The role of TRPV6 in breast carcinogenesis

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PhD Thesis

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Thesis abstract

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Abstract (the text has to fit into the space below)

TRPV6 is a calcium entry channel that is strongly expressed in breast adenocarcinoma tissue. In this PhD-study we further confirmed this observation by analysis of breast cancer tissues, which indicated that TRPV6 mRNA expression was upregulated compared to normal breast tissue. While TRPV6 is known to be expressed in the cancer tissue, its role as a calcium channel in breast carcinogenesis is poorly understood. Therefore, we investigated how TRPV6 affects viability, apoptosis, and calcium transport in breast cancer cell lines. TRPV6 expression can be regulated by estrogen, progesterone, 1,25-vitamin D, and tamoxifen (a selective estrogen receptor modulator that is widely used in breast cancer therapy) in T47D cells. Knockdown of TRPV6 in this cell model leads to a significant decrease of cell viability. Moreover, the effect of tamoxifen on cell viability was enhanced when TRPV6 expression was silenced with siRNA. Interestingly, tamoxifen reduced expression of TRPV6 in T47D cells and is able to inhibit its calcium transport activity (IC_{50}=7.5 µM) in Xenopus oocytes. We examined the effect of tamoxifen on TRPV6 function and intracellular calcium homeostasis in MCF-7 breast cancer cells transiently transfected with EYFP-C1-TRPV6 in detail. TRPV6 activity was measured with fluorescence microscopy using Fura-2. Tamoxifen decreased the transport rates of calcium and barium in transfected cells by 50%. This inhibitory effect was not blocked by the estrogen receptor antagonist, ICI 182,720 and a similar inhibition effect was also observed in MDA-MB-231 estrogen receptor negative cells. The effect of tamoxifen was completely blocked by activation of protein kinase C. Inhibiting PKC with calphostin C decreased TRPV6 activity but did not alter the effect of tamoxifen. These findings illustrate how tamoxifen might be effective in estrogen receptor negative breast carcinomas and suggest that the mechanisms of tamoxifen and PKC inhibitors used in breast cancer therapy might involve TRPV6 mediated calcium entry. This PhD-study highlights a possible role of TRPV6 as a therapeutic target in breast cancer therapy.

For my family

Table of contents

ABBREVIATIONS

VOCCs	voltage-operated calcium channel
ROCCs	receptor-operated calcium channel
SOCCs	store-operated calcium channel
TRP	transient receptor potential
PM	plasma membrane
ER	estrogen receptor
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 formucolipin
TRPN	transient receptor potential for no mechano-receptor potential
SNARE	*s*oluble *N*-ethylmaleimide-sensitive-factor *a*ttachment *re*ceptor
Src kinase	sarcoma kinase
PI3K	phosphoinositide-3- kinase
2-APB	2-aminoethoxydiphenylborate
CNS	central nervous system
PUFA	polyunsaturated fatty acid
4α-PDD	4α-phorbol 12,13-didecanoate
PACSIN	for PKC and CK2 substrate in neurons
mRNA	messenger RNA
DMEM	dimethylsulfoxide
FBS	fetal bovine serum
DMSO	dimethylsulfoxide
EYFP	enhanced yellow fluorescence protein
TG	thapsigargin
PARP1	poly (ADP-ribose) polymerase-1
PIP_2	phosphatidylinositol biphosphate
TMD	transmembrane domains
PLC	phospholipase C
PKC	proteinkinase C
PTH	parathyroid hormone
vitamin D3	1, 25 dihydroxy vitamin D3
NFAT	nuclear factor of activated T-cells
siRNA	small interfering RNA
DCT	distal convoluted tubule
CNT	connecting tubule
VDR	vitamin D receptor
HEK cells	human embryonic kidney *293* cells
VDDR-I	vitamin D dependency rickets type I
HER2	human epidermal growth factor receptor 2
CaM	calmodulin
GST pull-down *assay*	glutathione S-transferase pull-down *assay*
SERM	selective estrogen receptor modulators

CaR	Ca^{2+} sensing receptor
WNK3	WNK lysine deficient protein kinase 3
PTP1B	protein tyrosine phosphatase-1B
S100A10	S100 calcium binding protein A10
LNCaP	origin: lymph node carcinoma of the prostate
PC-3	origin: prostate cancer

1. Introduction

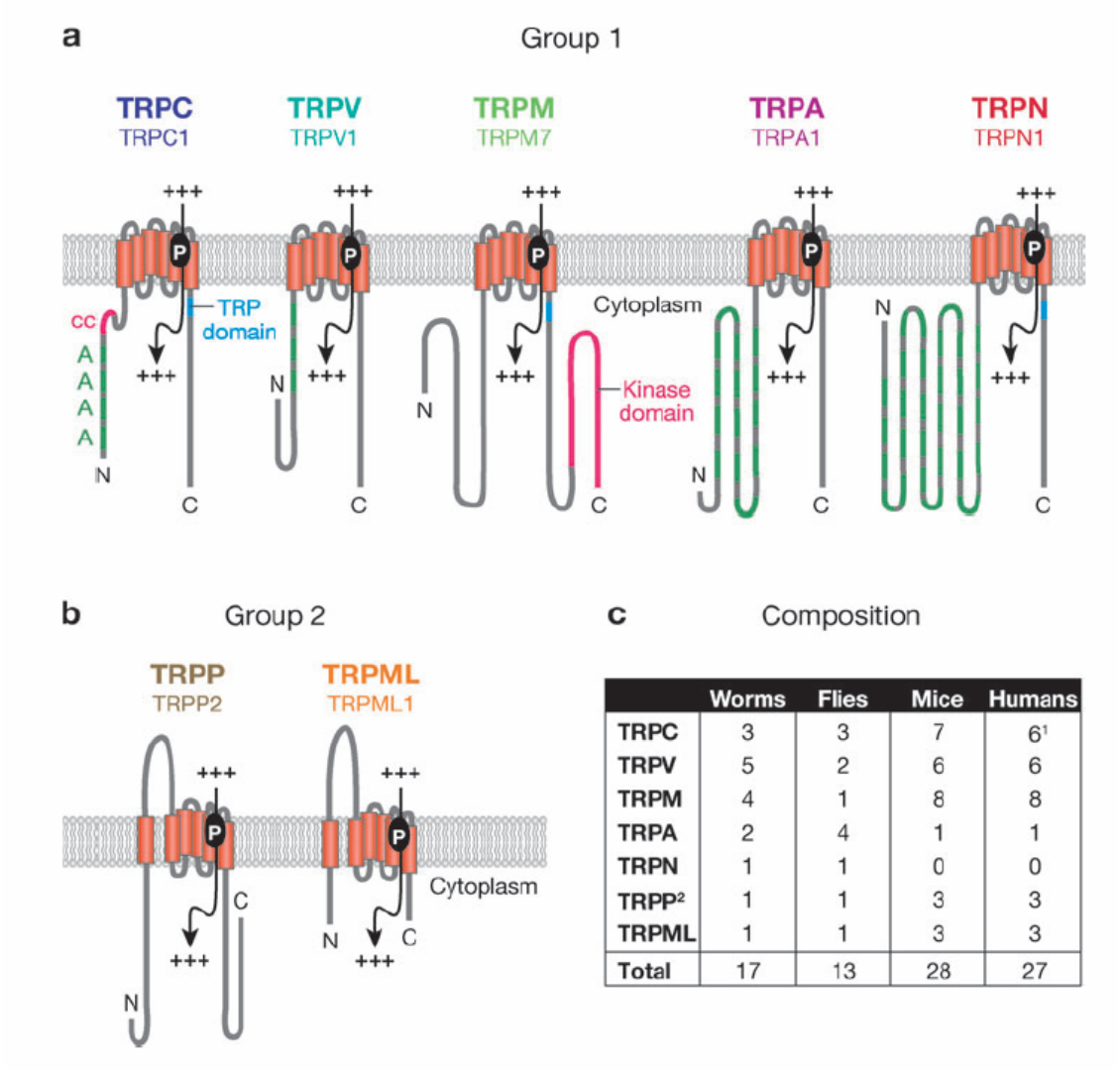
1.1 Calcium Entry Channels

The channels that mediate calcium influx form the extracellular space into the cell can be grouped into four categories: (I) Voltage-operated calcium channels (VOCCs). These channels are mainly found in excitable cells and they act in response to membrane depolarization and can be regulated by second messengers, drugs and toxins (1;2). (II) Voltage-independent receptor-operated calcium channels (ROCCs) that function after stimulation of a membrane receptor. (III) Store-operated calcium channels (SOCCs) are activated by depletion of sarcoplasmatic reticulum and endoplasmatic reticulum calcium stores (3). (IV) Epithelial calcium entry channels that allow apical calcium entry in absorptive and secretory tissues (4).

1.2 TRP channels

Category (IV) and in part category (III) belong to the Transient Receptor Potential (TRP) superfamily. These channels can be activated by light, sound, chemical, temperature, touch stimuli or hyperpolarisation. They can also be receptor activated by e.g. PIP_2, activated by specific ligands like capsaicin (5). The different activation mechanisms and the diversity of cation selectivity of members of the TRP family allow the detection of different types of changes in the close surrounding of the channels.

All the TRP channels have a common structure that contains six transmembrane domains (TMD) with a pore region between TMD five and TMD six and an intracellularly localized amino- and carboxyl-terminus (Figure 1). Based on homology and structure, they can be divided into two groups.



	Worms	Flies	Mice	Humans
TRPC	3	3	7	6[1]
TRPV	5	2	6	6
TRPM	4	1	8	8
TRPA	2	4	1	1
TRPN	1	1	0	0
TRPP[2]	1	1	3	3
TRPML	1	1	3	3
Total	17	13	28	27

Figure 1: The TRP superfamily. (*a*) Single members from each of the five group 1 subfamilies. (*b*) Single members from each of the two group 2 subfamilies. The following domains are shown: A, ankyrin repeats; cc, coiled-coil domain; protein kinase domain; TRP domain. Also shown are transmembrane segments (*vertical rectangles*) and the pore loop region (P) which allows the passage of cations (+++). (*c*) Composition of the TRP superfamily in worms, flies, mice, and humans. [1]Human *TRPC2* is a pseudogene and is not counted. [2]TRPP1-like proteins are not counted (6).

Group 1 includes the TRPV, TRPM, TRPA, TRPN, and TRPC subfamily. These subfamilies show the closest sequence homology to the Drosophila TRP channel that was first identified in 1989 (7). Amongst them the classical TRPC channels show the highest homology to Drosophila TRP. Interestingly, some members of the TRPM family are classified as chanzymes that possess a C-terminal enzyme domain (8). The TRPM channels also differ from the other subfamilies since they exhibit no ankyrin repeats in their structures. The members of the TRPA subfamily play a major

role in thermosensation (9-11). Not much information is available about TRPN channels, which are not expressed in mammals.

A specific feature of the members of group 2 (TRPP and TRPML) is that they additionally contain a large extracellular loop between the first and second TMD. Both members were identified because their mutation causes human diseases. A mutation in TRPP causes autosomal dominant polycystic kidney disease and one in TRPML muculipidosis type IV(12-15). Other known TRP channel mutations are present in group 1. Mutations in TRPC6 can result in glumerulosclerosis and a mutation in TRPM6 leads to hypomagnesemia (16-19).

1.3 TRPV channels

1.3.1 TRPV1

The TRPV channels were first identified through expression cloning. Application of this method resulted in the identification of TRPV1 (20) Subsequently, the other members of the TRPV family were identified:

Capsaicin, the active component of chili peppers that produces a sensation of burning, was applied to identify the TRPV1 channel (21). This channel is weakly calcium selective and outwardly rectified (22). Other stimuli that can activate TRPV1 are heat (≥43°C) enhanced by low pH (23), anandamide (24), camphor (25), piperine (26), and garlic (27). TRPV1 is expressed in distinct dorsal root ganglia neurons, where it reacts to environmental changes (28) and it plays an essential role in inflammatory thermal hyperalgesia (29). It is also involved in mechanically evoked purinergic signaling in the bladder, gastrointestinal motility, hearing modulation, and satiety (30-33). TRPV1-knockout mice show impaired nociception and pain sensation (34). It was demonstrated that TRPV1 can be potentiated by an activation of phospholipase C (PLC) with proanalgesic agents, a subsequent decrease in PIP_2 levels, and phosphorylation through protein kinase C (PKC) (35). Important for TRPV channel activity is also the insertion and retention of the channel in the plasma membrane. TRPV1 can interact with members of the SNARE-dependent exocytic pathway and lead to PKC-mediated translocation of TRPV1 to the plasma membrane (36). Insertion of TRPV1 in the plasma membrane can be also triggered

by proalgesic factors such as nerve growth factors. This mechanism functions via phosphorylation of the channel on a tyrosine residue by Src kinase (37).

1.3.2 TRPV2, TRPV3 and TRPV4

A similar activation mechanism through heat is known for TRPV2, TRPV3, and TRPV4. TRPV2 is weakly calcium selective and activated at high temperatures >52ºC (38), whereas TRPV3 and TRPV4 are already activated at warm temperatures around 33ºC (39). Its activity is increased by application of 2-aminoethoxydiphenylborate (2-APB) (40). It was found that TRPV2 function can be up regulated by phosphoinositide-3-kinase (PI3K) activation (41) and cell swelling induced by hypotonicity (42). TRPV2 activity is regulated by insertion of the channel into the plasma membrane from internal vesicles, which can be stimulated by insulin-like growth factor-I treatment shown in an *in vitro* system (43). Protein expression of TRPV2 was detected in the CNS (44), the myenteric plexus, and in the nodose ganglion (45).

In cell culture models, PLC stimulation (46), application of PUFAs (47), 2-APB (48), menthol, and some spices (49) induced a weakly calcium selective cationic conductance over TRPV3 (50). After heat activation TRPV3 currents are strong outwardly rectified and display a sensitization following repeated activation (51). TRPV3 protein is widely expressed in humans (52), but only in the skin of mice (53), where it might function in keratinocytes. Interestingly, there is evidence for an interaction of TRPV3 with TRPV1 (54) and TRPV2 (55), but the functional consequences are so far unidentified.

Moderate heat, hypotonic challenge, and 4α-PDD can activate the calcium permeable, constitutively active, and outwardly rectified channel TRPV4. An induction of TRPV4 by extracellular hypotonicity requires the interaction of TRPV4 with aquaporin 5 (56). Another mechanism of TRPV4 activation works over cytochrome P450-dependent formation of epoxyeicosatrienoic acids. This mechanism is triggered by anandamide and arachidonic acid (57). TRPV4 is in contrast to TRPV1, TRPV2, and TRPV3 not activated by 2-APB (58). TRPV4 KO mice show hearing loss and an impaired pressure, temperature, and osmotic sensitivity (59-62). Surface expression of TRPV4 involves its interaction with PACSINs (63) and glycosylation in the pore loop (64).

1.3.3 TRPV5 and TRPV6

Different to the other TRPV channels are TRPV5 and TRPV6 (5;65;66). TRPV5 was identified via expression cloning from rabbit kidney (67) and TRPV6 from rat duodenum by Hediger and colleagues (68).

They cannot be activated by heat and they exhibit the highest Ca^{2+} selectivity (P_{Ca}:P_{Na} > 100) of all mammalian TRP channels (66;69-71). In the absence of extracellular calcium they are also permeable to monovalent cations (72). TRPV5 is mainly expressed in the kidney and responsible for calcium reabsorption via epithelial cells (73). Around 65% of the filtered calcium is passively reabsorbed in the proximal tubule, while 20% is reabsorbed by claudin-16 over tight junctions via a paracellular pathway. The final reabsorption of calcium occurs in the distal convoluted tubule (DCT) and the connecting tubule (CT) in a highly regulated manner via TRPV5 to maintain an appropriate total body calcium homeostasis (74). TRPV5 has, beside the general TRP structure, six ankyrin repeats at the amino-terminus that are important for protein-protein interactions (75). The insertion of TRPV5 into the plasma membrane regulates its activity (76). Hydrolysis of extracellular sugar residues between TMD1 and TMD2 at an evolutionary conserved asparagine (N_{358}) by the β-glucuronidase Klotho promotes retention of the channel in the plasma membrane (77). There are also three potential PKC phosphorylation sites that might play a role in regulating channel activity (78-80). The active conformation of TRPV5 is an assembly into homo-tetramers (81;82). Characteristic for TRPV5, which is constitutively active at physiological membrane potentials, is its high calcium selectivity and strong inward rectification (83). TRPV5 KO mice show dramatically reduced active Ca^{2+}-reabsoption in the DCT and CNT and waste six- to ten-fold more calcium than wild-type mice (84).

Shortly after the discovery of TRPV5, another TRP channel was found that shares 75% amino acid identity to TRPV5. In 1999, TRPV6 was identified by Hediger and colleagues from rat duodenum using expression cloning (85).

1.4.1 Structure of TRPV6

TRPV6 shares the basic structure of TRP channels, which consist of intracellularly localized NH_2- and COOH-terminal tails flanking six TMDs with a pore-forming loop between TMD5 and TMD6. On the NH_2-terminus of TRPV6 are 3-4 ankyrin repeats

depending on the prediction algorithm (86). TRPV6 has a tetrameric structure where four subunits surround a single pore and can assemble in homo- and hetero-tetramers with TRPV5 (87). It was shown that the ankyrin repeat at position 116-191 of the NH_2-terminus is very important for functional subunit assembly (88). A structural model of the TRPV6 outer pore by Voets et al. displayed that a mutation of an aspartate residue at position 542 can alter the pore diameter and is important for calcium binding (89). TRPV5 and TRPV6 are side by side on the human chromosome 7q35, have only a 22kb distance between each other, and comprise 15 exons (90). TRPV6 consists of 730 amino acids and its predicted molecular mass is around 83kDa (91).

1.4.2 Expression and function of TRPV6

TRPV6 protein was detected in the apical membrane of epithelial cells of the intestine. The most abundant expression is found in the duodenum, although staining could be detected in the colon as well. In the duodenal villi at the brush border membrane, mRNA and protein could be detected at the upper part of the villus on the luminal side (92).

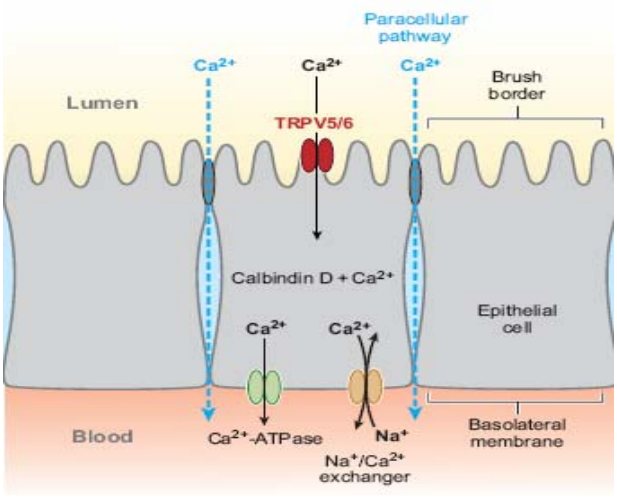


Figure 2. Mechanism of intestinal calcium absorption in the intestine and kidney (66).

TRPV6 plays a major role in intestinal calcium absorption. In the epithelial cells, calcium gets bound to calbindin D9k, which transports it to the basolateral membrane. There, calcium crosses the membrane via the Ca^{2+}-ATPase PMCA1b and the Na^{+}/ Ca^{2+} exchanger and enters the blood (66) (Figure 2).

