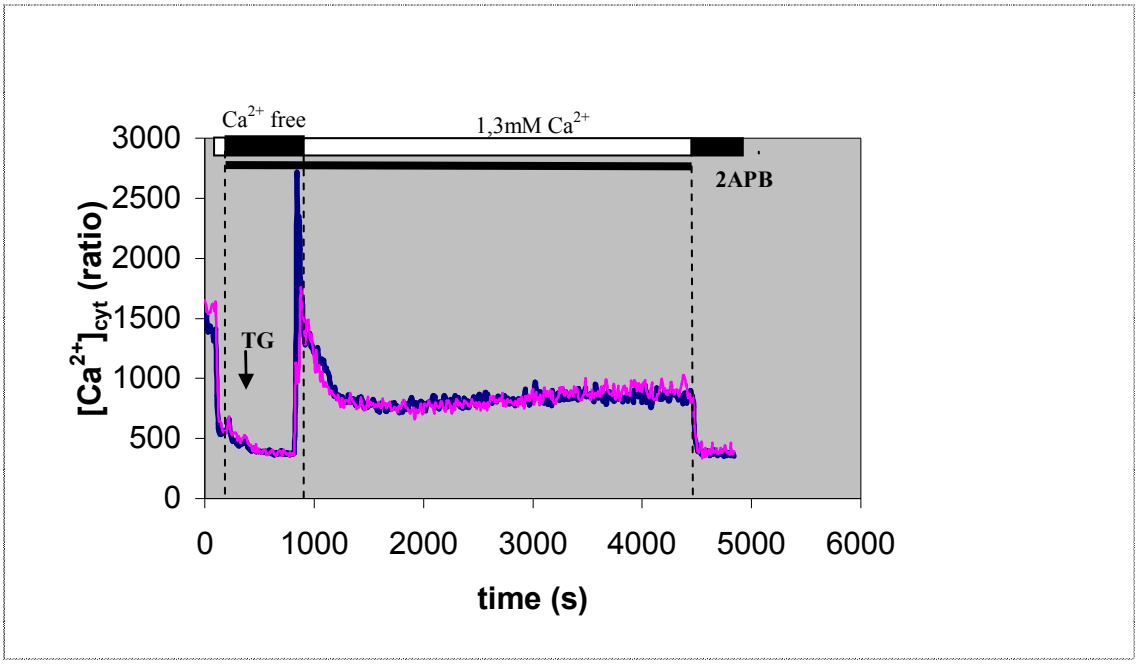

Figure 27: The increasing in Ca^{2+} plateau in HEK-293 cells co-transfected with TRPV6 and PTP1B (D181A). In these cells, the inactive substrate-trapping PTP1B(D181A) induced an increase in $[Ca^{2+}]_{cyt}$ 5,4±7,64%, n=28 cells, over 45 minutes.

which could not be further increased by DMHV. Mutation of this active site [PTP1B(D181A)] results in complete loss of phosphatase activity of PTP1B and should result in a similar increase of TRPV6-mediated Ca^{2+} entry as application of DMHV under the assumption that DMHV is specific (Figure 28). By quantifying the effect of DMHV on $[Ca^{2+}]_{cyt}$ we concluded that the increase in $[Ca^{2+}]_{cyt}$ by DMHV, which we found, was nearly only dependent on the inhibitory effect of DMHV on PTP1B (Figure 29), not on any potential side effects of the drug.

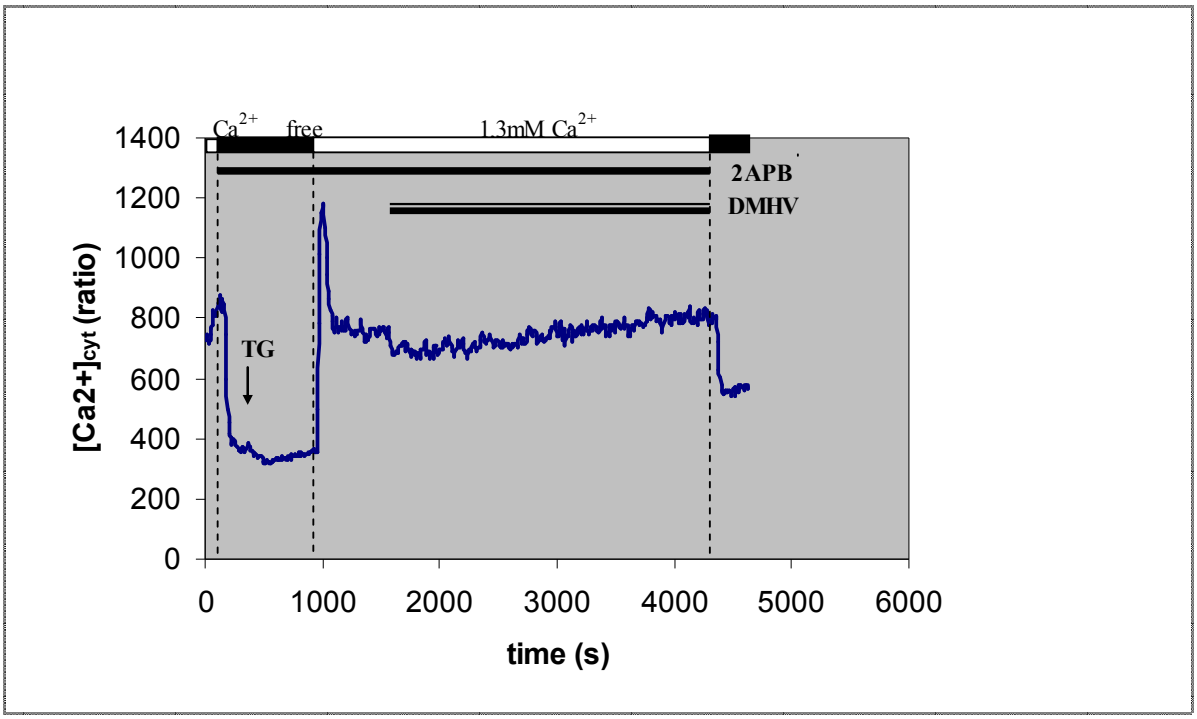

Figure 28: DMHV effect in HEK-293 cells co-transfected with TRPV6 and PTP1B (D181A). In the presence of DMHV (20µM), these cells showed an increase in $[Ca^{2+}]_{cyt}$ 6,8±8,9%, n=26 cells, over 45 minutes.