The organ that shows the highest expression level for TRPV6 is the human placenta, where levels 50-fold higher than in the duodenum can be measured (93). Using *in situ* hybridization, TRPV6 was found in trophoblasts and syncytiotrophoblasts (94). Recently, Suzuki et al., showed that it is involved in maternal-fetal calcium transport by comparison of TRPV6-knockout mice with wild-type mice (95). Protein signals were also detected at the secretory poles of mouse pancreatic acinar cells and at the inner surface of ductal epithelial cells in human mammary and sweat glands (96). Weber at al., could measure TRPV6 for the first time in the murine skin (97) and Lehen'kyi et al., found recently that TRPV6 is essential for Ca^{2+}-induced differentiation of human keratinocytes (98). TRPV6 is also expressed in the mouse inner ear and in human leukemia cells, where its function has not completely clarified thus far (99;100).

Studies of Bianco et al., with TRPV6 knockout mice demonstrated that TRPV6 is most likely to be the major calcium channel responsible for apical intestinal absorption (101). In the knockout mice the intestinal calcium absorption was drastically reduced, the animals weighed less, and were less fertile compared to wild-type mice. The blood levels of PTH and 1,25-vitamin D of the knockout mice were elevated, showing a secondary hyperparathyroidism caused by reduced calcium absorption. Consequently, these animals have a reduced bone density, which might be due to increased bone resorption or a possible role of TRPV6 in bone cells.

TRPV6 has special transport characteristics that are different to other calcium channels. One of them is that it is almost completely inwardly rectified (102). The other major characteristic of TRPV6 is its high calcium selectivity; permeation of Na^+ versus Ca^{2+} is 1:100. The permeability of other divalent ions decreases from: $Ca^{2+} > Ba^{2+} > Sr^{2+} > Mn^{2+}$ (103) (Figure 3).

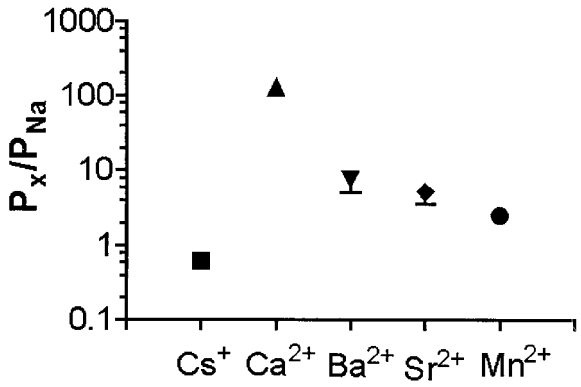


Figure 3: Relative permeability in CaT1-expressing CHO-K1 cells (to Na^+) of Cs^+ (0.6), Ca^{2+} (130), Ba^{2+} (7.5), Sr^{2+} (5.1) and Mn^{2+} (2.4) through CaT1 channels (n=8 for each) (Yue et al., 2001 Nature)

The K_m-value for Ca^{2+} is 0.44mM. Known nonspecific blockers of TRPV6 are ruthenium red, Gd^{3+}, and La^{3+} (104). Another nonspecific inhibitor of Ca^{2+}/cation channels in non-excitable cells is econazole (105). Schwarz et al., found 2006 in calcium imaging experiments with HEK cells transfected with TRPV6 that 600nM econazole reduces $[Ca^{2+}]_i$ (106). An nonspecific activator of TRPV6 is 2-APB, which could increase TRPV6 mediated currents with a concentration of 50µM (107). Until now, no specific inhibitors or activators are known.

1.4.2 Regulation of TRPV6

1. Regulation of channel abundance

Vitamin D3

Many studies demonstrated that vitamin D3 is very important for the regulation of TRPV6. First, TRPV6 promotor regions were identified that exhibit potential vitamin D3-response elements (108). Then, vitamin D3 application could enhance TRPV6 mRNA expression in *in vitro* models with Caco-2 intestinal cell lines (109;110). *In vivo* a dose of vitamin D3 increased duodenal TRPV6 mRNA levels in mice (111). Another study showed the time dependency of this response, which takes place 3-6 hours after stimulation (112). Studies with vitamin D3-receptor knockout mice revealed that these mice have significantly down regulated duodenal TRPV6 mRNA levels (113).

Dietary calcium

Dietary calcium can also regulate TRPV6 expression. To study this, VDR-knockout mice and 1α-hydroxylase knockout mice were used because in wild-type mice the vitamin D levels would be affected as well and single effects could be distinguished. High dietary calcium intake restored the reduced expression level of intestinal TRPV6 in 1α-hydroxlase knockout mice and led to a normalization of the calcium level in the plasma (114). In a similar way, dietary calcium up regulated the decreased level of duodenal TRPV6 mRNA expression in vitamin D3 receptor knockout mice (115). Interestingly, the CaR (calcium sensing receptor) does not stimulate TRPV6 activity, in contrast to findings obtained with TRPV5 (116).

Short chain fatty acids

Fukushima et al., found recently that short-chain fatty acids induce TRPV6 expression in rats and Caco-2 cells (117).

Parathyroid hormone

Parathyroid hormone (PTH) is secreted when the extracellular calcium level decreases. It acts on calcium reabsoption in the kidney and on calcium resorption from the bone to keep up the extracellular Ca^{2+} concentration. PTH also stimulates 1 alpha-hydroxylase that is necessary for vitamin D3 synthesis. Therefore, PTH plays at least an indirect role in TRPV6 regulation.

Oestrogens and androgens

Similar to vitamin D3, an estrogen-responsive element was found in the promoter sequence of TRPV6 (118). An *in vivo* study showed that in ovariectomized rats and 1α-hydroxylase knockout mice administration of 17β-estradiol led to an up regulation of duodenal TRPV6 mRNA and a normalization of plasma calcium (119). Another study demonstrated that in ER-knockout mice duodenal TRPV6 mRNA expression is lower than in wild-type mice and that it can be increased by estrogen treatment (120). TRPV6 regulation by androgens was shown first in a study of Peng et al., in prostate adenocarcinoma cell lines. The TRPV6 mRNA level was decreased by androgen and induced by an androgen receptor antagonist in LNCaP prostate cancer cells (121). Recent results of Lehen'kyi et al., suggest also, that TRPV6 expression in LNCaP cells is regulated by the androgen receptor (122).

2. Modulation of channel activity

The channel activity of TRPV6 can be modulated first by the intracellular calcium concentration. If the intracellular calcium level increases, a rapid inactivation of TRPV6 occurs (123). This rapid inactivation by transported calcium gets mediated over an activation of PLC and a subsequent depletion of PIP_2 (124). Secondly, the extracellular pH likely plays a role in activity modulation of TRPV6, because it was shown to be important for the calcium uptake through TRPV5 in Xenopus oocytes (125). The third factor was found by Nilius et al., when they demonstrated that influx of calcium and the intracellular calcium concentration induced feedback inhibition of the channel that is controlled by the intracellular calcium concentration in a micro domain close to the channel (126). Crucial for this process is a tyrosine T702 in the calmodulin binding site. Niemeyer et al., demonstrated that the phosphorylation of this tyrosine by PKC prevents calmodulin binding to the channel (127). Another group showed that inhibition of tyrosine phosphatase PTP1B increases TRPV6 activity (128) (Figure 4). WNK3 is a member of the With No Lysine (K) family of serine/threonine kinases. By co-expressing WNK3 and TRPV6 in Xenopus oocytes, it was found that WNK3 enhanced Ca^{2+}-influx and Na^{+}-current mediated by TRPV6 (129).

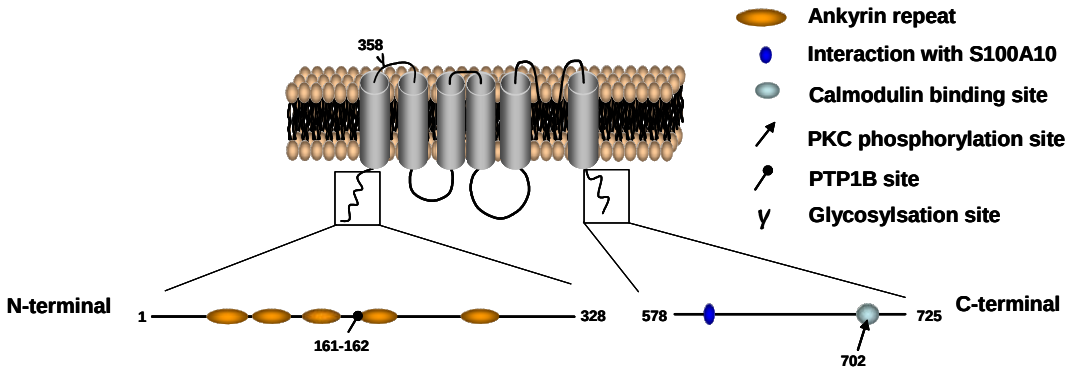


Figure 4. Structure of TRPV6 with special sites for activity modulation

3. Regulation through trafficking

TRPV6 expression at the cell surface is important for Ca^{2+} transport. As already noted, Klotho can hydrolyze extracellular sugar residues of TRPV5 resulting in cell

surface trapped TRPV5 (130). Lu et al., found that klotho and β-glucuronidase treatment also increases the activity of TRPV6 (131).

4. Regulation by associated proteins

S100A10/Annexin 2

S100A10 was found to be an auxiliarly protein of TRPV6 using the yeast two-hybrid system (132). It is predominatly complexed with annexin 2 that binds to actin to interact with the membrane-cytoskeleton (133). Van de Graf et al., showed that S100A10, annexin 2, and TRPV6 are co-expressed in the small intestine (134). The important motive for the association of S100A10 with TRPV6 is the five amino acid containing, highly conserved, sequence VATTV at the carboxy-terminus of the channel. Regarding the function of annexins, the S100A10/Annexin 2 complex may regulate the translocation of the TRPV6 channel into the plasma membrane.

Calmodulin

Calmodulin (CaM) binds to the carboxy-terminus of human TRPV6 in a calcium-dependent manner (135). When the calmodulin-binding site was removed from the carboxy-terminus of TRPV6, the channel had a significantly reduced slow-inactivation (136). Another piece of evidence was that HEK cells heterologously co-expressing calcium-insensitive CaM mutants with TRPV6 exhibited a significantly diminished calcium current (137).

Rab11a

Van de Graaf detected recently that Rab11a, a small GTPase important for trafficking, is associated with TRPV6 (138). TRPV6 co-localizes with Rab11a in the kidney in vesicular structures underlying the plasma membrane. The interaction was shown with a GST-pulldown assay and co-immunoprecipitation (139) and might indicate that Rab11a is important for TRPV6 trafficking to the membrane.

1.4.3 Diseases associated with the dysfunction of TRPV6

Vitamin D-deficiency rickets

Vitamin D-deficiency rickets type I (VDDR-I) is characterized by very low levels of vitamin D3 in the plasma that cause hypocalcemia, richets, osteomalacia, growth

retardation, and failure to thrive. The reason for the low vitamin D3 levels is a mutation in the 1alpha-hydroxylase gene (140). 1alpha-hydroxylase knockout mice exhibit lower levels of TRPV6 mRNA expression in the intestine than wild-type mice (141). It is very likely that channel abundance leads to impaired renal and intestinal calcium absorption and consequently to hypocalcemia in VDDR-I.

Hypercalciuria and kidney stone diseases

One characteristic of TRPV6 knockout mice is calciuria with bone abnormalities similar to clinical hypercalciuria that might be possibly connected to idiopathic hypercalciuria. Another hypothesis is that urine calcium levels correlate with TRPV6 gene mutations or polymorphisms. It is possible that hypercalciuria is one risk factor for kidney stone disease (142). It was also demonstrated that silencing mutations of TRPV5 and TRPV6 can hypothetically lead to primary renal as well as absorptive idiopathic hypercalciuria (143). Suzuki et al, found recently that the ancestral gain of function haplotype consisting of three non-synonymous polymorphisms in TRPV6 is important in calcium stone formation in hypercalciuria (144).

Osteoporosis

Estrogen deficiency after menopause results in a negative calcium balance that can induce osteoporosis (145). As mentioned before, estrogen regulates TRPV6 expression and TRPV6-knockout mice show certain symptoms of osteoporosis like reduced bone density (146). Therefore, there might be a linkage of TRPV6 and osteoporosis.

Cancer

Recent studies indicate that TRPV6 plays a role in certain cancers of epithelial origin. Schwarz et al., demonstrated 2006 that the heterologous expression of TRPV6 in HEK cells leads to increased cell proliferation (147). It was also found that cancer cell lines express higher amounts of TRPV6 than normal cells e.g. LNCaP and PC-3 prostate cancer cells, SW480 colorectal cancer cells, and K-562 chronic myelogenous leukaemia cell lines (148). Furthermore, Zhuang and colleagues detected that TRPV6 is higher expressed in tumor tissue compared to normal tissue of prostate, breast, colon, thyroid, and ovary (149) (Figure 5).

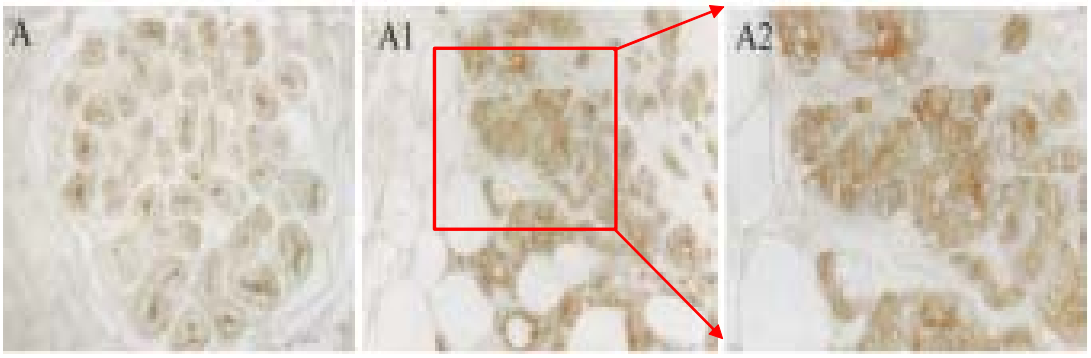


Figure 5. Immunohistochemical staining of CaT1 in human normal and tumor tissue.
A. Positive staining of the epithelial ductal cells in the normal human mammary gland
A1. and A2. Staining of mammary adenocarcinoma demonstrated a stronger positive reaction than its normal counterparts. Magnified views of expression patterns of human TRPV6 in A1 tumor tissues are shown in A2. (Original magnifications: A, A1,x200; A2,x400) (185).

Most studies published so far concentrate on TRPV6 in prostate cancer. As mentioned before, there is higher expression in cancer tissue compared to normal tissue (150). The grade of TRPV6 expression in prostate cancer correlates with the Gleason grade (151). The mechanistic details of the role of TRPV6 in prostate carcinogenesis were investigated by Lehen'kyi et al., 2007. They showed that knock-down of TRPV6 via siRNA led to decreased proliferation in LNCaP prostate cancer cells. Furthermore, they demonstrated that the calcium uptake in LNCaP cells is mediated by TRPV6 via a subsequent activation of nuclear factor of activated T-cell transcription factor (NFAT). They concluded that the up regulation of TRPV6 expression in prostate cancer serves as a mechanism to increase proliferation rate, cell survival, and apoptosis resistance (152). These findings might indicate that TRPV6 expression could be used as a marker of the clinical outcome of prostate cancer (153-155). Other known TRP channels that are highly expressed in cancer cells are TRPM8 and TRPM1. Their protein expression is changed from normal to tumorigenic stage. The TRPC1, TRPC6, TRPM5, and TRPV1 are also increased in cancer tissues (156-158). Certainly, some of the mentioned channels might be diagnostic markers and they might have also potential as drug targets.

1.5 Pathophysiology and therapeutic perspectives of breast carcinomas

In 2006, breast cancer had the highest incidence rate of all cancers in Europe. It was ranked third among all cancer deaths (159). Stella Kyriakides, the president of

Europa Donna, the European Breast Cancer Coalition stated that: “Within the European Union, every 2.5 minutes a woman is diagnosed with breast cancer. Every 7.5 minutes a woman dies from the disease” (160). For the development of additional therapies and new drugs, a better understanding of the physiology and pathophysiology of mammary gland is indispensable.

Breast cancer treatment

For the treatment of breast cancer four types of standard therapies are used. The first option for most patients with breast cancer is to have surgery to remove the tumor. Secondly, there is radiation therapy that applies high-energy x-rays to kill cancer cells. A third therapy option is the chemotherapy that uses drugs to stop the growth of cancer cells by killing them or stopping them from dividing. The forth treatment possibility is hormone therapy, which removes hormones or blocks their action and stops cancer cell proliferation. These therapies are often applied in combinations. There are also new types of treatments being tested in clinical trials: one is the sentinel lymph node biopsy followed by surgery. Another approach is to use high-dose chemotherapy with stem cell transplant that should replace blood-forming cells destroyed from the cancer treatment. The application of monoclonal antibodies as adjuvant therapy is also being investigated. The treatment is to administrate antibodies that can identify substances that are important for cancer cells and stop their growth. Trastuzumab (Herceptin) is a monoclonal antibody that blocks the effects of the growth factor protein HER2, which transmits growth signals to breast cancer cells. About one fourth of patients with breast cancer have tumors that may be treated with trastuzumab combined with chemotherapy. The administration of tyrosine kinase inhibitors might be also promising. They inhibit signals that the tumor needs to grow. Lapatinib is a tyrosine kinase inhibitor that blocks the impact of HER2 protein and other proteins inside tumor cells. It may be used to treat patients with HER2-positive breast cancer that has progressed following treatment with trastuzumab (161).

Hormone therapy

Exogenous estrogens and anti-estrogens are used for hormone-replacement therapy and as anti-cancer agents (162). There are three classes: the agonists, the mixed agonist-antagonists, and pure antagonists. The mixed agonist-antagonists are also

called selective estrogen receptor modulators (SERM). They exhibit a tissue specific activity (163). SERMs bind to the ER and change the biologic activity of the receptor (164). After discovery of the linkage between estrogen and breast cancer, the use of anti-estrogens as breast cancer drugs started. The first anti-estrogen, tamoxifen, was developed to inhibit the estrogen function. But tamoxifen is not a pure antagonist, it belongs to the SERMs because it works as antagonist in breast cancer cells, but functions as agonist in some other tissues like the liver. Tamoxifen is the standard hormonal therapy for breast cancer.

Tamoxifen

Tamoxifen is a lipophilic prodrug that can be easily absorbed in the intestine. In the blood, it is to 98% bound to albumin. In the gastrointestinal tract and in the liver it gets highly metabolized into the less active form N-desmethyl-tamoxifen and the two most active forms, 4-hydroxy-tamoxifen and endoxifen (165). The hydroxylated metabolites result from first pass metabolism in the liver and enter the blood stream over entero-hepatic circulation (166). 4-Hydoxy-tamoxifen and endoxifen have the same affinity for the ER as estrogen (167). 4-Hydroxy-tamoxifen possesses a higher ER-binding affinity than tamoxifen (168). Both metabolites can induce apoptotic cell death in ER-positive MCF-7 and ER-negative MDA-MB-231 and BT-20 breast cancer cells at micromolar concentrations (169). Tamoxifen binds to the two isoforms of the estrogen receptor ERα and ERβ with the same affinity (170). The tissue distribution and function of ERα and ERβ is variable, e.g. ERβ has an anti-proliferative effect in many tissues and ERα is more proliferative (171). But the interaction of SERMs with the ER is very complex and dynamic and until now not fully understood. It is known that tamoxifen can affect the intracellular Ca^{2+} homeostasis in cells. For example, tamoxifen inhibits voltage-gated Ca^{2+} current (L- and T-type) and contractility in vascular smooth muscle cells in rats (172). The effect of tamoxifen is concentration dependent. At nanomolar concentrations tamoxifen leads to growth arrest, whereas at µM concentrations cell death is induced in cell cultures. The effects at nM concentrations were largely reversible by addition of estrogen, but those at µM concentrations were not (173). Many clinical studies showed that steady state concentrations of tamoxifen can be up to 1µM and mean intra-tumor concentration are even higher, about 4µM (174). In ER-negative tumors,

the therapeutic efficacy of tamoxifen has been obtained at doses 4- to 8- fold above those used for ER-inhibition. The anti-tumor effect of tamoxifen is thus believed to be a combination of genomic (ER-mediated) and non-genomic (non-ER-mediated or other signaling pathways) mechanisms. The signaling proteins in the non-ER-mediated pathways include PKC, TGF-β, calmodulin, c-myc, ceramide, and MAP kinases (175).

1.6 Role of calcium in cancer biology

Calcium is important for many cellular processes, including the ones that are crucial for tumorigenesis, such as proliferation and apoptosis. Necessarily involved are calcium channels, pumps and exchangers, that are responsible for the fine tuning of the calcium homeostasis. The intracellular free calcium concentration is very important for proliferation and apoptosis and can be influenced by the calcium transport proteins. There is evidence that specific pumps or channels are up or down regulated in certain cancer types (176). One example for an up regulation is the increased expression of TRPM8 in prostate cancer (177;178). In contrast, SERCA3 is down regulated in tissue samples of colon cancer patients (179).

The extracellular free calcium is maintained at approximately 1.2mM, whereas the cytosolic calcium is approximately 100nM and can reach values of 1μM (180). Therefore, changes in free calcium concentration can be very potent and play a role in many processes in tumorigenesis. One of them is cellular motility. Another one is angiogenesis, where calcium is a key regulator of signaling pathways (181;182). In DNA damage response, calcium is important for genomic stability and cell survival, e.g. it can influence PARP1 activity (183). Transcription can be regulated directly via calcium oscillations that affect for example NFAT (nuclear factor of activated T-cells) (184). Interestingly, telomerase, that stabilizes the telomers, can inhibit the calcium effector S100A8 (185). Calcium signaling is also implicated in differentiation of cancer cells via the calcium sensing receptor (186). Excessive calcium accumulation in the mitochondria is one major characteristic for apoptosis and necrosis and is followed by mitochondrial membrane permeabilization (187). Finally, calcium is a key regulator of the cell cycle. It can increase proliferation by regulating the activity of ras

(188). Even direct modulation of cell-cycle is possible by calcium. It can activate transcription of genes important for Go-G1 transition and for the phosphorylation of retinoblastoma protein in late G1 (189) .

2. Hypothesis and Aim

There is almost no information available about the calcium entry mechanism and regulation of the intracellular calcium in breast cancer cells. Studies performed thus far have focused on the role of TRPV6 in prostate cancer. Therefore, I investigated how TRPV6 affects proliferation, apoptosis, and calcium transport in breast cancer cell lines. Additionally, the hormonal effects of estradiol, progesterone, and 1,25-vitamin D on TRPV6 expression were determined in T47D breast cancer cells. The apparent importance of calcium to breast tumor survival may lead to a therapeutic approach particularly apt for the treatment of breast tumors that are independent of estrogen and progesterone. These late stage tumors are highly metastatic and lethal and there is currently no effective treatment strategy. Understanding the relationship between calcium uptake induced through TRPV6 and the progression of breast tumor types might unveil TRPV6 as a novel target for anti-cancer drug development.

The second aim of my studies is to investigate the effect of the most common breast cancer treatment drug, tamoxifen, on TRPV6 mediated calcium uptake in breast cancer cells and to find the mechanism of regulation and the subsequent implication of this for intracellular calcium homeostasis. Tamoxifen at lower dose induces a cytostatic, anti-proliferative effect via ER (estrogen receptors). However, at higher dose it is cytotoxic which is not ER-dependent, but involving alteration of intracellular calcium homeostasis. This latter effect is probably responsible for the side effects (thrombosis=altering platelet functions) and for its effectiveness in ER-negative breast cancer cells. But the exact mechanism of how tamoxifen acts in cancer cells is not clarified thus far. Since the calcium channel TRPV6 shows increased expression in breast cancer, I hypothesized that it is also involved in tamoxifen mediated effects. Our findings might help clarify an additional possible mechanism of how tamoxifen may function in breast cancer therapy.

3. Results

3.1. “The role of TRPV6 in breast carcinogenesis” Bolanz KA, Hediger MA, Landowski CP. Mol Cancer Ther. 2008 Feb;7(2):271-9. Epub 2008 Feb 1.

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Abstract

TRPV6 is a calcium entry channel that is strongly expressed in breast adenocarcinoma tissue. In this study we further confirmed this observation by analysis of breast cancer tissues, which indicated that TRPV6 mRNA expression was upregulated between 2- and 15-fold compared to the average in normal breast tissue. While TRPV6 is known to be expressed in the cancer tissue, its role as a calcium channel in breast carcinogenesis is poorly understood. Therefore, we investigated how TRPV6 affects the viability, apoptosis, and calcium transport in the breast cancer cell line T47D. Hormones can also affect the tumor development; hence we determined the effects of estradiol, progesterone, and 1,25-vitamin D on TRPV6 transcription. Interestingly, the estrogen receptor antagonist tamoxifen reduced expression of TRPV6 and is able to inhibit its calcium transport activity (IC_{50}=7.5 µM). The *in vitro* model demonstrated that TRPV6 can be regulated by estrogen, progesterone, tamoxifen, and 1,25-vitamin D and has a large influence on breast cancer cell proliferation. Moreover, the effect of tamoxifen on cell viability was enhanced when TRPV6 expression was silenced with siRNA. TRPV6 may be a novel target for the development of calcium channel inhibitors to treat breast adenocarcinoma expressing TRPV6.

Introduction

Ionized calcium (Ca^{2+}) is a central signaling ion that is critical for controlling growth, proliferation, and survival of normal and malignant cells. In cellular processes, calcium is a key modulator of numerous enzymes located in the cytosol, organelles, and nucleus. Ca^{2+} cannot be degraded like other second messenger signals. Therefore, the intracellular calcium levels are tightly regulated on multiple levels by plasma membrane ion channels, ion exchangers and pumps, as well as by the release of calcium from the endoplasmic reticulum and nuclear envelope (1, 2).

One such plasma membrane channel is the TRPV6 calcium entry channel. TRPV6 is a Ca^{2+} permeable ion channel that was identified using expression cloning by Hediger and colleagues in 1999 (3). TRPV6 plays a central role in total body calcium homeostasis and its regulation directly affects intestinal calcium absorption, renal calcium excretion, and bone metabolism (4). TRPV6 appears to play a major role in facilitating the entry of Ca^{2+} into absorptive epithelial cells. This is evident in TRPV6 knockout (KO) mice, which exhibit defective intestinal Ca^{2+} absorption, increased urinary Ca^{2+} excretion, and decreased bone mineral density. Regulation of TRPV6 is controlled by 1,25-vitamin D, estrogen, and dihydrotestosterone (5-7).

While TRPV6 functions to maintain normal calcium homeostatsis, it also appears to play a role in tumor development and progression. TRPV6 was observed to be upregulated in tissue samples originating from prostate, breast, thyroid, colon, ovary, and pancreatic tumors (8). The channel has been localized to the apical membrane where it delivers calcium into the cells. Furthermore, it has been shown that TRPV6 is most strongly expressed in advanced stages of prostate cancer, while there is little to no expression evident in healthy tissue and benign prostate hyperplasia (7, 9). The transcript levels in both studies correlated positively with tumor progression and aggressiveness as indicated by the pathological stage and Gleason scores of the prostate tumors. Endogenous store operated channels play important roles in the apoptosis of LNCaP prostate cancer cells (10, 11). Numerous studies have linked enhanced endoplasmic reticulum (ER) Ca^{2+} accumulation to proliferation and/or apoptosis in prostate cancer (12-14). A study by Schwarz et al., (2006)

demonstrated that TRPV6 clearly increases the rate of Ca^{2+} dependent cell proliferation in HEK cells (15).

In mammary adenocarcinoma tissue, immunohistological analysis showed a clear enhancement in TRPV6 expression over normal tissue, suggesting it may play some role in the tumor development (8). Data from epidemiological studies suggest that higher intake of dietary calcium, which lowers 1,25-vitamin D levels in the blood, reduces the breast cancer risk in premenopausal women (16, 17). Calcium also appears important to breast cancer progression, since bone metastases occur in up to 70% of patients with advanced breast cancer (18). The metastatic cells at the bone are thus exposed to increased levels of free extracellular Ca^{2+} released from the mineralized bone matrix.

The most common hormonal treatment for hormone receptor positive breast adenocarcinoma is the selective estrogen receptor modulator tamoxifen. It blocks estrogens from signaling through the estrogen receptor (ER) and reduces the growth signals to the cells. The activity of tamoxifen can be observed best in breast tumors that express ER and/or progesterone receptor (PR). The highest response rates are observed in tumors expressing both ER and PR (70%), with lower response rates in ER negative/PR positive tumors (45%), and ER positive/PR negative tumors (34%) (19, 20). The lowest tamoxifen response rates are found in ER negative/PR negative tumors (<10%). However, a portion of the supposed non-responders may actually be attributable to false negative assay data (21, 22).

Little is known about the calcium entry mechanisms by which intracellular calcium is regulated in breast cancer cells. Therefore, in this study we investigated how TRPV6 affects proliferation, apoptosis, and calcium transport in the breast cancer cell line T47D. Additionally, the hormonal effects of estradiol, progesterone, and 1,25-vitamin D on TRPV6 expression were determined in the breast cancer cells. The apparent importance of calcium to breast tumor survival may lead to a therapeutic approach particularly apt for the treatment of breast tumors that are independent of estrogen and progesterone. These late stage tumors are highly metastatic and lethal and there is currently no effective treatment strategy. Understanding the relationship between calcium uptake induced through TRPV6 and the progression of breast tumor types might unveil TRPV6 as a novel target for anti-cancer drug development.

Materials and methods

Materials

T47D cells were obtained from NIH Culture Collection. Plastic 6-well culture dishes, 96-well culture dishes, and T75 cultivation flasks were obtained from BD Falcon. RPMI-1640 cell culture medium, fetal bovine serum (FBS), trypsin, and penicillin/streptomycin were from Gibco. Trizol reagent for RNA isolation was purchased from Invitrogen. TaqMan Universal Master Mix for real time PCR was from Applied Biosystems. siRNAs and HiPerFect transfection reagent were obtained from Qiagen. 1α,25-dihydroxyvitamin D3, β-estradiol, and progesterone were purchased from Sigma. Tamoxifen was obtained from Acros Organics. Human Breast Cancer Rapid-Scan Gene cDNA Panel was purchased from Origene.

Cell culture

A human breast cancer cell line T47D was used in this study. Tumor cells were grown in T75 culture flasks in RPMI medium supplemented with 10% FBS and antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin) in a humidified atmosphere with 5% CO_2. Cells used in these studies were between passages 30 to 40. The cells were split twice a week.

siRNA treatment

For TRPV6 knockdown the following siRNAs were used:
siRNA1 (target sequence): CTG CAT GTC AGA GCA CTT TAA
siRNA2 (target sequence): AAC CTG CTG CAG CAG AAG AGG
siRNA control: AAT CAT CTA AGC TGG CTT TGC
The cells were seeded in 2 ml of culture medium at 400,000 cells per well in 6-well plates. After 24 h, siRNA was diluted in phenol red free medium without FBS to give a final siRNA concentration of 5 nM (20 µM stock). Then, HiPerFect was added according to the manufacturer's protocol and the mixture was incubated for 10 min at room temperature and added drop-wise onto cells. Cells were incubated with siRNAs for 72 h before isolating RNA using the Trizol RNA isolation method according to manufacturer's protocol.

Hormonal treatments

For hormone treatment, T47D cells were seeded at 400,000 cells per well in 6-well dishes and grown for 48 h. Cells were serum-starved for 24 h prior to treatment. Then, cells were treated in the above mentioned culture medium without FBS for the duration of the studies. After pilot studies to determine appropriate concentrations, 100 nM 1,25-dihydroxyvitamin D (100 µM stock), 10 nM estradiol (10 µM stock), 100 nM progesterone (100 µM stock), and 1 µM tamoxifen (1 mM stock) were all dissolved in ethanol and applied to the cells, along with ethanol only controls, for 24 to 72 h. Dose response studies during a 24 h treatment time were performed with estradiol and 1,25-dihydroxyvitamin D. Total RNA was harvested after 24, 48, and 72 h of treatment. Three independent experiments were performed with every treatment in triplicate. TRPV6 mRNA expression was determined by real time PCR.

Real time PCR

cDNA was prepared for every sample by reverse transcription of total RNA using TaqMan Reverse Transcription Reagents (Applied Biosystems) according to the manufacturer's manual. For all experiments mRNA expression was measured by real time PCR using an Applied Biosystems 7500 Real Time PCR System. Reactions consisted of 1x Mastermix, 0.9 µM forward and reverse primers, and 0.2 µM dual-labeled fluorescent probes each for TRPV6 and β-actin. The sequences of the forward and reverse primers for TRPV6 were 5′-GGT TCC TGC GGG TGG AA-3′ and 5′-CCT GTG CGT AGC GTT GGA T-3′ respectively, with the resulting amplicon being 62 bp with a T_m of 60°C. The sequence of the probe for TRPV6 was 5′-ACA GGC AAG ATC TCA ACC GGC AGC-3′. The sequences of the forward and reverse primers for β-actin were 5′-CCT GGC ACC CAG CAC AAT-3′ and 5′-GCC GAT CCA CAC GGA ATA CT-3′ respectively, with the resulting amplicon being 69 bp with a T_m of 60°C. The sequence of the probe for β-actin was 5′-ATC AAG ATC ATT GCT CCT CCT GAG CGC-3′. The specificity of all primers was confirmed by BLAST search. Primer Express (Applied Biosystems, Foster City, CA) was used for designing primers for TRPV6 and β-actin. All primers were designed to cross exon-exon boundaries of the coding sequence. Primers were optimized and validated for the comparative Ct method, as described in the manufacturer's manual. ABI Prism SDS software version 1 was used for the analysis of the amplification plots. The TRPV6

expression values were normalized to β-actin and then compared. The results are shown as fold change ± SD.

Cell viability and apoptosis

Cell viability experiments were carried out in 96-well culture plates with an initial cell number of 2500 cells per well to determine the influence of different hormones on cell growth. To determine the effect of siRNA on cell viability, the cells were seeded at 5000 cells per well. After the cells were plated for 24 h, siRNA treatment was done for 72 h with 5 nM siRNA. Prior to optical density measurements, plates were kept continuously in cling film during incubation and the wells on the outer edge were not used for measurements. After incubation time, the number of living cells was determined by the Cell Proliferation Kit II (XTT) (Roche) following the manufacturer's instructions. Briefly, 50 µl of XTT labeling mixture (including the electron-coupling reagent, N-methyl dibenzopyrazine methyl sulfate) was added to each well and plates were incubated for 4 h. The absorbance was measured by an ELISA reader (Vmax microplate reader, Molecular Devices) at 450 nm with a reference wavelength at 650 nm.

Apo-one homogenous caspase-3/7 assay (Promega, Madison, WI) was done according to manufacture's protocol. Cells and siRNAs were prepared as described above with the viability assay. The profluorescent substrate was added to the cells for 3 hours before measuring the caspase activity on the Flexstation II (485 nm excitation and 527 nm emission). The percentage of apoptotic cells was calculated relative to the untreated control cells.

Ca^{2+} uptake activity measurement

5000 cells per well were seeded in a black 96-well plate with 50 µl culture medium per well. After cells have been plated for 24 h, siRNA treatment was performed with 5 nM siRNA as described previously. Subsequently, after 72 h the calcium uptake activity was observed in real-time using the FLIPR calcium 3 assay kit (Molecular Devices) according to the manufacturer's manual. Briefly, 50 µl of calcium free loading buffer was added to the cell medium. Then 100 µl of calcium 3 dye was

added and incubated with the cells for 1 h at 37°C. The plate was put into the Flexstation II, EGTA (5 mM final) was added to the cells to deplete intracellular calcium stores and finally calcium solution (10 mM final) was added to the cells to observe calcium entry. Fluorescence units (RFU) per second was calculated from the slope of the uptake curve to show the calcium uptake activity after siRNA treatment. Calcium uptake activity mediated by TRPV6 was also performed in Xenopus oocytes using $^{45}Ca^{2+}$, as done previously (3).

Reverse transcription PCR

The Promega Access RT-PCR System was used according to the manufacturer's instructions for detection of calcium channel expression in T47D cells. After an initial heating step at 45°C for 45 min, the reactions were heated for 2 min at 94°C as a denaturing step. Subsequently, the cycle included a 30 second denaturing step, annealing at 60°C for 15 seconds, and extension reaction for 30 seconds at 68°C. 24 cycles resulted in optimal linear amplification of products. A final extension was done for 5 minutes at 68°C. Samples were stored at -20°C before electrophoresis on a 1% agarose gel.

The following primers were used:

TRPC1A:

forward 5′-TCTGCCCAAAGGCCATTG-3′

reverse 5′-GGGTATACTACTCTCCTCCATATTTTCTTC-3′

TRPC3:

forward 5′-CGGCCGCACGACTATTTC-3′

reverse 5′-CCAGCCCCTTGTAGGCATT-3′

TRPC4:

forward 5′-TGGCATGAAATATGGCTCAGTT-3′

reverse 5′-CGAGAGTTCTGATTCTGCTCTTACTATC -3′

TRPC5:

forward 5′-TGAGTTCAAGGCCGAGTATGAG-3′

reverse 5′-TCTCGATGGTTGAGGATGATCTC-3′

TRPC6:

forward 5′-TTCTCCCATGATGTGACTCCAA-3′

reverse 5′-GAGGCCGTTCAATCCTAGCA-3′

TRPC7:

forward 5′-GGGCATGCTGAATTCCAAA-3′

reverse 5′-TCTGGTGGGCTTGCTCAAAG-3′

TRPV5:

forward 5′-GCATTGTCAACTTCGCCTTC-3′

reverse 5′-GATCATTGTGGTTCTCAACC-3′

TRPV6:

forward 5′-CCTGTGCGTAGCGTTGGAT-3′

reverse 5′-GGTTCCTGCGGGTGGAA-3′

Statistical data analysis

Statistical significances between treatments and controls were analyzed with Student's t-test using GraphPad Prism 3.0. Differences in the comparison tests lower than $p < 0.01$ were considered significant.

Results

In vitro TRPV6 knockdown

The effect of TRPV6 knockdown on TRPV6 mRNA expression in T47D breast cancer cells was determined via real time PCR (Figure 1A). The TRPV6 messenger RNA level was significantly reduced in T47D cells after 72 hours by two different TRPV6 specific siRNA molecules. The most effective siRNA molecule was siRNA2, which reduced the TRPV6 message by 47%. The siRNA1 had nearly the same effect on TRPV6 expression, subsequently reducing the mRNA by 40%. The control siRNA did not significantly change TRPV6 expression and was comparable to untreated controls.

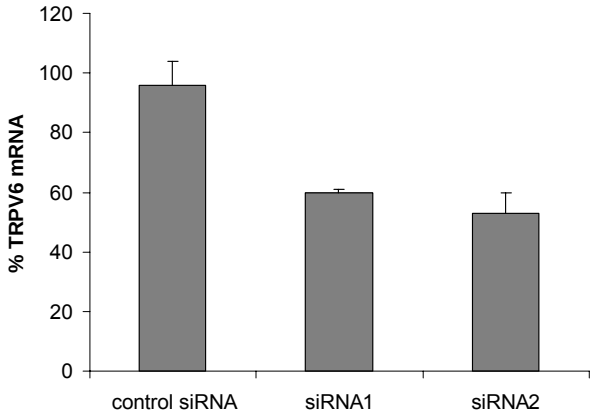

Figure 1. A, TRPV6 expression knockdown in T47D breast cancer cells after 72 hour treatment with two specific siRNAs (5 nM) and one siRNA control (5 nM), compared to one untreated control. TRPV6 was measured by real time PCR and standardized via β-actin.