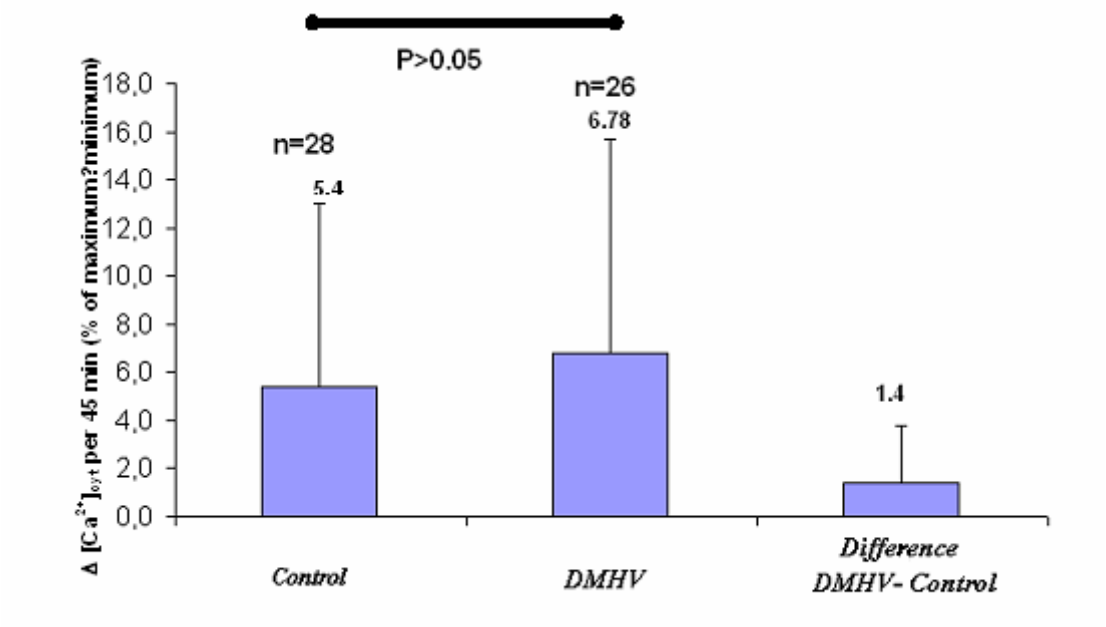

Figure 29: Measuring the effect of DMHV in HEK-293 transfected with TRPV6 and PTP1B (D181A). By comparing the differences in $[Ca^{2+}]_{cyt}$ in the presence and absence of DMHV, the DMHV induced an increase in $[Ca^{2+}]_{cyt}$ by 1,40± 2,37%.

As previously described, the inhibitory effect of DMHV on PTP1B transiently co-transfected with TRPV6 in HEK-293 cells induced a slow increase in $[Ca^{2+}]_{cyt}$. But in a more direct approach co-expression of PTP1B (D181A) in TRPV6-transfected HEK-293 cells also manifested itself in an increase in Ca^{2+} influx through TRPV6 channel (Figure 27). By comparing each with the difference in $[Ca^{2+}]_{cyt}$ over 45 minutes in HEK-293 cells co-transfected with both of TRPV6 and PTP1B in the absence of DMHV we concluded nearly similar effect (Figure 30).

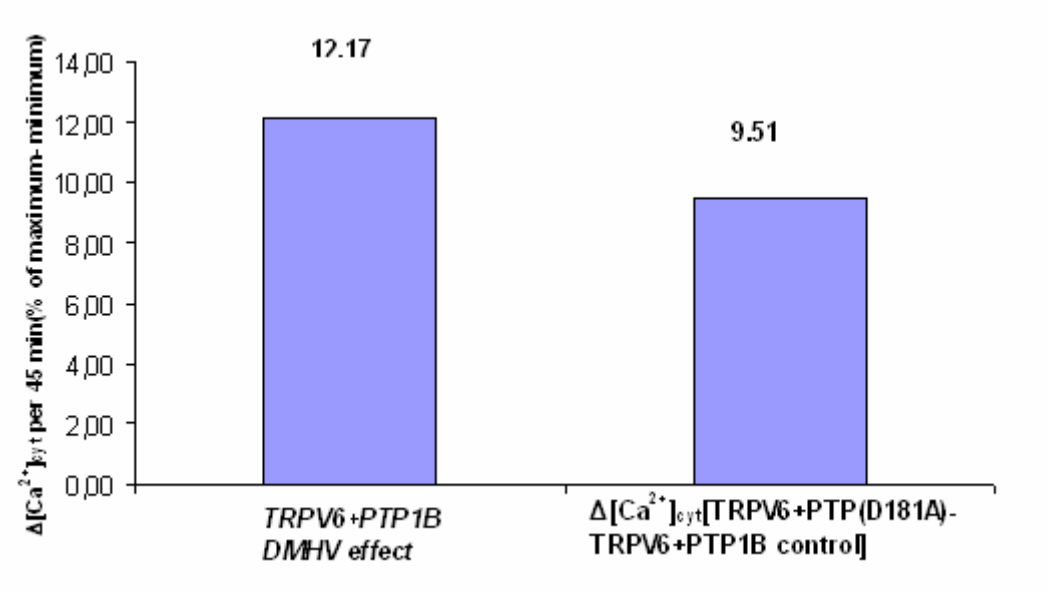

Figure 30: Effect of PTP1B inhibition on $[Ca^{2+}]_{cyt}$. Column **1** shows the increase in $[Ca^{2+}]_{cyt}$ after inhibition of PTP1B by DMHV, whereas column **2** shows the increase after transfection cells with PTP1B (D181A) without DMHV.