After the treatment of T47D cells for 72 hours in 96-well plates with both siRNAs, the XTT viability assay was performed. Figure 1B shows the percentage of growth reduction, measured as viable cells, of siRNA treated cells compared to control treated ones. The knockdown of TRPV6 led to a decrease in cell proliferation compared to siRNA treated controls, to 62% and 67%, respectively. There was no effect of the control siRNA treatment on the growth of the T47D cells. The anti-estrogen tamoxifen (10 μM) comparatively reduced the number of viable cells to 34%, compared to vehicle only treated cells. Dosing the cells with a combination of tamoxifen (10 μM) and siRNA1 (5 nM), in the presence of serum, diminished the

number of viable cells even further (19%), demonstrating more effectiveness than either treatment alone.

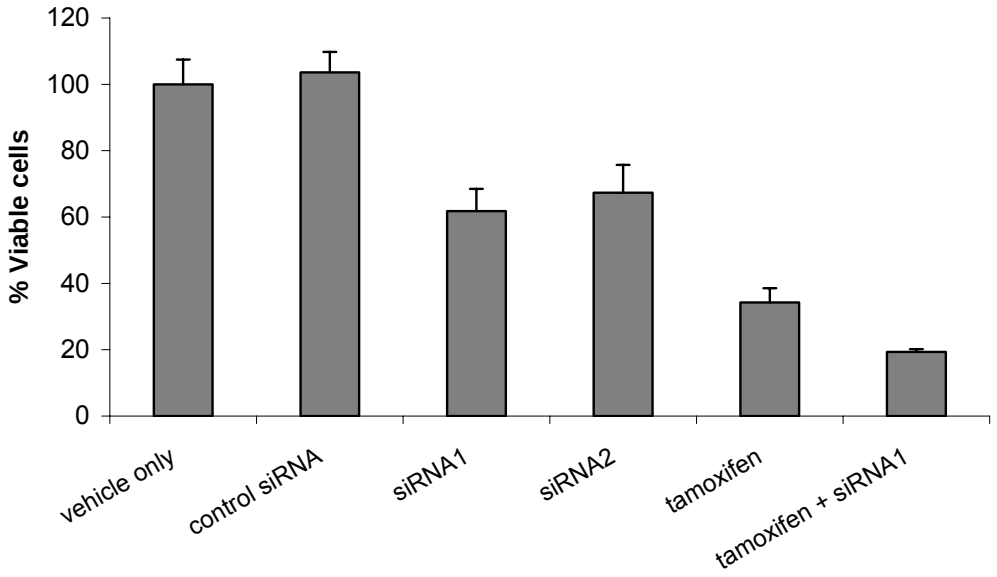

Figure 1. B, TRPV6 expression knockdown effect on T47D breast cancer cell viability. Determination was performed with XTT viability assay 72 hours after siRNA (5 nM), tamoxifen (10 μM), or a combination of siRNA (5 nM) and tamoxifen (10 μM) treatment compared to siRNA control or vehicle only control**.** All treatments were done in presence of serum.

The treatment of T47D cells with siRNA did not induce a large amount of apoptosis, as indicated by caspase-3/7 assay (Figure 1C). Knocking down TRPV6 expression slightly increased the amount of apoptotic cells by 7%, compared to control siRNA. Both siRNAs had similar effects on apoptosis. The treatment of cells with control siRNA produced a minimal increase in apoptotic cells, 4% more than the untreated control.

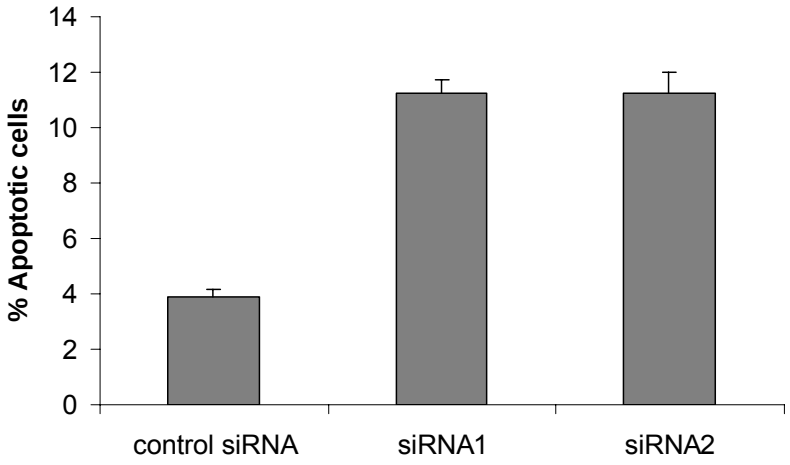

Figure 1. C, Apoptosis assay after treatment of T47D breast cancer cells with siRNAs (5 nM) for 72 hours. Apoptosis was measure by caspase-3/7 assay. *p< 0.01, significant difference between untreated cells and control siRNA; **p<0.01, significant differences between control siRNA and TRPV6 siRNAs (two-tailed t-test).

The reduced calcium channel activity after TRPV6 knockdown demonstrates that siRNA treatment affects the function of the channel and illustrates the importance of the channel in this cell line. The knockdown led to a decrease in calcium uptake activity to 47% for siRNA1 and 48% for siRNA2, compared to control. The uptake activity was measured in real-time and displayed in fluorescent units/sec (Figure 1D).

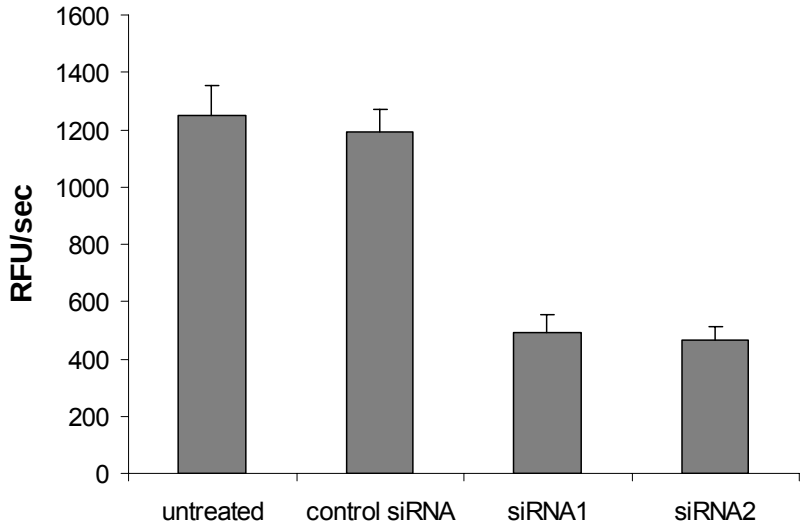

Figure 1. D, Calcium uptake activity measured by fluorescence-based FLIPR calcium 3 assay after 72 hours of TRPV6 knockdown with siRNAs (5 nM). Calcium uptake activity is shown in fluorescent

units/sec measured as the slope of the uptake rates. *p< 0.01, significant differences between control siRNA and TRPV6 siRNAs (two-tailed t-test). Columns, mean; bars, SD; N=3.

TRPV6 expression in breast cancer

To investigate the levels of TRPV6 expression in breast cancer tissues, a commercial cDNA panel was used to quantitate expression by real time PCR. Twelve cDNA samples from breast cancer tissue and eleven samples from normal mammary tissue were included in the analysis. The majority of samples originated from invasive ductal carcinoma tissue (N=9), while sample #3 (invasive lobular carcinoma), sample #5 (invasive mixed tubular carcinoma), and sample #9 (adenoid cystic carcinoma) were the only exceptions. The TRPV6 expression from the 11 normal mammary tissues was averaged and used to compare expression from individual carcinoma tissue samples. Thus, the data in figure 2 is represented as fold difference over normal. From this analysis TRPV6 mRNA expression in 7 of 12 patients was detected to be up-regulated between 2- and 15-fold over the average in normal breast tissue (Figure 2). In 8 samples, TRPV6 transcript levels were upregulated on average 4.6-fold compared to the normal tissue average. Further, the coefficient of variance (CV) of TRPV6 expression in normal breast tissue was 8% (N=11). TRPV6 expression in the breast cancer samples was over twice as variable as in normal tissue, showing a CV of 17% (N=12).

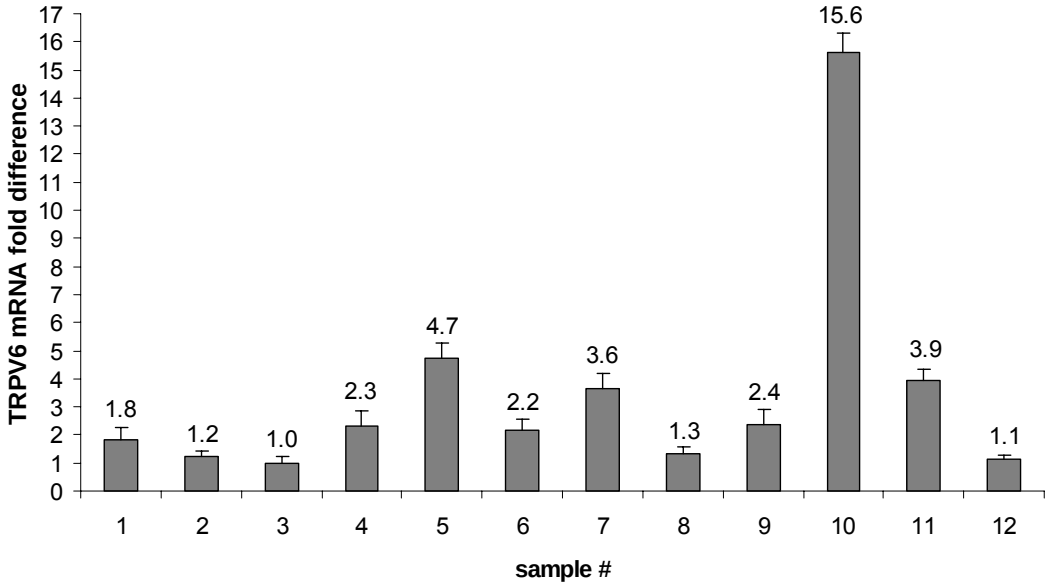

Figure 2. TRPV6 mRNA expression in 12 breast cancer tissue samples compared to the average of 11 normal samples based on real time PCR. TRPV6 was measured by real time PCR and standardized via β-actin. The data is represented as fold difference compared to the average expression from 11 normal breast tissue samples. Columns, mean; bars, SD; N=6.

In vitro hormone effects on TRPV6 expression

The most common treatments for breast tumors are hormonal treatments that act on the estrogen receptor to control breast cancer cell growth. Therefore, we investigated whether estrogen and progesterone could regulate the expression of TRPV6. The influence of these two hormones and controls on TRPV6 mRNA expression was determined via real-time PCR. T47D cells were treated with different concentrations of estradiol, progesterone, and 1α,25-dihydroxyvitamin D3. Additionally tamoxifen, an estradiol receptor antagonist which is used in breast cancer therapy, and 5-fluorouracil, a compound used in several cancer therapies, were assessed for their affects on TRPV6 transcription regulation.

Dose and time experiments were done to determine adequate concentration and treatment times. Figure 3 shows the TRPV6 mRNA expression compared to vehicle treated controls after 24, 48, and 72 hour treatments with estradiol (10 nM), progesterone (100 nM), a combination of both estradiol and progesterone, 1α,25-dihydroxyvitamin D (100 nM), tamoxifen (1 μM), and 5-fluorouracil (1 μM). After 24 hours the effect of progesterone on TRPV6 expression was only slightly different compared to vehicle treated control. A time dependent increase in expression was seen with estradiol after 24 hours, wherein after 72 hours the TRPV6 mRNA increased 69%. Treatment with progesterone also had a mild effect on increasing TRPV6 expression up to 56% after 72 hours. Both hormones applied together stimulated induction of TRPV6 levels 96% over control when cells were treated for 72 hours.

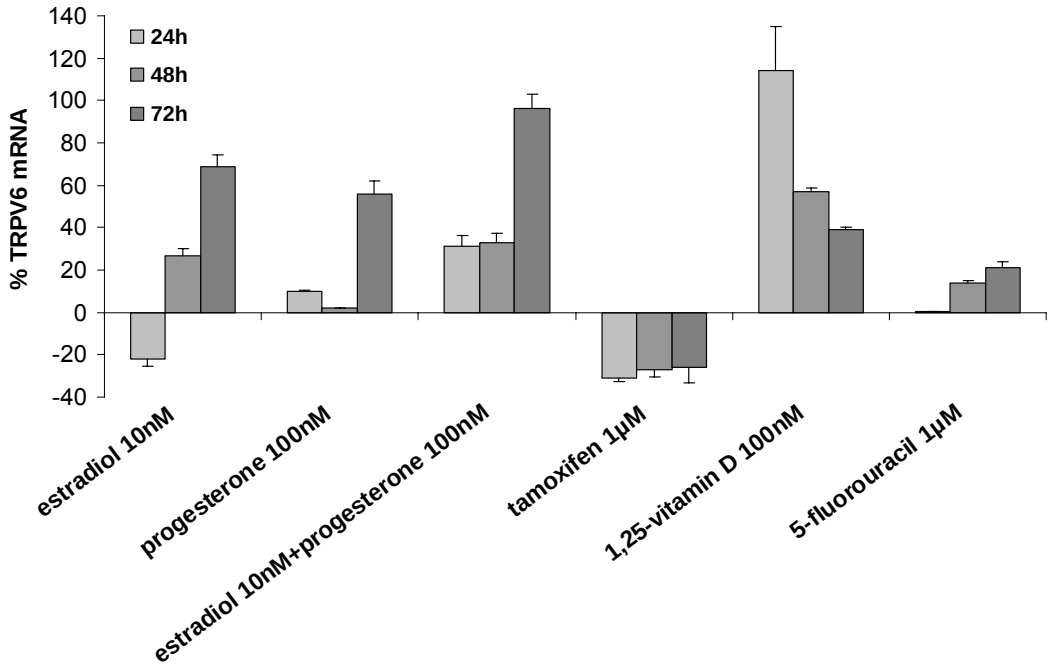

Figure 3. TRPV6 mRNA expression changes after treatment with estradiol (10 nM), progesterone (100 nM), both estradiol and progesterone, 1α,25 dihydroxyvitamin D (100 nM), tamoxifen (1 μM), and 5-flurouracil for 24, 48, and 72 hours as determined by real time PCR. Cells were starved of serum 24 hours prior to treatment and kept without serum for the duration of the study. Data is percent change compared to vehicle only treated cells. Columns, mean; bars, SD; N=3.

The estrogen receptor antagonist tamoxifen inversely affected TRPV6 expression at all time points tested. Tamoxifen moderately reduced the TRPV6 mRNA by 31% at 24 hours and was slightly less effective after 48 and 72 hours when used at 1 μM. A log dose response study was performed with 24 hours of treatment time from which it was evident that reducing the tamoxifen concentration lessens the effect on TRPV6 mRNA (Figure 4A). Dosing the cells with 10 μM tamoxifen reduced TRPV6 mRNA by 35%. The highest dose tried with tamoxifen was 50 μM; however, the cells became apoptotic. At 100 nM, tamoxifen only slightly reduced (12%) the TRPV6 expression level.

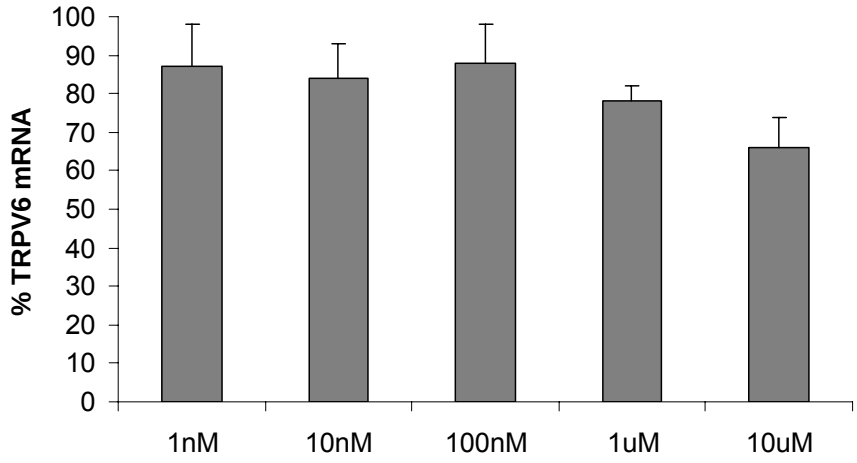

Figure 4. A, Dose dependent effect of tamoxifen (100 nM, 10 nM, 1 nM, and 100 pM) on TRPV6 mRNA expression in T47D cells after 24 hours determined via real-time PCR. Columns, mean; bars, SD; N=3.

The hormone 1α,25-dihydroxyvitamin D (100 nM) was on its own far more potent at stimulating TRPV6 mRNA expression (114%) when the T47D cells were treated for 24 hours (Figure 4B). However, the stimulatory effect seen reduced over time and lead to only 39% induction after a 72 hour treatment. A dose dependent relationship can be seen during a 24 hour treatment as seen in figure 4B. With log reductions of 1α,25-dihydroxyvitamin D concentration, the stimulation effect nearly disappears at 100 pM (14%).

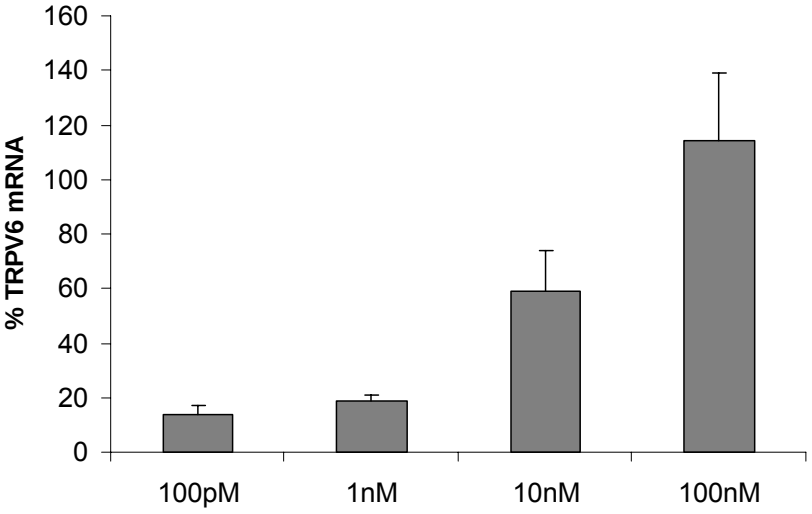

Figure 4. B, Dose dependent effect of 1α,25 dihydroxyvitamin D (100 nM, 10 nM, 1 nM, and 100 pM) on TRPV6 mRNA expression in T47D cells after 24 hours determined via real time PCR. Data shown is percent change in TRPV6 mRNA compared to vehicle only control.

As a control, 5-fluorouracil was used as another anticancer compound with an estrogen receptor independent mechanism (Figure 3). After 24 hours with 5-fluorouracil (1 μM), the treatment had no effect on TRPV6 mRNA level. There was a slight upregulation, 14 and 21%, after 48 and 72 hours.

Tamoxifen effect on TRPV6 calcium transport activity

TRPV6 expressing oocytes were used to probe the inhibition effect of tamoxifen on calcium transport. The drug showed a dose responsive blocking ability on TRPV6 mediated $^{45}Ca^{2+}$ uptake into oocytes (Figure 5). Tamoxifen inhibits the channel with an IC_{50} value of 7.5 μM, which suggests that it may have an additionally beneficial mechanism to slow the growth of breast cancer cells. The IC_{50} value was determined using nonlinear data fitting (GraphPad Prism version 3.0).

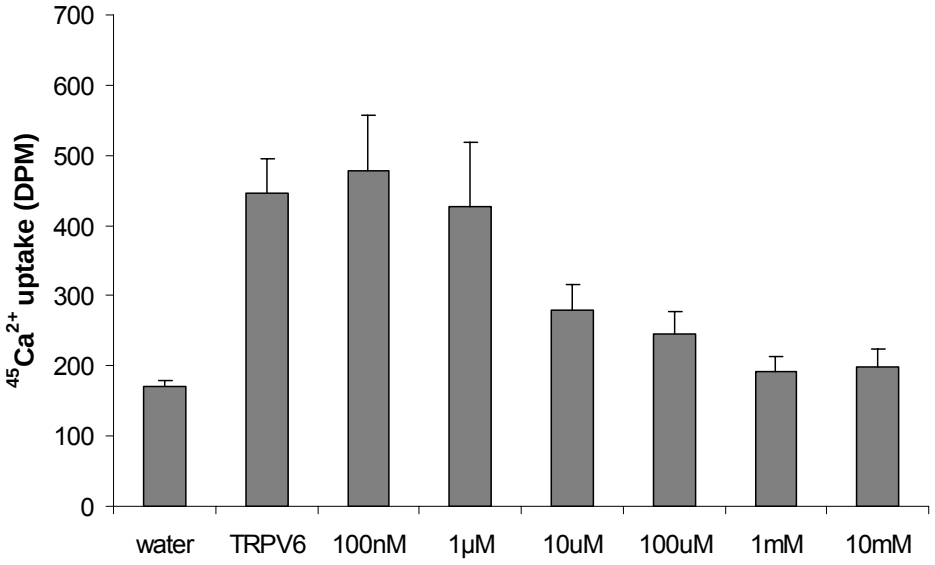

Figure 5. Tamoxifen inhibits TRPV6 calcium transport activity of $^{45}Ca^{2+}$ into Xenopus oocytes expressing TRPV6. Log dilutions of tamoxifen were used to inhibit TRPV6 mediated calcium uptake into oocytes. Columns, mean; bars, SD; N=6.

Calcium channel expression in T47D cells

To investigate the mRNA predominance of TRPV6 in T47D breast cancer cells, the expression of it and other calcium channels in this cell line were determined with

reverse transcription PCR and gel electrophoresis. Figure 6 shows the relative expression levels of the calcium channels: TRPC1, TRPC3, TRPC4, TRPC5, TRPC6, TRPC7, TRPV5, and TRPV6 in T47D cells. From this analysis TRPC4, TRPC6, and TRPV6 are the most predominately expressed calcium channels on the RNA level.

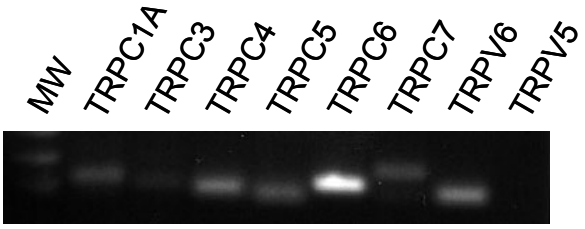

Figure 6. Relative RNA expression levels of calcium channels as determined by semiquantitative RT-PCR. MW (lane 1), TRPC1A (lane 2), TRPC3 (lane 3), TRPC4 (lane 4), TRPC5 (lane 5), TRPC6 (lane 6), TRPC7 (lane 7), TRPV6 (lane 8), and TRPV5 (lane 9) in T47D cells. Amplicons range in size from 100-150 bp.

Discussion

The TRPV6 calcium entry channel has previously been shown to be linked to prostate tumor progression and development. The role of TRPV6 in the progression of tumor cells is not completely clear, but appears to involve accumulation of cellular calcium to promote cancer cell growth or survival.

Previously, it has been demonstrated that TRPV6 appeared to be upregulated on the protein level in breast adenocarcinoma tissue (8). In this report we further investigated the expression level of TRPV6 in breast cancer tissue from patients as compared to normal mammary tissue. In the 12 patient samples analyzed by real time PCR, 8 samples had TRPV6 transcript levels that were upregulated on average 4.6-fold compared to the normal tissue average. This further strengthens our earlier observation where we seen upregulation on the protein level (8). There are associated ER and PR values for each cancer tissue sample (data not shown), but we were unable to find any clear, statistically significant correlation between these values and TRPV6 expression.

We investigated this relationship under more controlled conditions to see how estrogen receptor may regulate TRPV6 in breast cancer cells in vitro. When stimulated with estrogen for 24 hours, the RNA transcript of TRPV6 was slightly reduced as compared to vehicle only treated cells. Likewise, when prostate cancer cells were treated with dihydrotestosterone for a similar period of time, the transcription of TRPV6 also decreased (7). In contrast, when activated longer with estrogen from 48 to 72 hours, the cells increased the transcription of TRPV6 up to 69%. It has long been known that estrogen promotes T47D cell proliferation (23, 24). Long term upregulation of TRPV6 appears to be part of this cell growth mechanism. The anti-estrogen, tamoxifen showed interesting activity upon TRPV6 expression at all time points tested. Blocking the estrogen receptor with tamoxifen caused the TRPV6 transcript levels to reduce. The limited signaling that can occur from ER seems to lead to lower TRPV6 expression. And with increasing concentrations of tamoxifen, this effect becomes even more pronounced. These observations suggest that the ER is able to regulate TRPV6 expression to possibly promote increased calcium entry into the cells. When used at low concentrations (0.1-1 μM) tamoxifen

has been shown to induce G1 cell cycle arrest in T47D cells, thereby stopping cell proliferation (23, 25). Correspondingly the T74D cells reduced TRPV6 expression, which acts negatively to inhibit the cell growth. Larger doses of tamoxifen over 5 μM have been reported to induce apoptosis in T47D cells (26). In our studies we have observed that the activity of tamoxifen was enhanced by knocking down TRPV6 expression. We would expect a stronger enhancement if the expression was further knocked down. However, affecting the channel activity may be done more effectively through designing TRPV6 specific chemical inhibitors. These studies demonstrate that a combination therapy using tamoxifen and a TRPV6 inhibitor would be a beneficial approach to treating breast cancer cells.

Estrogen is known to induce increased calcium absorption via upregulating TRPV6 in female mouse intestine via a vitamin D independent mechanism (6). During pregnancy the increased calcium absorption is needed to compensate for increased nutritional demands. Likewise, 1,25-vitamin D is also a known TRPV6 stimulator as shown in this study and previous studies (27). At low concentrations (0.05 nM-0.8 nM) vitamin D promotes growth (28), whereas increasingly higher concentrations cause cell cycle block in G1 phase and subsequent apoptosis (29) (30). In our experiments, we see that TRPV6 expression reduces significantly after 24 hours after 1,25-vitamin D (100 nM) treatment. Thus, this effect seems to be in coordination with the T47D cell reducing its proliferation.

Additionally, we observed that tamoxifen can not only downregulate TRPV6 mRNA expression, but it can directly inhibit the calcium channel activity. We observed this affect when we expressed TRPV6 in Xenopus oocytes, giving an IC_{50} value of near 7.5 μM. This is intriguing given the fact that it is an estrogen receptor inhibitor. However, it does share several structural similarities with known T-type calcium channel inhibitors such as a tertiary nitrogen, aromatic groups, and an ether bond. In support of this, it can be found in the literature that both tamoxifen and raloxifene are able to affect calcium channel activity. In whole cell patch clamp studies in vascular smooth muscle cells, tamoxifen reduced the current through L-type calcium channels with an ID_{50} of 2 μM and through T-type channels 10 μM (31). The anti-estrogen SERM, raloxifene, is also known to affect voltage sensitive L-type calcium channel activity (32) (33, 34).

Tamoxifen is also known to be effective in ~10% ER and PR negative patients (19, 20). This might suggest that tamoxifen is directly acting on the TRPV6 calcium channel in those patients. Therapeutic concentration of tamoxifen in the breast cancer tissue can reach low μM values (35). Therefore, IC_{50} concentration for TRPV6 inhibition measured in oocytes is in a similar range to what has been seen in tumor tissues, and thus may be clinically relevant in the affect of the drug. Regardless, it would be interesting to collect data on TRPV6 expression along with receptor values when assessing the effectiveness of tamoxifen in patients. More specific TRPV6 inhibitors could be employed to regulate the growth of breast tumor cells expressing TRPV6.

In the T47D breast cancer cells, TRPV6 is one of several potential calcium entry channels expressed, but TRPV6 is the only calcium specific entry channel present. The importance of TRPV6 could be seen after siRNA knockdown studies, since the proliferation rate of the cancer cells was reduced by 60% and calcium transport was reduced by 50%. Importantly, the siRNA knockdown of TRPV6 was not complete in these experiments, so the full influence in these cells could not be fully assessed. There are two other potential calcium permeable channels, TRPC4 and TRPC6, which could also be contributing to the calcium uptake into these cells. However, these channels are receptor-operated, nonselective cation channels (36). Specifically, TRPC6 behaves as a nonselective cation channel that is activated by diacylglycerol in a membrane-delimited fashion, independently of protein kinase C (37). The TRPC6 channel is not considered to be store-operated and only has limited Ca^{2+} permeability relative to monovalent cation permeability (38). We cannot rule out their contributions at this time, but our results indicate that TRPV6 has a major influence on cellular calcium entry.

The breast cancer cells used in this study were slowed by reduced calcium channel expression and lower calcium uptake potential. The stable expression of TRPV6 in the kidney cell line HEK-293 promoted increased cellular proliferation and calcium accumulation (15). Clearly, the calcium state inside cells can influence their proliferation rate. Furthermore, previous studies in prostate cancer cell lines indicate that TRPV6 plays a significant role in prostate cancer calcium influx (39, 40).

In T47D cancer cells, TRPV6 also contributes a rather significant portion of the calcium entry activity and leads to a large reduction (~60%) in the proliferative ability of the cells. Further, it was found that treatment with TRPV6 specific siRNAs produced a small increase (~7%) in apoptosis in the breast cancer cells, as indicated by a caspase-3/7 assay. Therefore, the net result indicates that the cancer cells are growing less rapidly, rather than dying from reduced calcium uptake. Silencing TRPV6 in the breast cancer cells may be causing cell cycle arrest, which may explain the less rapid cell proliferation. We have not provided direct evidence for this, but it has been shown that TRPV6 silencing in the prostate cancer cell line, LNCaP, results in a reduced number of cells entering S-phase (39). Similar to our study, the LNCaP cells show a higher rate (~15%) of apoptosis after specific TRPV6 siRNA knockdown (39). From their study in LNCaP it was suggested that calcium entry via TRPV6 not only maintains an increased proliferation rate, but increases cellular survival and provides resistance to apoptosis. Here we see similar results; however, in T47D cells TRPV6 seems to provide less resistance to apoptosis. This may be due to the presence of TRPC4, TRPC6, or other entry channels permeable to calcium or insufficient TRPV6 knockdown. Therapeutic treatments aimed at inhibiting the TRPV6 channel would thus keep the cancer cells from rapidly growing. For instance, it has been shown that calcium channel inhibitors that likely block TRPV6 are able to restrict the growth of prostate cancer cells in a cytostatic manner and when administered to mice show no toxicity (41).

In this study we have shown that TRPV6 is expressed at elevated messenger levels in breast cancer patient samples. In our in vitro model, estrogen, progesterone, and 1,25-vitamin D were all able to regulate TRPV6 transcription. Interestingly, tamoxifen acts negatively on two levels to reduce both the expression and activity of TRPV6. This suggests that TRPV6 might be involved in the antiproliferative activity of the widely used breast cancer treatment tamoxifen. However, further clinical data would be needed to address this hypothesis. Regardless, TRPV6 appears be able to control the proliferative ability of T47D breast adenocarcinoma cells. The activity of tamoxifen was enhanced by combination treatment with TRPV6 silencing siRNA. Our studies indicate that TRPV6 may be suitable target for development of specific chemical inhibitors. Furthermore, when hormonal therapies are no longer effective,

inhibitors for TRPV6 may be of increased value particularly when the tumors become ER or PR negative.

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3.2. Tamoxifen inhibits TRPV6 activity via estrogen receptor independent pathways in TRPV6 expressing MCF-7 breast cancer cells

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ABSTRACT

The epithelial calcium channel TRPV6 is up regulated in breast carcinomas compared to normal mammary gland tissue. The selective estrogen receptor modulator tamoxifen is widely used in breast cancer therapy. Previously, we showed that tamoxifen inhibits calcium uptake in TRPV6-transfected *Xenopus* oocytes. In this study, we examined the effect of tamoxifen on TRPV6 function and intracellular calcium homeostasis in MCF-7 breast cancer cells transiently transfected with EYFP-C1-TRPV6. TRPV6 activity was measured with fluorescence microscopy using Fura-2. The basal calcium level was higher in transfected cells as compared to non-transfected cells in calcium containing solution but not in nominally calcium free buffer. Basal influxes of calcium and barium were also increased. In transfected cells, 10 μM tamoxifen reduced the basal intracellular calcium concentration to the basal calcium level of non-transfected cells. Tamoxifen decreased the transport rates of calcium and barium in transfected cells by 50%. This inhibitory effect was not blocked by the estrogen receptor antagonist, ICI 182,720. Similarly, a tamoxifen induced inhibition effect was also observed in MDA-MB-231 estrogen receptor negative cells. The effect of tamoxifen was completely blocked by activation of protein kinase C. Inhibiting PKC with calphostin C decreased TRPV6 activity but did not alter the effect of tamoxifen. These findings illustrate how tamoxifen might be effective in estrogen receptor negative breast carcinomas and suggest that the mechanisms of tamoxifen and PKC inhibitors used in breast cancer therapy might involve TRPV6 mediated calcium entry. This study highlights a possible role of TRPV6 as therapeutic target in breast cancer therapy.

Key words: TRPV6, epithelial calcium channel, tamoxifen, breast cancer, estrogen receptor, cancer therapy

INTRODUCTION

Breast cancer is still ranked third among all cancer deaths and had the highest incidence rate of all cancers of both sexes in Europe in 2006 (190). The survival chances of breast cancer patients depend to a great extent on the expression level and responsiveness of steroid hormone receptors in cancer tissues. Tumors that are estrogen (ER) and progesterone receptor (PR) positive have a higher treatment success than those which are non-responsive to estrogen and progesterone (191). The most widely used anti-estrogen therapy is the application of tamoxifen, which belongs to the selective estrogen-receptor modulators. Tamoxifen competitively inhibits estradiol binding to the ER and evokes a series of events such as conformational changes of the ER and dimerization and dissociation of heat shock proteins. This triggers binding of the ER to estrogen responsive elements and transcriptional regulation. Additionally, many coregulator proteins can interact with the ER, but the exact mechanism of how tamoxifen effects the tumors and what role calcium plays in this process is not yet completely understood (192). There is also evidence that tamoxifen is cytotoxic for estrogen receptor negative breast cancer cells and that it can be effective in one-third of estrogen receptor negative tumors (46; 102). A study by Bollig et al., 2007, showed that tamoxifen up regulates protein phoshatase 1 alpha (PP1alpha) via an estrogen receptor independent pathway (193). Altered changes in intracellular Ca^{2+} were shown to be induced by tamoxifen at micromolar concentrations and to trigger death of breast cancer cells (45; 60; 97; 139).

Changes in intracellular calcium homeostasis are a crucial step in tumor formation in every type of cancer because they influence several cellular functions such as

cellular motility, differentiation, proliferation, and apoptosis. Furthermore, calcium is a key regulator of the cell cycle (194). Interestingly, there is an up- or downregulation of specific calcium channels or pumps associated with certain types of cancer. For instance, the plasma membrane ATPase 2 (PMCA2) is overexpressed in certain breast cancer cell lines such as ZR-75-1 compared to non-tumorigenic 184B5 cells (Monteith et al., 2005). In prostate cancer tumors, the expression level of TRPM8 mRNA is significantly increased in malignant tissue compared to healthy tissue (Fuessel et al., 2003). One other example is the altered expression of TRPV6 in prostate, pancreatic, thyroid, colon, ovary, and breast cancer (195).

TRPV6 belongs to the transient receptor potential (TRP) channels. The "TRPV" vanilloid subfamily comprises six channels of which TRPV5 and TRPV6 are relatively selective epithelial calcium channels expressed in renal tubular cells or in the apical membrane of enterocytes, respectively. TRPV6 was discovered in 1999 in rat duodenum using expression cloning (196). Structurely, TRPV6 comprises 730 amino acids and contains six transmembrane domains and a short hydrophobic region between TM5 and TM6, which functions as the pore-forming region. The COOH terminus contains a calmodulin binding site and the NH_2 terminus has several ankyrin repeats on the intracellular side (120). TRPV6 is predominantly expressed in the duodenum where calcium is absorbed. *In situ* hybridization showed localization in the epithelial cells and on the villi tips (195). Additionally, TRPV6 expression was detected in pancreatic acinar cells, in mammary gland duct cells, sweat glands, skin, and placenta (197). Examination of TRPV6 transport by electrophysiology revealed that the channel mediates passive Ca^{2+}-transport with high calcium selectivity and an apparent K_m value for Ca^{2+} of 0.44mM. The cation permeability decreases in the order $Ca^{2+} > Ba^{2+} > Sr^{2+} > Mn^{2+}$ (198). At the present time, there is no selective

blocker for TRPV6 mediated calcium influx available. Non-specific blockage with ruthenium red, Gd^{3+}, and La^{3+} was observed (199). TRPV6 mRNA expression is regulated by dietary calcium, 1,25-vitamin D3, dihydrotestosterone, and estrogen (133; 156; 178). The intestinal calcium absorption is significantly reduced in TRPV6 knockout mice (9). This demonstrates the importance of TRPV6 as a major calcium uptake pathway of dietary calcium in the intestine.

The exact function of TRPV6 in exocrine tissues is still unknown and its role in cancer is not clear thus far. Numerous studies are published on the role of TRPV6 in prostate cancer. A correlation of TRPV6 expression to the prostate cancer Gleason grade was detected in different cancer stages, whereas there was only little expression in normal prostate tissue (200). Furthermore, certain prostate cancer cell lines like LNCaP and PC-3 express high amounts of TRPV6 mRNA (201). A study in LNCaP cells demonstrated that TRPV6 can influence the proliferation rate, the cell cycle, and the expression of the proliferating cell nuclear antigen (PCNA). TRPV6 is the main channel responsible for calcium uptake in this cell line and it can activate the nuclear factor of activated T-cells (NFAT). Lehen'Kyi *et al* demonstrated in the same study that the androgen receptor is involved in TRPV6 regulation in a ligand-independent way. They found that AR knockdown by siRNA decreased TRPV6 mRNA and protein levels, but the ligands DHT, an AR-selective agonist, and Casodex, a selective antagonist, had no significant effect on TRPV6 mRNA expression (202).

This confirms earlier results of studies using HEK293 cells, which revealed an increased proliferation rate if the cells were stably expressed with TRPV6 (203). Of great importance is the fact that TRPV6 mRNA and protein expression is not only

increased in prostate cancer, but also in human carcinomas of the colon, thyroid, ovary, and breast (133; 195; 208).

In the ductal epithelial cells of the mammary gland, TRPV6 was found to be expressed in the apical membrane (195).

Our former study confirmed that TRPV6 is expressed at higher levels in breast cancer samples compared to non-tumorous samples (204). We also showed that TRPV6 expression in T47D breast cancer cells is increased by estrogen, progesterone, and 1,25-vitamin D3. In contrast, tamoxifen was found to downregulate TRPV6 expression in cancer cells and to inhibit radioactive calcium uptake into TRPV6-expressing *Xenopus* oocytes. Therefore, TRPV6 might be involved in the mechanism of tamoxifen in breast cancer cells. In T47D cells, TRPV6 is able to control proliferation as we showed using the siRNA expression knockdown. The studies indicated that it might be clinically useful to develop specific TRPV6 inhibitors as breast cancer drugs. Especially, in the case of estrogen receptor negative tumors, TRPV6 targeting could be promising (205).