3.6 The site of tyrosine dephosphorylation in TRPV6

In the second step, we next sought to identify the site of the tyrosine phosphorylation in TRPV6 through site-directed mutagenesis studies. Preliminary sequence analysis of TRPV6 detected a potential tyrosine phosphorylation site of the amino acids Tyr(161,162), whereas sequence alignment indicated juxtaposed tyrosine residues. Therefore, on the tyrosines present in TRPV6, site-directed mutagenesis was performed to replace these residues with phenylalanine (Y161F,Y162F). As shown in Fig, 31 we found that regulated Ca^{2+} influx (DMHV effect) was not only abolished in the TRPV6(Y161/162F) mutant but it even showed DMHV-induced inhibition of Ca^{2+} influx below the control (0 line) (Ca^{2+} influx in the absence of DMHV).

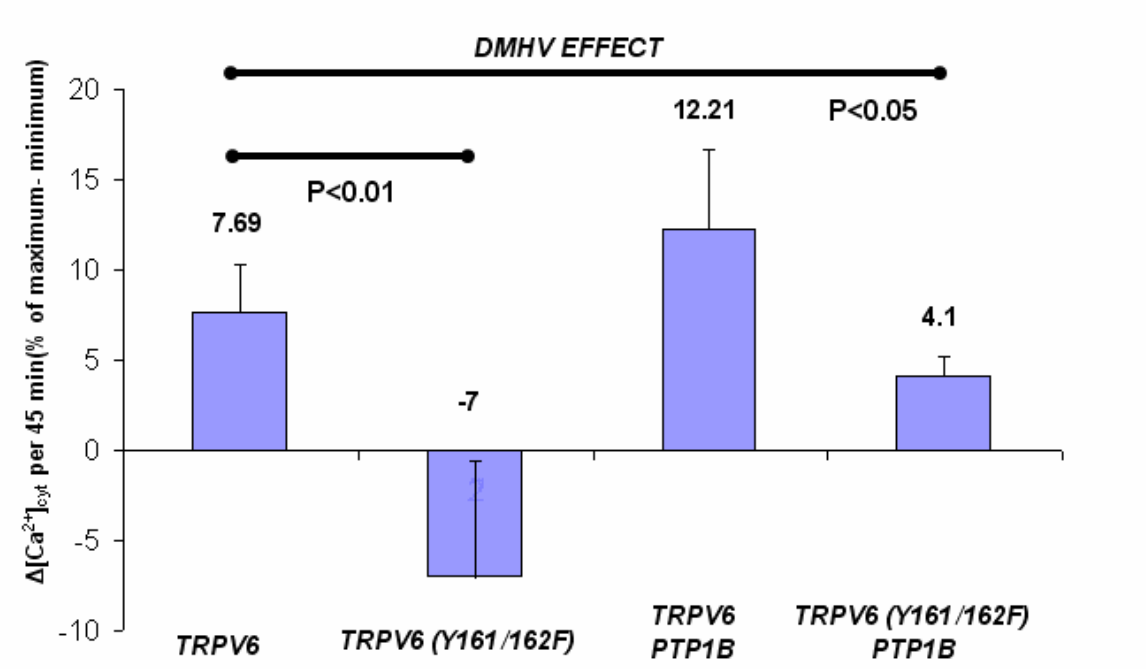

Figure 31: DMHV effect in TRPV6(Y161/162F). the first column shows DMHV effect in the presence of TRPV6-transfected cells, the second column shows the decrease in $[Ca^{2+}]_{cyt}$ in TRPV6(Y161/162F)-transfected cells in the presence of DMHV, the third column shows the increase in $[Ca^{2+}]_{cyt}$ in cells co-transfected with TRPV6 and PTP1B which was inhibited in the fourth column when cells were transfected with the TRPV6(Y161/162F)mutant.

In another approach, we performed experiments on cells which had been transfected with the TRPV6(Y161/162F) mutant and PTP1B. DMHV (20 µM) induced an increase in $[Ca^{2+}]_{cyt}$ but as shown in figure 31, it was lower than the increase which DMHV induced in HEK-293 cells transfected with TRPV6 and PTP1B.

To localize other tyrosines in TRPV6 which might be involved in the regulation of TRPV6-mediated Ca^{2+} influx, we have used TRPV6 fragments in which TRPV6 had been truncated at the N-terminal or the C-terminal ends. Deletion of N-terminal amino acids up to 1-37 in TRPV6 gave the same results as the full-length TRPV6 protein concerning Ca^{2+} influx. At the same time, it is interesting that deletion of the C-terminal amino acids 695-727 yielded a protein showing a regulated Ca^{2+} influx (DMHV effect) which was even higher than that measured with the full length TRPV6 (Figure 32).