As a continuation of our published article on the role of TRPV6 in breast cancer, in the present study, our goal was to demonstrate the inhibitory effect of tamoxifen on TRPV6 mediated calcium uptake in breast cancer cells and to investigate the mechanism and the subsequent effect on intracellular calcium homeostasis. Our findings help to clarify a potential mechanism of how tamoxifen may be useful in breast cancer therapy.

RESULTS

Expression of TRPV6 in MCF-7, T47D ER+ and MDA-MB-231 ER- breast cancer cells

First, we tested the expression of TRPV6 mRNA in three widely used human breast cancer cell lines. TRPV6 mRNA was found to be expressed at a high level in T47D cells whereas MCF-7 and MDA-MB-231 cells showed very low levels of TRPV6 (Fig. 1A). The amount of TRPV6 mRNA expressed in T47D was comparable to the mRNA level in LNCaP prostate cancer cells (data not shown). Immunoprecipitation of TRPV6 followed by Western blot technique revealed that T47D cells express higher levels of TRPV6 protein compared to the other two cell lines (Fig. 1B). When we examined the level of the expressed TRPV6 protein qualitatively in these three cell lines using immunofluorescence microscopy, our observation was similar as shown in Fig. 1C.

Fig. 1A-B

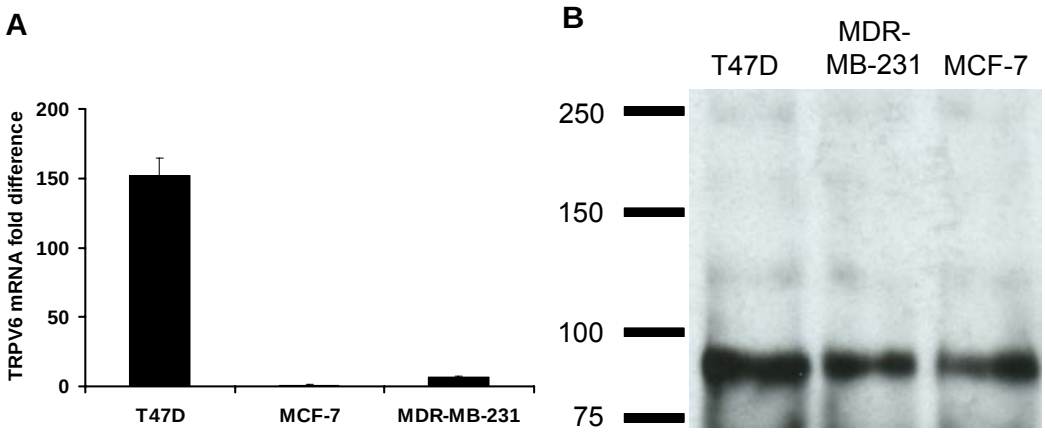


Fig. 1C

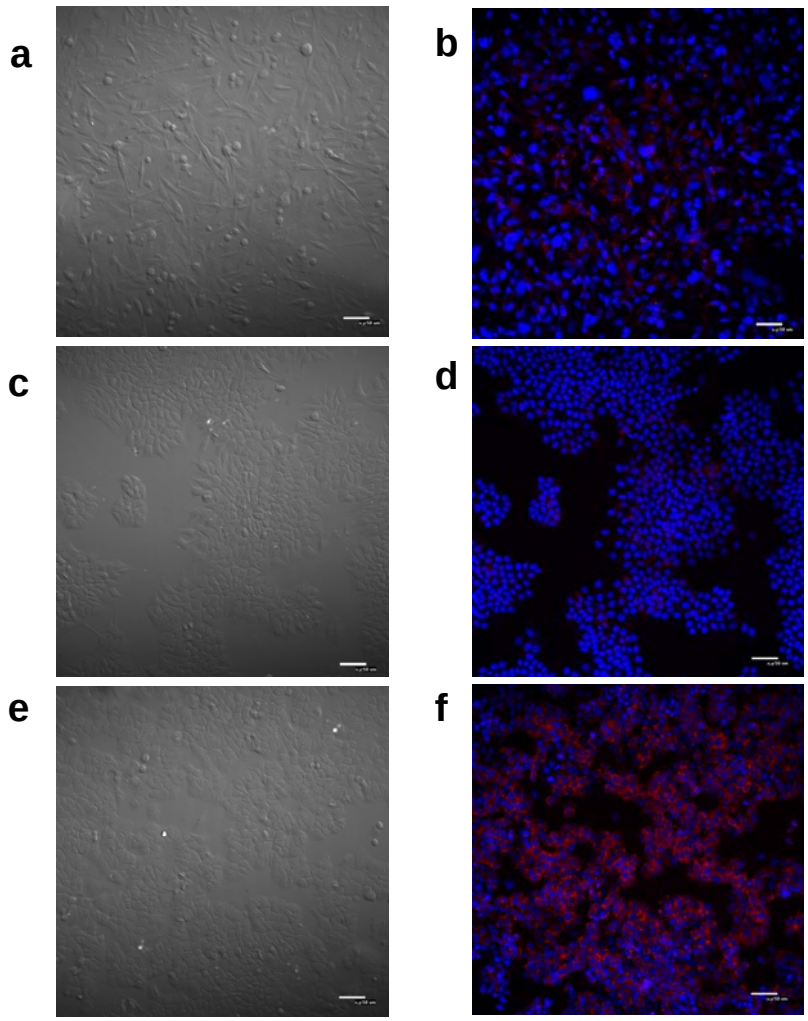


Figure 1: (A) Comparison of TRPV6 mRNA expression (fold difference) in MCF-7, MDA-MB-231 cells and T47D human breast cancer cells detected with real-time PCR. (B) Comparison of TRPV6 protein in T47D, MDA-MB-231, and MCF-7 cells measured with immunopreciptiation and Western blotting. (C) Phase contrast images (*left*) and immunofluorescence staining (*right*) of TRPV6 in MDA-MB-231 cells (a,b),MCF-7(c,d), and T47D cells (e,f). Images were taken with a 20x objective (scale bar 20 µM).

Furthermore, in T47D cells TRPV6 showed co-localization with PMCA, suggesting that it is expressed at the plasma membrane (Fig. 2).

Fig. 2

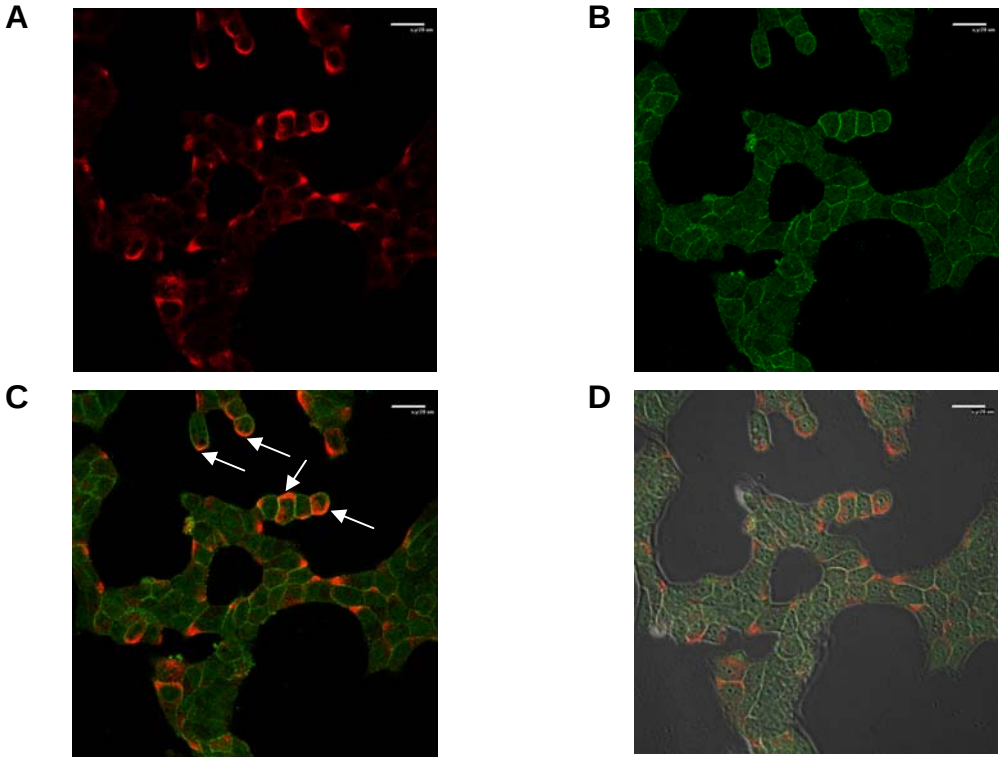


Figure 2: Immunofluorescence double staining with anti-TRPV6 (A) and anti-PMCA antibodies (B) in T47D cells. Arrows point at the co-localization of TRPV6 and PMCA in the merged (C) image. The overlay of the phase contrast and the merged image is presented on Panel D. Images were taken with a 20x objective (scale bar 20 μM).

Effect of tamoxifen on calcium entry in T47D cells

The basal calcium influx was determined by measuring the rate of the increase of the fura-2 fluorescence ratio following administration of 1 mM calcium. In T47D cells, this calcium influx was very small (Figs. 3A and 3C). When we investigated the effect of 10 μM tamoxifen on basal calcium influx, we found a remarkable increase in calcium entry (Figs. 3B and 3C). Additionally, tamoxifen induced a large transient rise in intracellular calcium in nominally calcium-free buffer (Fig. 3B).

A B Fig. 3

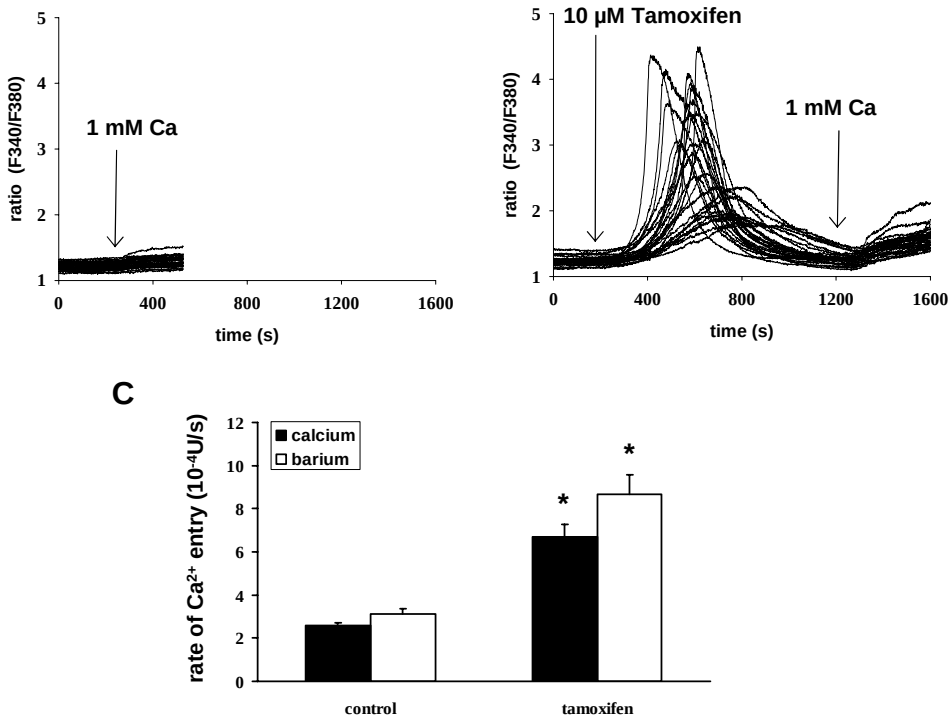


Figure 3: Representative tracings (Panel A, and B) and bar graph (Panel C) showing the effect of 10 µM tamoxifen citrate on basal barium and calcium influxes in T47D cells. Number of separate experiments >6; *, $p<0.05$.

This effects can be explained by the fact that tamoxifen induces calcium depletion of the endoplasmic reticulum calcium stores which in turn activate store-operated calcium channels (SOCCs) expressed in the plasma membrane. This phenomenon was observed in several other cell types. Unfortunately, the activation of SOCCs masks any possible effect of tamoxifen on TRPV6. Therefore, we had to turn to another approach namely overexpressing EYFP-tagged TRPV6 in MCF-7 cells.

Effect of tamoxifen on TRPV6 mediated calcium entry calcium entry in MCF-7 cells

MCF-7 cells were chosen for transfection of TRPV6 because they express only very low levels of endogenous TRPV6 (Fig. 1A). Confocal, fluorescence images of

transfected MFC-7 cells suggested that exogenous TRPV6 is expressed at the plasma membrane (Fig. 4 arrows).

Fig. 4

A **B**

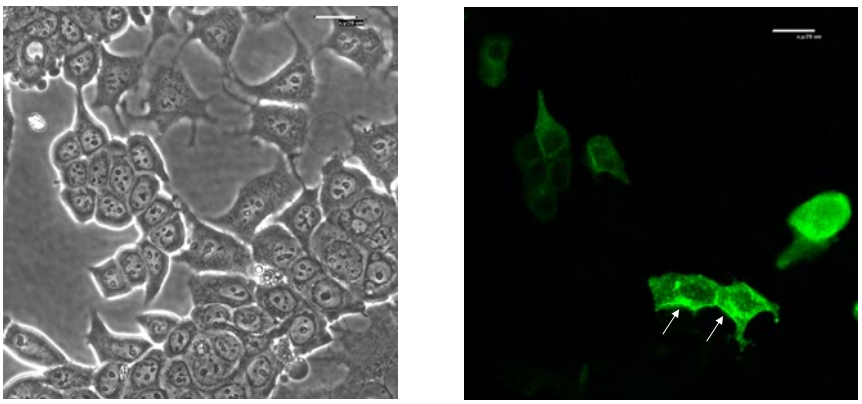
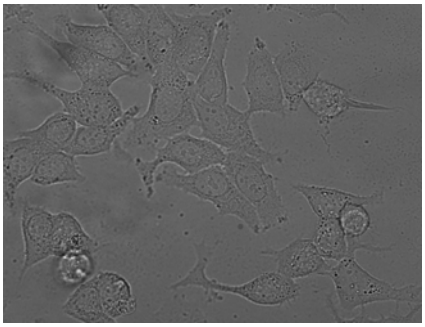


Figure 4: Phase contrast (A) and fluorescence EYFP-image (B) to display localization of EYFP-C1-TRPV6 after transient transfection in MCF-7 cells. Images were taken with a 60x objective (scale bar 20 µM). Arrows point at plasma membrane localization.

The transfected cells were identified based on EYFP fluorescence (Fig. 5A und 5B).

A **Fig. 5**



B **C**

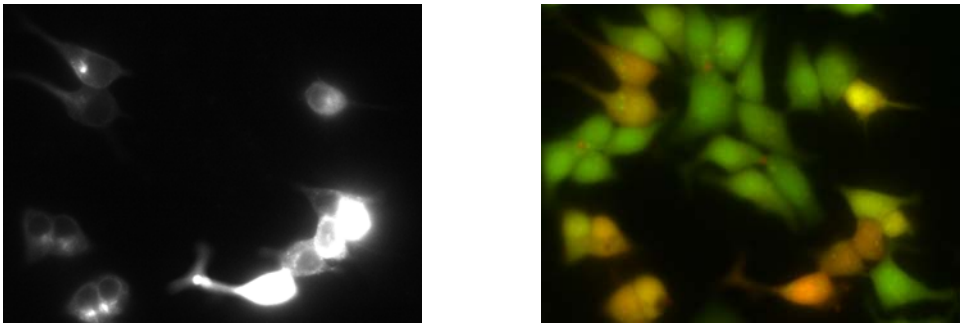


Figure 5: Phase contrast image (A), EYFP-image (B), and Fura-2 ratio image (C) of EYFP-C1-TRPV6 expressing MCF-7 cells. Note that TRPV6 expressing cells have higher basal intracellular calcium level.

As expected, the basal intracellular calcium concentration was highly increased in transfected cells, compared to non-transfected cells (ratio of 2.027 ± 0.023 vs 1.23 ± 0.004; $P < 0.001$) (Figs. 5C, 6A and 6B). When extracellular calcium was omitted this difference was no longer detectable (1.16 ± 0.004 vs 1.116± 0.002). Furthermore, basal calcium and barium influx was 100- and 9- fold larger in transfected cells compared to non-transfected cells, respectively (Figs. 6C and 6D).

Basal intracellular calcium concentrations and the basal calcium influx rate correlated closely, as shown in Figs. 6E and 6F. Both of the influxes were almost completely inhibited by 100 µM Gd^{3+}, a non-specific TRPV6 inhibitor (data not shown). Our functional data and confocal images provided solid evidence that the expressed EYFP-TRPV6 is fully functional at the plasma membrane.

Fig. 6A-B

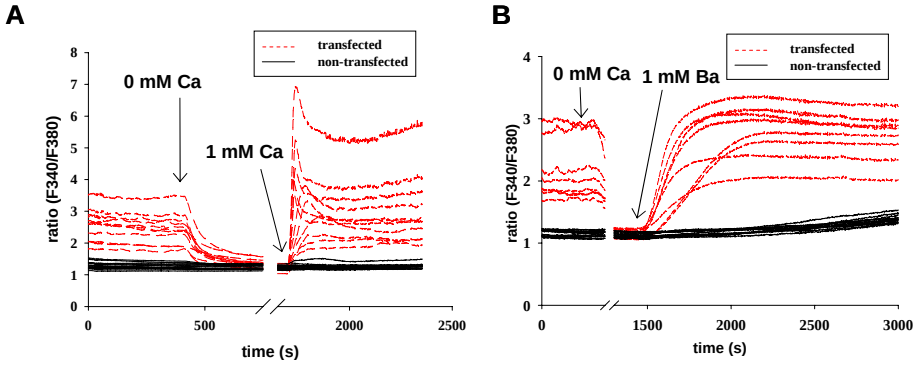


Fig. 6C-D

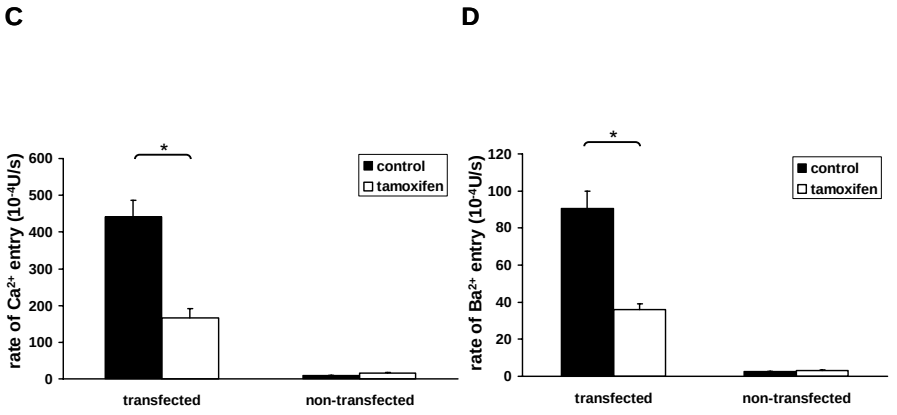


Fig. 6E-F

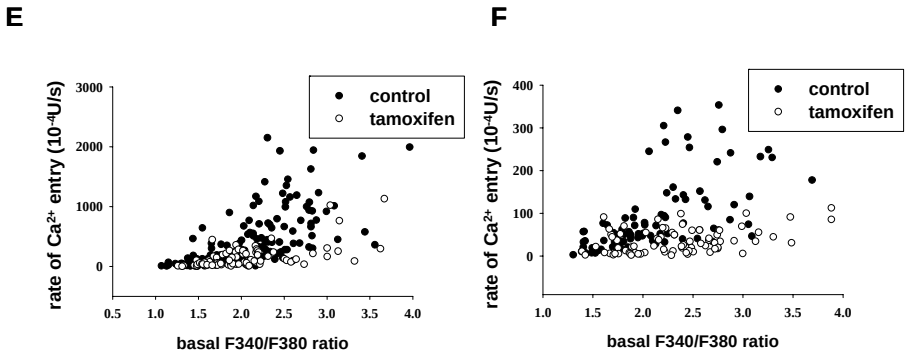


Figure 6: Representative tracings showing Ca^{2+} (Panel A), Ba^{2+} (Panel B) influxes in MCF-7 cells using the Ca^{2+}-sensitive fluorescent dye fura-2. First, extracellular Ca^{2+} was removed followed by administration of 1 mM external Ca^{2+} or Ba^{2+} when the fura-2 ratio was stabilized. The rate of the increase of the fura-2 ratio in response to re-addition of the particular divalent cation was analyzed. Summary data in Panels C and D show the rate of changes in Fura-2 ratio after re-addition of calcium or barium w/o 10 μM tamoxifen citrate treatment for 10 min in transfected and non-transfected cells. Panels E and F depict the correlation between basal calcium or barium entry and normal intracellular calcium concentration in transfected cells. *, $p<0.05$ control vs tamoxifen group.