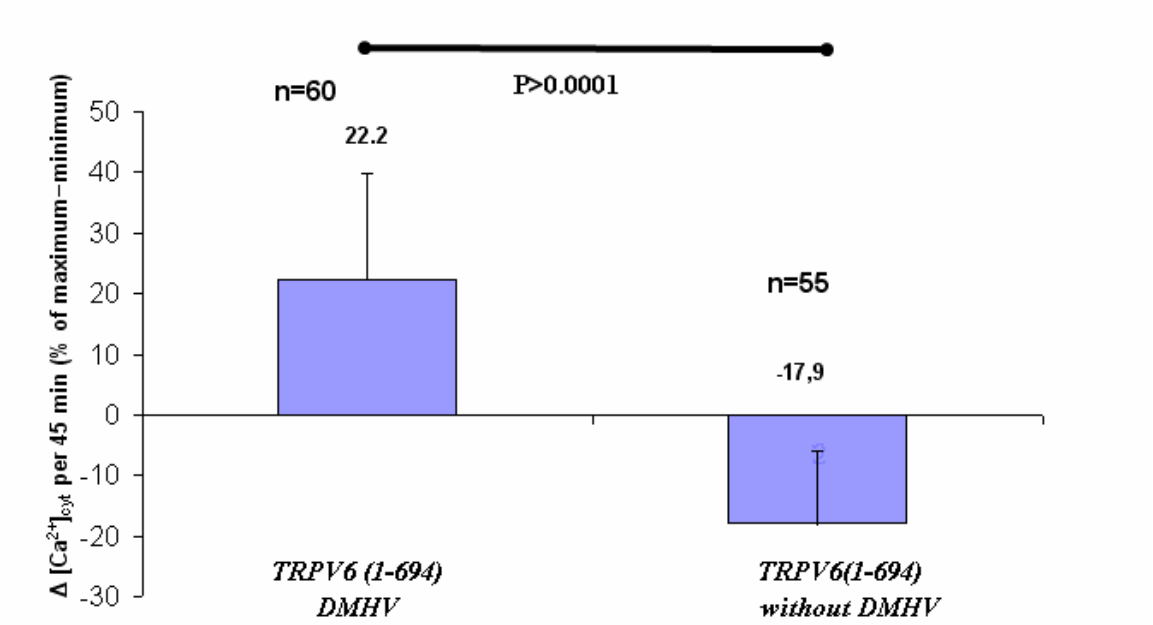

Figure **32: DMHV effect on the fragment TRPV6(1-694)**. First column shows a big increase in $[Ca^{2+}]_{cyt}$ in the presence of DMHV in HEK-293 cells transfected with TRPV6(1-694) and the second column shows a huge decrease in $[Ca^{2+}]_{cyt}$ in the absence of DMHV.

Chapter 4

Discussion

In the present study, I demonstrate that TRPV6 channel activity can be modulated by conditions which most likely result in tyrosine phosphorylation or dephosphorylation. The dephosphorylation seems to be induced by PTP1B. TRPV6- mediated Ca^{2+} influx has been investigated in transiently transfected HEK-293 cells. The characterization was performed by use of the Ca^{2+} indicator Fura-2 and Video-Imaging Technique. This method determined Ca^{2+} entry into HEK-293 cells as changes of the cytosolic Ca^{2+} concentration. Recent studies have shown that several protein kinases are involved in the regulation of *Drosophila* TRP ion channels. TRPV1 activity is modulated by protein kinase C (Premkumar, L.S., and Ahern, G.P. 2000). Cyclic AMP-dependent protein kinase A potentiates TRPV1 by direct phosphorylation (Bhave, G et al. 2002), TRPV1 is a potential target for cellular tyrosine kinase-dependent phosphorylation (Xiaochun Jin et al. 2004). TRPC6, TRPM7 and TRPV4 are also regulated by Src family kinase-dependent tyrosine phosphorylation (Chihiro Hisatsune et al. 2004), (Jiang X et al. 2003), (Xu H et al. 2003). Here, we describe the first evidence that PTP1B interacts with TRPV6 and therefore increases TRPV6-mediated Ca^{2+} influx via tyrosine dephosphrylation. However, in this study, we showed that the PTP1B inhibitor DMHV induced an increase in the Ca^{2+} influx which is expected to be induced by the activation of TRPV6 channels by phosphorylation. We can be sure that TRPV6 is involved because other transport mechanisms, which together with TRPV6 channels determine the amplitude of Ca^{2+} influx, remain unaffected. These mechanisms include endogenous CRAC channels which were blocked by 2-APB, transport by Ca^{2+} ATPases. Mitochondrial Ca^{2+} homeostasis, and activity of K^{+} channels might be involved but it was unexpected since untransfected HEK-293 cells did not show an increase in Ca^{2+} influx in the presence of DMHV. In addition, in our cells, co-transfection of TRPV6 with PTP1B resulted in an almost twofold increase in the Ca^{2+} influx which suggests a significant role for PTP1B in the regulation of TRPV6 currents.

On the other hand, the effect of DMHV could be observed in the absence of other chemicals, indicating that neither TG nor 2-APB induced this described increase in cytosolic Ca^{2+} as a potential side effect. This verification was important because 2-APB can affect multiple processes involved in cellular Ca^{2+} homeostasis such as inhibition of inositol 1.4.5-triphosphate ($InsP_3$)-mediated Ca^{2+} release and inhibiting ATP-evoked Ca^{2+} signals (Claire M. Peppiatt et al. 2003).

Direct interaction of the protein tyrosine phosphatase PTP1B and its predicted target TRPV6 could be demonstrated in living cells by “bimolecular fluorescence complementation” (BiFC) (Sternfeld, Krause et al. 2005). Also, DMHV most likely mediates its inhibitory effect on PTPases through its vanadium center. The multicoordinated vanadium center mimics a phosphate ion and thus can compete with tyrosine phosphate residues for binding to PTPases. Kinitics and molecular modeling studies indicate that DMHV strongly interacts in particular with PTP1B (Cuncic C et al. 1999) (Nxumalo F et al. 1998). Our data strongly suggest a direct role of PTP1B in TRPV6 regulation through tyrosine dephosphorylation, but not by any protein-protein interaction, which was verified when HEK-293 cells were co-transfected with TRPV6 and PTP1B(D181A). Flint had discovered that a mutation of the invariant catalytic acid (Asp-181 in PTP1B) converted an extremely active enzyme into a “ substrate trap”. Expression of this D181A mutant of PTP1B resulted in an enzyme that competes with endogenous PTP1B for substrates (Andrew J. Flint et al. 1997) but do not have the catalytic function. However, when HEK-293 cells transfected with the substrate-trapping mutant PTP1B(D181A), a long-lasting sustained calcium elevation could be observed and no significant difference was detected in the presence of DMHV which indicated that endogenous PTP1B was inhibited in the presence of the mutant PTP1B(D181A). Only a small but insignificant increase in cytosolic Ca^{2+} was induced in the presence of DMHV. This could be due to an incomplete inhibition of endogenous PTP1B by the mutant PTP1B(D181A), whereas DMHV inhibits PTPase activity by direct interaction with the catalytic region of the enzyme whereas PTP1B(D181A) proteins compete with the endogenous PTP1B for the same target. Another possibility is that other protein tyrosine phosphatases could be involved in the regulation of TRPV6 channel.

After describing the process by which in nonexcitable cells, Ca^{2+} enters in response to the release of Ca^{2+} from intracellular calcium stores, this process was termed either “Capacitative calcium entry” or “store−operated Calcium entry” (Putney,J.W.,Jr. 1997). The Ca^{2+} release-activated Ca^{2+} (CRAC) channel refered to the store-operated channel in the rat basophilic leukemia (RBL) and Jurkat T-lymphocyte cell lines. However, the molecular identity of the CRAC channel is still unknown and its identification is of significant importance. The best candidate was TRPV6 which, interestingly, when expressed in CHO cells manifested the pore properties of CRAC channel (Yue et al. 2001). In addition, Hsu et al. (2003) suggested that CRAC influx is modulated by tyrosine phosphorylation and dephosphorylation which involves the tyrosine phosphatase PTP1B. Therefore, we expected tyrosine phosphorylation

as be a prerequisit for regulation of TRPV6 by tyrosine phosphorylation and dephosphorylation. Depletion of the intracellular Ca^{2+} store was necessary. Our data showed that in HEK-293 cells, co-transfection of TRPV6 with PTP1B resulted in the same DMHV-induced increase of TRPV6-mediated Ca^{2+} influx in the presence or absence of filled stores. Therefore, we suggested that tyrosine dephosphorylation of TRPV6 is not a part of the store-operated mechanism, which is then in agreement with the fact that many differences between CRAC and TRPV6 exist and that both proteins do not seem to be identical (Heike Kahr et al. 2004).

For identification of particular tyrosine residues as sites for PTP1B activity, it was of interest that of the whole tyrosine residues in murine TRPV6, two residues Tyr(161,162) were identified as a candidate dephosphorylation site. Mutation of this site abolished the DMHV-mediated long-lasting calcium elevations in HEK-293 cells. Furthermore, DMHV also leads to Ca^{2+} influx inhibition below the control. On the other hand, truncation of TRPV6 by aa 695-727 leads to a drastical increase in regulated Ca^{2+} influx, indicating an inhibitory role of C-terminal located amino acids including Y725. We assume that DMHV-induced phosphorylation of Y725, the only tyrosine in this sequence, might be responsible for this inhibition. But the fact that the truncated TRPV6Δ695-727 lacks the C-terminal domain required for Ca^{2+}-calmodulin binding and this binding domain may also be responsible for Ca^{2+} induced inhibition of Ca^{2+} influx through TRPV6 (Bodding and Flockerzi. 2004) intrigue this suggestion.

In summary, activation and inactivation of TRPV6-mediated Ca^{2+} entry involve complex regulatory mechanisms, including intracellular Ca^{2+}-dependent inactivation, calmodulin inactivation, as well as the activation by PKC-dependent phosphorylation. Tyrosine phosphorylation and dephosphorylation are most likely novel regulatory mechanisms of TRPV6 channel activity. Our data provide evidence that protein tyrosine phosphatase PTP1B is important for downregulation of TRPV6 and suggest a direct interaction whereas TRPV6 channels themselves are the target for PTP1B. Indentification of the tyrosine residues of TRPV6 dephosphorylated by PTP1B help in understanding the mechanical insight into how tyrosine dephosphorylation regulates TRPV6 channel properties.

References:

1. Andersen JN, Mortensen OH, Peters GH, Drake PG, Iversen LF, Olsen OH, Jansen PG, Andersen HS, Tonks NK, Moller NP: Structural and evolutionary relationships among protein tyrosine phosphatase domains. Mol Cell Biol. 21:7117-36, 2001.

2. Berridge MJ, Peter Lipp, Martin D. Bootman: The versatility and universatility of calcium Signaling. Nature Review Mol cell Biol.1:11-21, 2000.

3. Bhave G, Zhu W, Wang H, Brasier DJ, Oxford GS, Gereau RW: cAMP-dependent protein kinase regulates desensitization of the capsaicin receptor (VR1) by direct phosphorylation. Neuron. 35:721-31, 2002.

4. Bilmen JG, Michelangeli F: Inhibition of the type 1 inositol 1,4,5-trisphosphate receptor by 2-aminoethoxydiphenylborate. Cell Signal. 14:955-60, 2002.