Incubation with 10 μM tamoxifen for 10 minutes induced a transient calcium increase followed by a sustained decrease in intracellular calcium. The intracellular calcium in transfected cells finally reached the calcium level of non-transfected cells (Fig. 7A). In nominally calcium free medium, the tamoxifen-induced calcium increase was reduced to insignificant levels (Fig. 7B).

Fig. 7

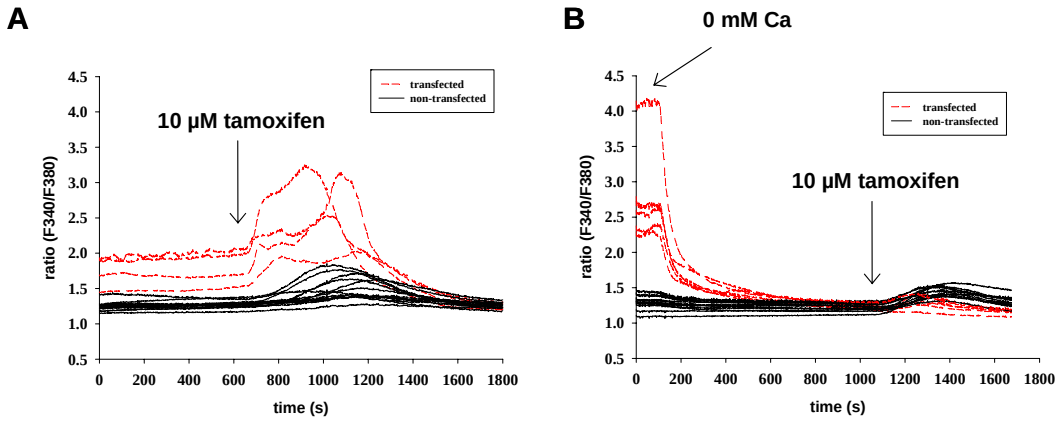


Figure 7: Representative tracings showing the effect of 10µM tamoxifen citrate on intracellular [Ca^{2+}] ([Ca^{2+}]$_i$) in EYFP-C1-TRPV6 expressing MCF-7 cells in the presence of 1 mM extracellular Ca^{2+} (A) or in nominally calcium free solution (B).

The application of 10 µM tamoxifen for 10 min induced a prominent decrease in initial calcium and barium influx rate down to 37.6% (ratio of 440.84 ± 45.84 vs. 165.77 ± 26.39), respectively 39.7% (ratio of 90.75 ± 9.00 vs. 36.04 ± 3.20) (Fig. 6C and 6D). Tamoxifen at 1 µM for 1h also decreased TRPV6 activity to 24.4 % (ratio of 440.84 ± 45.84 vs. 107.63 ± 16.04) but not when applied for 10 minutes (Fig. 8). Furthermore, the tamoxifen metabolite, 4-hydroxy-tamoxifen (10 µM for 10 min), decreased TRPV6 activity to 17.77% (ratio of 440.84 ± 45.84 vs. 78.32 ± 13.74) (Fig. 8).

Fig. 8

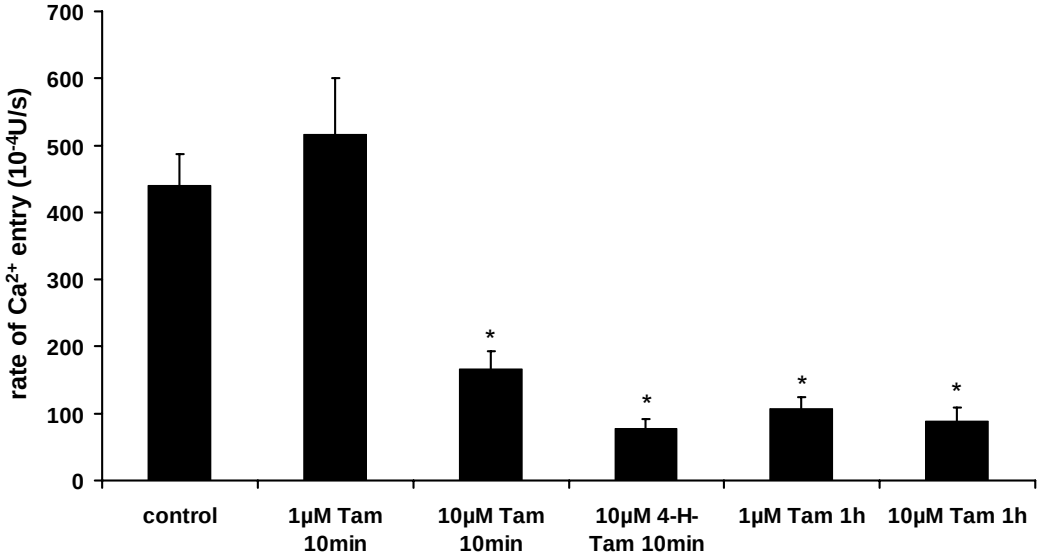


Figure 8: Summary data show the rate of changes in the fura-2 ratio after re-addition of calcium in control group and in response to treatment with 1 µM, 10 µM tamoxifen (Tam), and 10 µM 4-hydroxy-tamoxifen (4-H-Tam) treatment for 10 min and with 1 µM and 10 µM tamoxifen treatment (Tam) for 1h in MCF-7 cells transfected with TRPV6. *, $p<0.05$ treatment vs control group

Determination whether the estrogen receptor is involved in the observed effect of tamoxifen

When the estrogen receptor antagonist ICI 182,780 (1 µM) was applied together with tamoxifen to MCF-7 cells, we observed no significant change of the tamoxifen induced inhibition in the presence of ICI 182,780 (Fig. 9A). To confirm these findings, we examined the effect of 10 µM tamoxifen in the estrogen receptor negative, human breast cancer cell line, MDA-MB-231, transiently transfected with EYFP-C1-TRPV6. Tamoxifen exerted a similar effect like in transfected MCF-7 cells. The initial calcium influx was decreased to 30.5% by tamoxifen in transfected cells compared to the control group (ratio of 542.28 ± 157.58 vs. 165.73 ± 32.29) (Fig. 9B).

Fig. 9A

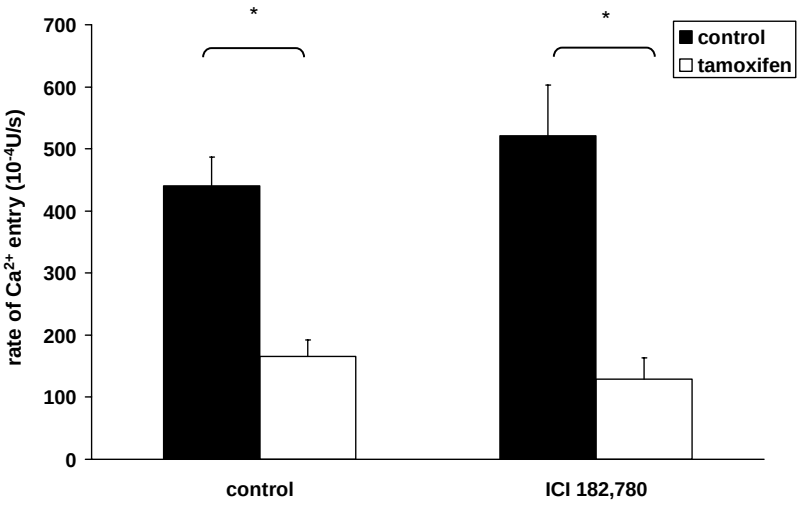


Fig. 9B

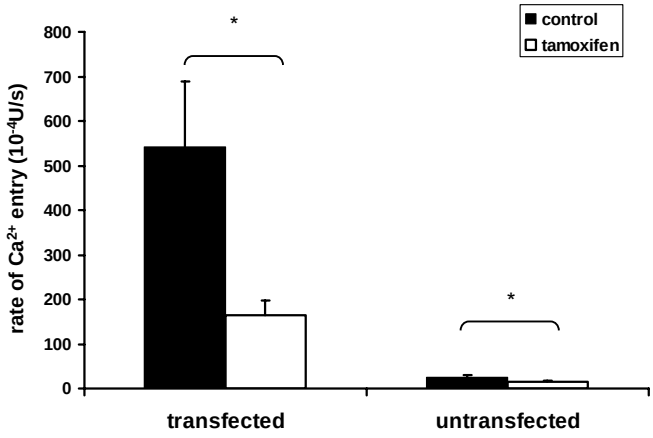


Figure 9: (A) Summary data showing the effect of a pretreatment in transfected MCF-7 cells with the estrogen receptor antagonist ICI 182.780 (1 µM) for 10 min on TRPV6 activity with and without tamoxifen. Data are the means of at least 25 cells from 6 separate experiments. (B) Summary data showing the effect of 10 µM tamoxifen on calcium influx in estrogen receptor negative MDA-MB-231 cells *, $p<0.05$ control vs tamoxifen group.

Determination whether PKC plays a role in the observed effect of tamoxifen

Since tamoxifen was shown to affect protein kinase C (PKC) activity in MCF-7 cells and several possible serin/threonine phosphorylation sites can be predicted on

TRPV6, we tested whether PKC inhibition could be involved in the effect of tamoxifen on TRPV6 activity (206). We found that activation of PKC with 200 nM PMA promptly elevated intracellular calcium in transfected cells, whereas the effect was much smaller in non-transfected cells. Furthermore, in some transfected cells, slow calcium oscillations could be observed (Fig. 10A). When extracellular calcium was removed, this robust calcium increase vanished. Also, basal calcium influx was significantly increased in the transfected cells (Fig. 10A). The inhibitory effect of tamoxifen was completely abolished in the presence of PMA (Fig. 10B). When we applied the PKC inhibitor calphostin C (100 nM), we observed that calphostin C alone or in combination with tamoxifen decreased the initial calcium influx in transfected MCF-7 cells to 50.4% and 46.4%, respectively (from 440.84 ± 45.84 to 178.57 ± 21.99, to 204.34 ± 30.56) (Fig. 10B) . Therefore, tamoxifen exerted no additional inhibitory effect when PKC was inhibited.

Fig. 10

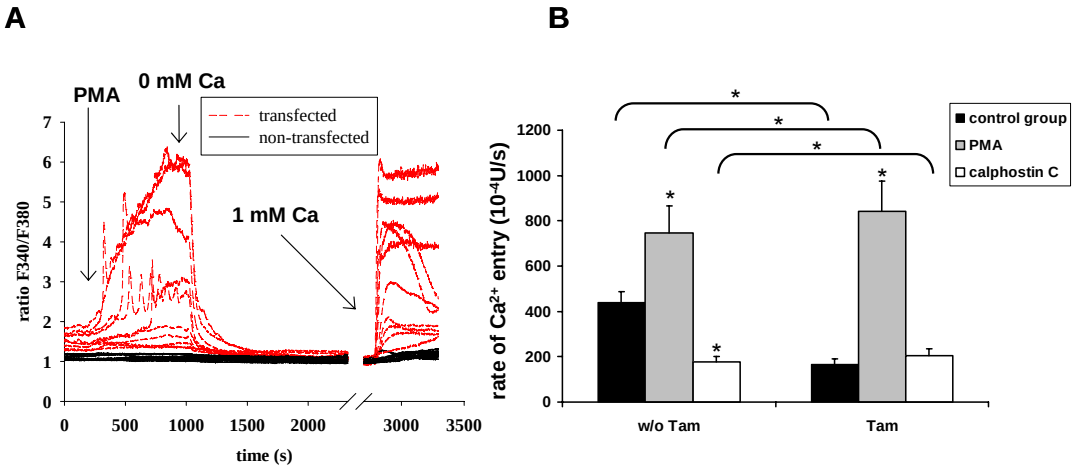


Figure 10: Panel A: Representative tracings showing changes in the calcium increase induced by inhibition of PKC with 200 nM PMA for 10 min. Panel B: Summary data showing the effect of the PKC activator PMA (200 nM) and the PKC inhibitor calphostin C (100 nM) on the inhibitory effect of 10 µM tamoxifen in MCF-7 cells transfected with TRPV6. Cells were pre-treated with calphostin C for 1h. Data are the means of at least 25 cells from 6 separate experiments. *, p<0.05 treatment vs control group

DISCUSSION

In spite of intensive efforts towards development of novel therapeutic approaches for the prevention and treatment of breast cancer, the incidence rate of breast adenocarcinomas is still highest among all cancer types and the mortality rate was 3rd among all cancers in Europe in 2006 (207). Since intracellular calcium plays a critical role in many fundamental cellular processes such as proliferation, apoptosis, and secretion, calcium levels are spatially and temporally tightly controlled in cells. Disturbances in intracellular calcium homeostasis are a crucial factor in the process of tumor progression in all cancer types. Many studies have been conducted to evaluate how the regulatory system of intracellular calcium homeostasis and the associated calcium signaling pathways are altered in breast cancer cells compared to normal mammary gland cells. A recent review summarizes the possible roles of T-type calcium channels in breast cancer progression (208). The essential roles of the store operated calcium channel Orai1 and the signaling molecule Stim1 in breast cancer metastasis formation was recently reported (209). Monteith and his group observed that the expression of different isoforms of the PMCA is changed in breast cancer cells and that PMCA2 expression is significantly increased (83; 84). Furthermore, inhibition of PMCA using siRNA decreased proliferation of MCF-7 human breast cancer cells (210). In recent years, there has been accumulating evidence that the TRPV6 epithelial calcium entry channel is involved in breast cancer progression. In human and mouse breast cancer samples, TRPV6 protein expression was increased (195).

In addition, our laboratory recently demonstrated that a knockdown of TRPV6 expression using siRNA decreased basal calcium influx and proliferation of T47D

human breast cancer cells (211). As a novel finding, we also found that tamoxifen, at micromolar levels, inhibits TRPV6-mediated calcium influx in human TRPV6-expressing *Xenopus* oocytes. In the present study, we addressed the mechanism of this inhibitory effect and its consequences on intracellular calcium homeostasis using transfected breast cancer cell lines.

We selected two widely used human, estrogen receptor positive breast cancer cell lines, T47D and MCF-7, and MDA-MB-231 which are estrogen receptor negative. First, we examined the expression of TRPV6 in these cell lines. The mRNA levels were low in MCF-7 and MDA-MB-231 and high in T47D cells. The expression of TRPV6 in T47D cells was comparable to LNCaP prostate cancer cells which are known to have high TRPV6 levels (data not shown). Immunofluorescence staining, and Western blotting of immunoprecipitated samples also showed much higher expression of TRPV6 in T47D cells compared to MCF-7 or MDA-MB-231 cells. Unfortunately, in T47D cells, a high dose of tamoxifen above 1 μM induced depletion of the ER calcium stores followed by a robust increase in store-operated calcium channel (SOCC) activity. Since, in non-depolarizing cells, the majority of the basal calcium influx occurs through SOCC, the possible effect of tamoxifen on native TRPV6 activity was masked. Tamoxifen also exerts the same effect on SOCC activity in several cell types such as human oral cancer cells, CHO-K1 cells, human osteosarcoma cells, and ZR-75-1 human breast cancer cells (32; 37; 76; 95). This effect was shown not to be mediated by estrogen receptors.

To evaluate the effect of tamoxifen on TRPV6, we increased the number of TRPV6 channels residing in the plasma membrane of the MCF-7 cells, which expresses a low level of endogenous TRPV6. To this end, we over-expressed EYFP-tagged human TRPV6 in MCF-7 cells to reach expression levels of TRPV6 comparable to

those of SOCCs. In such TRPV6 over-expressing cells, we observed a significantly higher basal calcium level and increased calcium and barium influxes. Confocal immunofluorescence studies confirmed that TRPV6 is expressed at the plasma membrane. A short, 10 minute incubation of 10 μM tamoxifen induced a much higher transient calcium response in transfected cells compared to non-transfected cells, the effect of which was diminished when extracellular calcium was omitted.

It should be noted that we selected this initial tamoxifen concentration because the IC_{50} value of tamoxifen was 7.5 μM in *Xenopus oocytes* (212).

In transfected MCF-7 cells, after transient calcium elevation, basal intracellular calcium returned to the same level as in non-transfected cells. As described above, this temporary calcium elevation is probably due to ER calcium store depletion and subsequent activation of SOCCs. In transfected cells, both basal calcium and barium influxes were reduced by tamoxifen to 37.6% and 39.7%, respectively. In non-transfected cells, there was either no effect (barium influx) or a small increase (calcium influx) in response to tamoxifen. 10 μM hydroxy-tamoxifen and prolonged incubation with lower (1 μM) dose of tamoxifen also reduced TRPV6 mediated calcium influx, suggesting that in tamoxifen-treated breast cancer patients, inhibition of TRPV6 could be involved in the anti-tumor effect of tamoxifen. After treatment of patients with 5 or 20 mg tamoxifen citrate daily for 28 days the concentration of tamoxifen can reach between 0.73 μM and 2 μM in the breast cancer tissue (213). In our experiments we used 10 μM tamoxifen and to further ensure the physiological significance also a lower concentration of tamoxifen (1 μM).

As we expected, estrogen receptors were not involved since inhibition of estrogen receptors with ICI 182,780 in ER+ MCF-7 cells was ineffective. Also, tamoxifen

reduced TRPV6 activity in the ER negative cell line, MDA-MB-231, to the same extent as in the ER positive cell line, MCF-7.

Previously, it was shown that tamoxifen is a potent PKC inhibitor *in vitro* (214). However, in MCF-7 cells, the reported findings on the effect of tamoxifen are controversial. Rowlands et al tested the inhibitory potential of several tamoxifen analogues in MCF-7 cells (215). In 2003, one group observed a reduction of PKC activity by tamoxifen, whereas in another report, an increased PKC activity and induction of PKC translocation to the plasma membrane in response to tamoxifen was observed (18; 22). The phosphorylation of T702 on TRPV6 by PKC was also demonstrated by Flockerzi and his group (216). This threonine residue is localized in the calmodulin binding site of TRPV6. Phosphorylation at this position blocks inactivation of the channel by calcium-calmodulin complex. In our experiments, activation of PKC by PMA completely abolished the inhibitory effect of tamoxifen on TRPV6. When the PKC inhibitor calphostin C (100nM) was applied, TRPV6 activity was diminished. Furthermore, tamoxifen had no inhibitory effect in the presence of calphostin C. It was demonstrated previously that calphostin C can reduce MCF-7 cell viability very potently (66). However, because TRPV6 mediated barium entry was also inhibited by tamoxifen, and because barium does not bind to calmodulin (217), we conclude that tamoxifen does not inhibit TRPV6 function through augmentation of calmodulin-mediated inactivation. Nevertheless, there are several other possible sites on TRPV6 predicted to be phosphorylated by PKC. Phosphorylation of one or more of these could enhance TRPV6 mediated ion influx.