5. Bodding M, Flockerzi V: Ca2+ dependence of the Ca2+-selective TRPV6 channel. J Biol Chem. 279:36546-52, 2004

6. Bootman MD, T. J. Collins, L. Mackenzie, H. L Roderick, M J. Berridge, C. M. Peppiatt**:** 2-aminoethoxydiphenyl borate (2-APB) is a reliable blocker of store-operated Ca2+ entry but an inconsistent inhibitor of InsP3-induced Ca2+ release. FASEB J. 16:1145-50, 2002.

7. Bouillon R, Van Cromphaut S, and Carmeliet G: Intestinal calcium absorption: molecular vitamin D Mediated mechanisms. J Cell Biochem 88: 332-339, 2003.

8. Boulay G, Brown DM, Qin N, Jiang M, Dietrich A, Zhu MX, Chen Z, Brinbaumer M, Mikoshiba K, Brinbaumer L: Modulation of Ca^{2+} entry by polypeptides Of the inositol 1,4,5-triphosphate receptor (IP3R) that bind transient receptor Potential (TRP): Evidence for roles of TRP and IP3R in store depletion-activated Ca^{2+} entry. Proc Natl Acad Sci 96: 14955-14960, 1999.

9. William A. Catterall: Structure and regulation of voltage-gated Ca^{2+} channels. Annu. Rev. Cell Dev. Biol. 16: 521-55, 2000.

10. Clapham DE: Calcium signaling Cell. 80:259-68, 1995.

11. Clapham DE, Loren W. Runnels, Carsten Strübing: The TRP ion channel family. Nature Rev Neurosci. 2: 387-96, 2001.

12. Cui J, Bian JS, Kagan A, Mcdonald TV: CaT1 contributes to the store-operated calcium current in Jurkat T-lymphocytes. J Biol Chem 277:47175-47183, 2002.

13. Cuncic C, Desmarais S, Detich N, Tracey AS, Gresser MJ, Ramachandran C: Bis(N,N-dimethylhydroxamido)-hydroxooxovanadate inhibition of protein tyrosine phosphatase activity in intact cells: comparison with vanadate. Biochem Pharmacol. 58:1859-67, 1999.

14. Denu, J.M, Dixon,J.E: Protein tyrosine phosphatases: mechanisms of catalysis and regulation. Curr Opin Chem Biol. 2:633-41, 1998.

15. Flint AJ, Tiganis T, Barford D, K.Tonks NK: Development of "substrate-trapping" mutants to Identify physiological substrates of protein Tyrosine phosphatases. Proc Natl Acad Sci U S A. 94:1680-5, 1997.

16. Fraser DR, Kodicek E: Unique biosynthesis by kidney of a Biological active vitamin D metabolite. Nature 228:764-766, 1970.

17. Gallin WJ, Greenberg ME: Calcium regulation of gene expression in neurons: the mode of entry matters. Curr Opin neurobiol 5:367-374, 1995.

18. Grynkiewicz G, Poenie M, Tsien RY: A new generation of Ca2+ indicators with greatly improved fluorescence properties. J Biol Chem. 260:3440-50, 1985.

19. Hardie RC, Minke B: the trp gene is essential for a light-activated Ca^{2+} channel in Drosophila photoreceptors. *Neuron* 8: 643–651, 1992.

20. Hisatsune C, Kuroda Y, Nakamura K, Inoue T, Nakamura T, Michikawa T, Mizutani A, Mikoshiba K: Regulation of TRPC6 channel activity by tyrosine phosphorylation. J Biol Chem. 279:18887-94, 2004.

21. Hoenderop JG, Voets T, Hoefs S, Weidema F, Prenen J, Nilius B, Bindels RJ: Homo- and heterotetrameric architecture of The epithelial Ca^{2+} channels TRPV5 and TRPV6. EMBO J.22:776-85, 2003.

22. Hoenderop JG, Nilius B, Bindels RJ: ECaC: the gatekeeper of transepithelial Ca2+ Transport. Biochim Biophys Acta.1600:6-11. 2002.

23. Hoenderop JG, Vennekens R, Müller D, Prenen J, Droogmans G, Bindels R, Nilius B: Functional and expression of the epithelial Ca^{2+} channel family: comparison of Mammalian ECaC1 and 2. J Physiol. 537:747-61. 2001

24. Hoenderop JG, Nilius B, and Bindels RJ: Molecular mechanism of active Ca^{2+} reabsorption in the distal nephron. Annu Rev Physiol 64: 529-549, 2002.

25. Hoth M, and Penner R: Depletion of intracellular calcium stores activates a calcium current in mast cells. Nature 355:353-356, 1992.

26. Hsu S, Schmidt A, Sternfeld L, Anderie I, Solis G, Hofer HW, Schulz I: Tyrosine phosphatase PTP1B modulates store-operated calcium influx. Cell Signal. 15:1149-56, 2003.

27. Jiang X, Newell EW, Schlichter LC: Regulation of a TRPM7-like current in rat brain microglia. J Biol Chem. 278:42867-76, 2003.

28. Jin X, Morsy N, Winston J, Pasricha PJ, Garrett K, Akbarali HI: Modulation of TRPV1 by nonreceptor tyrosine kinase, c-Src kinase. Am J Physiol Cell Physiol. 287:C558-63. 2004.

29. Kahr H, Schindl R, Fritsch R, Heinze B, Hofbauer M, Hack ME, Mortelmaier MA, Groschner K, Peng JB, Takanaga H, Hediger MA, Romanin C: CaT1 knock-down strategies fail to affect CRAC channels in mucosal-type mast cells. J Physiol. 557:121-32, 2004.

30. Lipp P, Thomas D, Berridge MJ, Bootman MD: Nuclear calcium signaling by individual Cytoplasmic calcium puffs. EMBO J. 16: 7166-73. 1997.

31. Montero M, Alonso MT, Carnicero E, Chuchillo-Ibanez I, Albillos A, Garcia AG, Garcia-Sancho J, Alvarez J: Chromaffin-cell stimulation triggers fast Millimolar mitochondrial $Ca1^{2+}$ transients That modulate sectretion. Nature cell Biol 2:57-61, 2000.

32. Moreau R, Hamel A, Daoud G, Simoneau L, Lafond J: Expression of calcium channels along the differentiation of cultured trophoblast cells from human term placenta. Biol Reprod. 67:1473-9, 2002.

33. Neer RM: The evolutionary significance of vitamin D, skin pigment, and ultraviolet light. Am J Phys Anthropol 43:409-416, 1975.

34. Eric. J. Nestler, Paul Greengard**:** Protein phosphorylation is of fundamental importance in biological regulation. Book. Basic Neurochemistry. Sixth Edition, Lippincott, Williams & Wilkins, 1999.

35. Niemeyer BA, Bergs C, Wissenbach U, Flockerzi V, Trost C: Competetive regulation of CaT- like-Mediated Ca^{2+} entry by protein kinase C and Calmodulin. Proc Natl Acad Sci USA 98:3600-3605, 2001.

36. Nijenhuis T, Hoenderop JG, van der Kemp AW, Bindels RJ: Localization and regulation of the epithelial Ca^{2+} channel TRPV6 in the kidney. J Am Soc Nephrol 14:2731-2740, 2003.

37. Nilius B, Prenen J, Vennekens R, Hoenderop JG, Bindels RJ, Droogmans G: Parmaological modulation of monovalent cation currents through the epithelial Ca^{2+} channel ECaC1. Br J Pharmacol 134:453-462, 2001.

38. Nilius B, Vennekens R, Prenen J, Hoenderop JG, Droogmans G, Bindels RJ: The single pore residue Asp542 determines Ca^{2+} permeation and Mg^{2+} block of the epithelial Ca^{2+} channel. J Biol Chem 276:1020-1025, 2001.

39. Nordin BE, Horsman A, Marshall DH, Simpson M, And Waterhouse GM: Calcium requirement and calcium therapy. Clin Orthop 140: 216-239, 1979. Pflugers Arch 445:584-588. 2003.

40. Nxumalo F, Glover NR, Tracey As: Kinetics and molecular modelling studies of the inhibition of protein tyrosine phosphatases by N,N-dimethylhydroxylamine complexes of vanadium(V). *J. Biol. Inorg. Chem.* 3: 534-542, 1998.

41. Parekh AB, Penner R: Store depletion and calcium influx. Physiol Rev 77:901-930, 1997.

42. Peng J-B, Chen X-Z, Berger UV, Vassilev PM, Brown EM, Hediger MA: A rat kidney-specific calcium transporter the distal nephron. J Biol Chem. 275:28186-94. 2000.

43. Peng JB, Chen XZ, Berger UV, Weremowicz S, Morton CC, Vassilev PM, Brown EM, Hediger MA: Human calcium transport protein CaT1. Biochem Biophys Res Commun 278:326-332, 2000.

44. Peng JB, Brown EM, and Hediger MA: Epithelial Ca^{2+} entry channel: trancellular Ca^{2+} transport and beyond. J Physiol 551: 729-740, 2003.

45. Peppiatt CM, Collins TJ, Mackenzie L, Conway SJ, Holmes AB, Bootman MD, Berridge MJ, Seo JT, Roderick HL: 2-Aminoethoxydiphenyl borate (2-APB) antagonizes inositol 1,4,5-trisphosphate-induced calcium release, inhibits calcium pumps and has a use-dependent and slowly reversible action on store-operated calcium entry channels.
Cell Calcium 34:97-108, 2003.

46. M. Prakriya, R.S. Lewis: Potentiation and inhibition of Ca(2+) release-activated Ca(2+) channels by 2-aminoethyldiphenyl borate (2-APB) occurs independently of IP(3) receptors.
J Physiol. 536:3-19, 2001.

47. Premkumar, L.S, Ahern, G.P: Induction of vanilloid receptor channel activity by protein kinase C.
Nature. 408:985-90, 2000.

48. Putney JW: Capacitative calcium entry
Landes Biomedical Publishing, Austin, TX, USA. 1997.

49. Reichel H, Koeffler HP, Norman AW: The role of vitamin D endocrine system in health and disease.
N Engl J Med 320:980-991, 1989.

50. Schindl R, Kahr H, Graz I, Groschner K, Romanin C: Store depletion-activated CaT1 currents in rat basophilic leukemia mast cells are inhibited by 2-aminoethoxydiphenyl borate. Evidence for a regulatory component that controls activation of both CaT1 and CRAC (Ca^{2+} release-activated Ca^{2+} channel) channels.
J Biol Chem.277:26950-8, 2002.

51. Sternfeld L, Krause E, Schmid A, Anderie A, Latas A, Al-Shaldi H, Kohl A, Evers K, Hofer HW, Schulz I: Tyrosine phosphatase PTP1B interacts with TRPV6 in vivo and plays a role in TRPV6-mediated calcium influx in HEK293 cells. Cell Signal. 17:951-60, 2004.

52. Suzuki M, Ohki G, Ishibashi K, Imai M: A single amino acid mutation results in a rapid inactivation of epithelial calcium channels. Biochem Biophys Res commun 291: 278-285, 2002.

53. Tonks. N.K., Diltz, C.D, Fischerl, E.H: Purification of the major protein-tyrosine-phosphatases of human placenta. J Biol Chem. 263:6722-30, 1988.

54. Tracey AS: Hydroxamido vanadates: aqueous chemistry and function in protein tyrosine phosphatases and cell cultures. J Inorg Biochem. 80:11-6, 2000.

55. Van Abel M, Hoenderop JG, van der kemp AW, van Leeuwen JP, Bindels RJ: Regulation of the epithelial Ca^{2+} channel in small intestine as studied by quantitive mRNA detetion. Am J Physiol Gastrointest Liver Physiol 285: G78-G85, 2003.

56. Van Cromphaut SJ, Rummens K, Stockmans I, Van Herck E, Dijcks FA, Ederveen AG, Carmeliet P, Verhaeghe J, Bouillon R, Carmeliet G: Intestinal calcium transporter genes are upregulated by estrogens and the reproductive cycle through vitamin D receptor-independent mechanisms. J Bone Miner Res 18:1725-1736, 2003.

57. Vanden Abeele F, Roudbaraki M, Shuba Y, Skryma R, Prevarskaya N: Store-operated Ca^{2+} current in prostate cancer epithelial cells. Role of endogenous Ca^{2+} transporter type 1. J Biol Chem 278:15381-15389, 2003.

58. Vassilev PM, Peng JB, Johnson J, Hediger MA, Brown EM: Inhibition of CaT1 channel activity by a noncomptetive IP_3 antagonist. Biochem Biophys Res commun 280:145-150, 2001.

59. Vennekens R, Hoenderop JG, Prenen J, Stuiver M, Willems PH, Droogmans G, Nilius B, Bindels RJ: Permeation and gating properties of the novel epithelial Ca^{2+} channel. J Biol Chem 275:3963-3969. 2000.

60. Voets T, Prenen J, Fleig A, Vennekens R, Watanabe H, Hoenderop JG, Bindels RJ, Droogmans G, Penner R, Nilius B: CaT1 and the calcium release-activated calcium channel manifest distinct pore properties. J Biol Chem. 276:47767-70. 2001.

61. Vriens J, Owsianik G, Voets T, Droogmans G, Nilius B: Invertebrate TRP proteins as functional Models for mammalian channels. Pflugers Arch. 449: 213-226, 2004.

62. Weber K, Erben RG, Rump A, Adamski J: Gene structure and regulation of the murine Epithelial calcium channel ECaC1 and 2. Biochem Biophys Res Commun 289:1287-1294, 2001.

63. Xu H, Zhao H, Tian W, Yoshida K, Roullet JB, Cohen DM: Regulation of a transient receptor potential (TRP) channel by tyrosine phosphorylation. SRC family kinase-dependent tyrosine phosphorylation of TRPV4 on TYR-253 mediates its response to hypotonic stress. J Biol Chem. 278:11520-7, 2003.

64. Yue L, Peng JB, Hediger MA, Clapham DE: CaT1 manifests the pore properties of the calcium-Release-activated calcium channel. Nature 410:705-709, 2001.

65. Zhuang L, Peng JB, Tou L, Takanaga H, Adam RM, Hediger MA, Freeman MR: Calcium-selective ion channel, CaT1, is apically localized in gastrointestinal tract epithelia and is aberrantly expressed in human malignancies. Lab Invest 82:1755-1764, 2002.

66. Zitt C, Strauss B, Schwarz, Spaeth EN, Rast G, Hatzelmann A, Hoth M: Potent inhibition of Ca2+ release-activated Ca2+ channels and T-lymphocyte activation by the pyrazole derivative BTP2. J Biol Chem. 279:12427-37, 2004.

Acknowledgments

I would like to acknowledge many people who have directly or indirectly contributed to the successful development of the work in this thesis. First, I would like to thank my adviser, Professor. Dr. Irene Schultz, for her invaluable guidance and support. Her questions and suggestions have always inspired me to think about this topic more thoroughly and at a deeper level.

I would like to thank PD. Elmar Krause for his help and support for me to continue my work, and for his wise advice on technical as well as non technical issues. Thi advice has helped me greatly in overcoming many obstacles.

I gratefully acknowledge Mrs. Anja Ludes for her generous help and support.

A very special thanks goes to Mrs. Gabi Mörshbächer, whose mastery and efficiency of administrative matters, took a lot of worries from my head.

I would like to thank the other members of the group for the pleasure of working with them and learning from them: PD. Andreas Schmidt, Dr Ines Anderie, Dr. Lutz Sternfeld,
Dr. Monika Bozem, Fei Yang, Ivan Bogeski, Ilona Rickel, Sabine Plant, Petra Hammes, Michael Konzmann, Valentina , Chritine Paulus.

I am deeply indebted to my family for their love and unconditional support.

Curriculum vitae

Name:	Hajaj Alshaldi Born 8. Mai 1974 Hama
Marital status	Single
School Education	
1980-1986	Primary school in Salamieh, school "Talbieh"
1986-1989	Preparatory school in Salamieh school "Ali Ben abi Taleb"
1989-1993	Secondary school in Salamieh, school "Ali Ben abi Taleb"
1993	School-leaving examination in science branch.
Highschool education	
1993/94-1998/1999	The study of medicine at Aleppo University
26.04.2000-15.05.2001	Resident for specialization in orthopedic in "Damascus" Hospital.
20.05.2001	Highschool assistant at Aleppo University, Department of physiology.
Mars 2003	Began dessination under the guidance of Prof. Dr. Irene Schultz and Priv. doz Elmar Krause in the Institut Of Physiology, University of Saarland.