In summary, we show that tamoxifen inhibits TRPV6 activity via an estrogen receptor independent way. This finding may help to explain the mechanism behind the

success in therapy of estrogen receptor negative breast tumors with tamoxifen. Although the detailed mechanism of how tamoxifen affects TRPV6 still needs to be elucidated, we conclude that protein kinase C activity modulates the inhibitory effect of tamoxifen. We also found that PKC activation enhances, whereas PKC inhibition decreases TRPV6 activity, and that these effects are not mediated by calmodulin. It is tempting to suggest that PKC inhibitors used for breast cancer treatment affect TRPV6 as well. Our findings suggest that TRPV6 might be a possible target for the development of new breast cancer medications.

MATERIALS AND METHODS

Materials

The breast cancer cell lines, MCF-7, MDA-MB-231 and T47D were obtained from ATCC, Health Protection Agency and NIH National Cell, respectively. Culture Center. Fugene 6 transfection reagent was used from Roche Applied Biosystems (Rotkeruz, Switzerland). The EYFP-C1-hTRPV6 vector was a generous gift from Prof. Christoph Romanin, Institute for Biophysics, Johannes Kepler Universität Linz.

Rabbit anti-human TRPV6 antibody was obtained from ProteinTech Group Inc. (Chicago, IL, USA). Mouse anti-human PMCA antibody was purchased from Abcam (Cambridge, UK). Hoechst 33342, goat anti-rabbit IgG conjugated to Alexa 594, goat anti-mouse IgG conjugated to Alexa 488, Fura-2 AM, cell culture medium RPMI-1640, and cell culture reagents were obtained from Invitrogen (Basel, Switzerland). The mounting medium Citifluor AF2 was from LucernaChem (Luzern, Switzerland). Tamoxifen citrate was purchased from Acros Organics (Geel, Belgium), and ICI 182,780 from Tocris Bioscience (Bristol, UK). Phorbol-12-myristate-13-acetate and calphostin C was purchased from Calbiochem (Nottingham, UK). All other chemicals were obtained from Sigma-Aldrich St Louis, MO, USA). Anti-rabbit IgG HRP conjugate (Promega, Madison, WI, U.S.A.), Amersham™ ECL™ Western Blotting Detection Reagents (GE Healthcare, Buckinghamshire, UK), Complete Mini (protease inhibitors) (Roche Diagnostics GmbH, Mannheim, Germany), Immobilon-P Transfer Membrane (Milian, Meyrin, Geneva,

Switzerland), and 1% Nonidet P-40 (Roche Diagnostics GmbH, Mannheim, Germany) were used for the Western blotting and immunopreciptation.

Cell culture

The human breast cancer cells lines MCF-7 and T47D were cultured in RPMI-medium supplemented with 10 % FBS, 1 mM HEPES and 1% penicillin/streptomycin. The cells were kept in a cell culture incubator at 5 % CO_2 at 37 °C and were passaged twice a week.

Real time PCR

cDNA was prepared for every sample by reverse transcription of total RNA isolated with Trizol using TaqMan Reverse Transcription Reagents (Applied Biosystems, Foster City, CA) according to the manufacturer's manual. For all experiments mRNA expression was measured by real time PCR using an Applied Biosystems 7500 Real Time PCR System. Reactions consisted of 1x Mastermix, 0.9 µM forward and reverse primers, and 0.2 µM dual-labeled fluorescent probes each for TRPV6 and β-actin. The sequences of the forward and reverse primers for TRPV6 were 5′-GGT TCC TGC GGG TGG AA-3′ and 5′-CCT GTG CGT AGC GTT GGA T-3′ respectively, with the resulting amplicon being 62 bp with a Tm of 60°C. The sequence of the probe for TRPV6 was 5′-ACA GGC AAG ATC TCA ACC GGC AGC-3′. The sequences of the forward and reverse primers for β-actin were 5′-CCT GGC ACC CAG CAC AAT-3′ and 5′-GCC GAT CCA CAC GGA ATA CT-3′ respectively, with the resulting amplicon being 69 bp with a Tm of 60°C. The sequence of the probe for β-actin was 5′-ATC AAG ATC ATT GCT CCT CCT

GAG CGC-3′. The specificity of all primers was confirmed by BLAST search. Primer Express (Applied Biosystems, Foster City, CA) was used for designing primers for TRPV6 and β-actin. All primers were designed to cross exon-exon boundaries of the coding sequence. Primers were optimized and validated for the comparative Ct method, as described in the manufacturer's manual. ABI Prism SDS software version 1 was used for the analysis of the amplification plots. The fold change ± SD in TRPV6 expression was normalized to β-actin.

Immunoprecipitation and Western blotting

Three confluent 100 mm cell culture dishes of T47D, MDA-MB-231 and MCF-7 cells were washed 3 times with ice-cold PBS. Cells were lysed in lysis buffer (composition: 10 mM Tris-HCl, 150 mM NaCl, 10 mM MgCl, 1% Nonidet P-40, and protease inhibitor cocktail) for 30 minutes at 4 ^{o}C under constant agitation. Samples were centrifuged at 12000 rpm for 20 minutes at 4 ^{o}C, and the protein concentration of the saved supernatant was adjusted to 2 mg/ml. Thereafter, 1 ml of the supernatant was incubated with 4 µg of rabbit anti-TRPV6 antibody for 1 hour under rotary agitation at 4 ^{o}C. The samples were then mixed with 30 µl of agarose protein G beads, and the mixture was incubated overnight at 4 ^{o}C. Beads were washed 3 times with lysis buffer and afterwards 40 µl of 2x loading buffer was added. The samples were boiled at 95 ^{o}C for 5 minutes and run on a 6% SDS-PAGE. The samples were transferred with a semidry method to a PVDF membrane, and blocked with 5% milk, 0.5% BSA, and 0.02% sodium azide in PBS overnight at 4 ^{o}C. The blocked membrane was probed with the same anti-TRPV6 antibody at 1:500 dilution for 2 hours at room temperature and washed 3 times in PBS with

0.1% Tween 20 (PBST). An HRP-conjugated, goat anti-rabbit secondary antibody (1:20000) was applied for 1 hour at room temperature. After 3 washes in PBST and one final wash in PBS, enhanced chemiluminescence was used to visualize the bands on the membrane.

Immunofluorescence

3 days before staining MCF-7 and T47D cells (150.000 cells / well) were seeded on poly-D-lysine coated cover slips in 6-well plates. All the following steps were performed at RT. First, the cells were rinsed twice with TBS and then fixed with 4 % PFA in TBS for 15 min. Subsequently, they were washed 3 times for 10 min with TBS and blocked with TBS supplemented with 10 % goat serum for 1 hour. Then, incubation with rabbit anti-TRPV6 (1:100) in TBS with 0.5 % BSA and 0.02% NaN_3 for 90 min followed by 3 washes with TBS for 10 min was performed. The cells were stained with mouse anti-PMCA (1:500) for 1h in the same buffer followed by the same washing procedure. The cells were incubated first with a goat anti-rabbit secondary antibody conjugated to Alexa 594 and then with a goat anti-mouse secondary antibody conjugated to Alexa 488 at 1:4000 dilution in TBS with 0.5 % BSA and 0.02% NaN_3 for 1h separated by 3 10 min washes with TBS. Then, the cells were washed 3 times for 10 min and mounted with CityFluor AF2. For staining of EYFP-TRPV6 transfected cells Fugene 6 was applied 48hours after seeding according to the manufacturer's manual. After transfection cells were incubated for 24 to 48 hours before fixing and mounting the cells as described above.

Confocal imaging

The slides were imaged with a confocal, laser scanning microscope setup using a Nikon Eclipse TE2000-E fully automatized inverted, epifluorescence microscope outfitted with Nikon D-Eclipse C1 laser confocal optics. The system equipped with a violet-diode (405 nm) and a multiline Argon (457-515nm) from Melles Griot, and a Helium/Neon (594 nm) lasers from JDS Uniphase. Nikon EZ-C1 3.6 confocal imaging software installed on a HP xw4400 workstation was used for image acquisition. Brightness and contrast were adjusted with ImageJ.

Ion imaging

For calcium imaging breast cancer cells (300.000 cells / well) were seeded on poly-D-lysine treated coverslips in 35mm-dishes. After 24 hours they were transfected with EYFP-TRPV6 plasmid using Fugene 6 according to the manufacturer's manual. After transfection, cells were incubated for 24 to 48 hours before ionic imaging.

Cell were loaded with 2.5 µg/ml Fura-2 acetoxy-methyl ester dissolved in DMSO containing PluroniC F-1278 20% in serum-free RPMI in cell culture incubator for 1 h. Cells were incubated in calcium containing Krebs buffer for 20 min. For calcium and barium measurement Fura-2 ratios (F340/F380) were monitored. All measurements were background corrected. The calcium imaging was performed on a Nikon Eclipse Ti-U microscope equipped with a Polychrome V+ monochromator (TILL Photonics). A Nikon 40x SFluor objective was used for visualization. Images were captured with a Hamamatsu Orca-ER monochrome CCD camera. Image acquisition and

analysis was performed using Simplepci version 6.2 from CImaging. Basal calcium or barium entry was measured as the rate of the increase of Fura-2 ratio in response to the administration of 1 mM divalent cation into nominally calcium free solution.

Statistics

Data are presented as mean ± SEM. Non-transfected groups were compared with Mann-Whitney rank sum test. Spearmans rank correlation test and Fisher's *z* transformation were used in case of testing significance between transfected groups. Differences in the comparison tests lower than $p < 0.05$ or $Z_{0.05} > 1.96$ were considered significant.

DISCLOSURE OF POTENITAL CONFLICTS OF INTERESTS

The authors have no conflict of interests.

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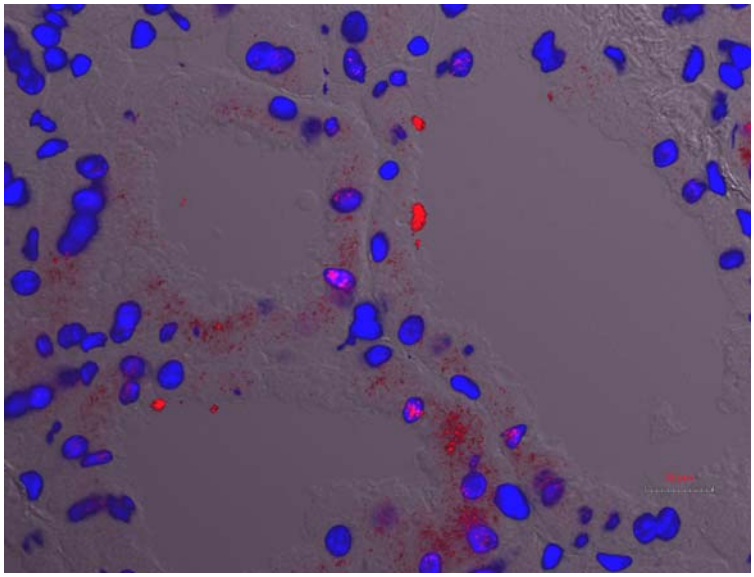
4. Additionally performed experiments and preliminary data

4.1 Physiological role of TRPV6 in the mammary gland

One other project of my PhD program was to investigate the physiological role of TRPV6 in the mammary gland. The goal was to compare the milk calcium content and the TRPV6 expression in the mammary gland of normal, early lactating and late lactating mice. To address the question of whether TRPV6 plays a role in the calcium transfer into the milk, I first wanted to determine whether there is a difference in milk calcium content between wild-type and TRPV6 knock-out mice. I performed experiments were milk, blood and tissue for RNA and protein analysis were taken. Then, I tried to isolate primary cells from the knockout and wild-type mouse mammary gland to compare calcium uptake via calcium imaging. Additionally, I performed cryo-sections and immuno-histochemistry with many different TRPV6 antibodies. Unfortunately, I failed to find any difference between knock-out mouse and wild-type control (figure 5).

A.



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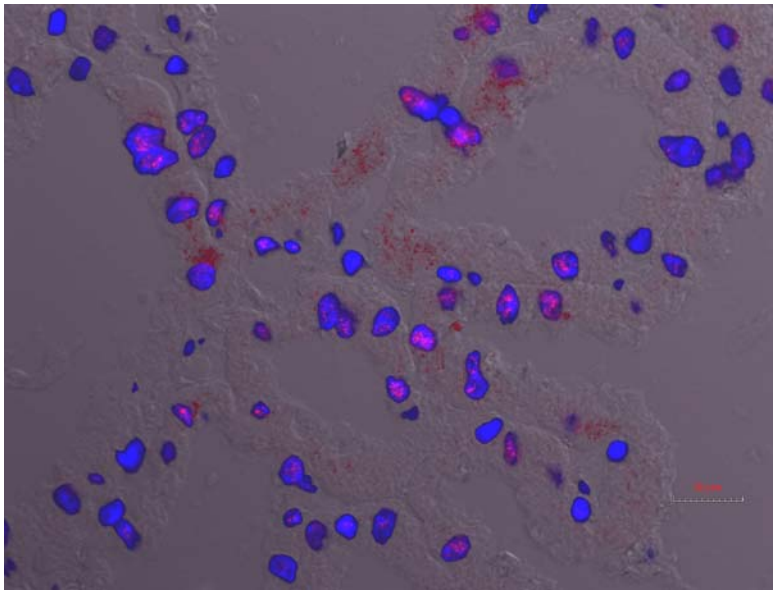


Figure 5: staining of mammary gland cryosections of wild-type (A) and knockout mouse (B) with Anti-TRPV6 antibody

Our lab also generated polyclonal antibodies against TRPV6, but the results using these antibodies were not promising. Also, there was an issue to get Western blots working. Even when using ultracentrifugation to isolate membrane preparations did not help to detect any specific protein band on Western blots. However, we could get a specific band using membrane surface biotinylation followed by isolation of labeled proteins (Pinpoint™ Cell Surface Protein Isolation Kit, Pierce, Rockford, IL, USA) of transiently overexpressed TRPV6 in HEK cells (figure 6). Recently, we could successfully perform immunoprecipitation of TRPV6 in T47D, MCF-7 and MDR-MB-231 cells (data shown in second paper).

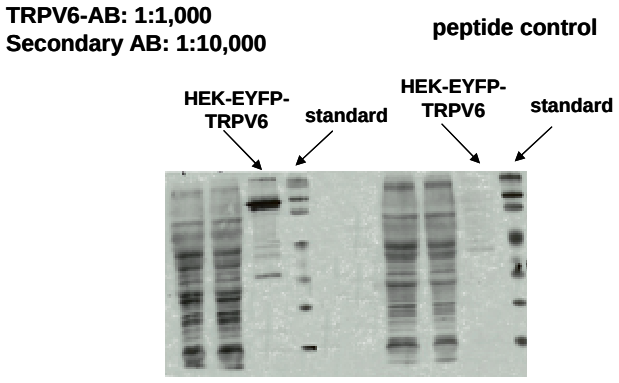


Figure 6: Western blot with anti-TRPV6 antibody of transiently TRPV6 expressing HEK cells after biotinylation compared with co-administration with peptide control

To move forward with this project, a real specific antibody against TRPV6 that works well in tissues would be necessary. Another problem of this project was that the knock-out mice compensate their reduced intestinal calcium uptake with bone resorption and therefore they have blood calcium levels similar to those of wild-type mice. The knock-out mice are also not as fertile as the wild-type mice which meant that it took very long to obtain lactating knock-out mice. Also, because we could not observe any difference in milk calcium content of knock-out and wild-type, and because all our knock-out mice strain got a pasteurella infection, we finally decided to suspend this project and to focus on *in vitro* experiments to evaluate the role of TRPV6 in breast cancer.

4.2 More findings on the role of TRPV6 in breast cancer progression

A) TRPV6 mRNA expression is increased in estrogen receptor- and progesterone receptor negative tumor tissue

I recently obtained additional results on the role of TRPV6 in breast cancer that might be important for further studies. One of these findings was that TRPV6 is not only higher expressed in breast cancer compared to normal tissue, but it in PR and ER negative cancer tissue compared to positive tissue at the mRNA level (figure 7).

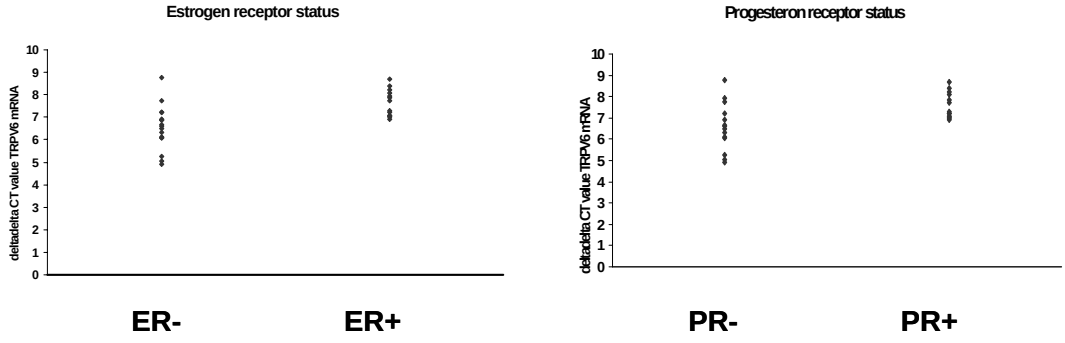


Figure 7. Real-time PCR data: delta delta CT values of breast cancer tissue samples analyzed on their TRPV6 mRNA content with real-time PCR and grouped into ER+ and ER-/PR+ and PR-.

B) Localization of TRPV6 in T47D breast cancer cells

We also investigated, for the first time, where TRPV6 is exactly localized in breast cancer cells. Interestingly, staining of small processes coming out of the membranes of T47D breast cancer cells was observed (figure 8).

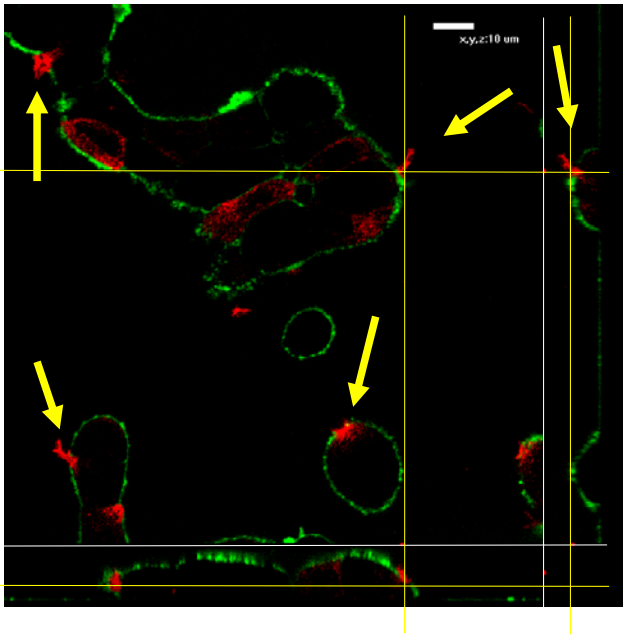


Figure 8: .T47D cells stained with anti-TRPV6 antibody (red) and biotin-steptavidin (green), 60x

C) TRPV6 seems to be expressed in the plasma membrane

Co-localization studies of TRPV6 and biotin-streptavidin also confirmed that TRPV6 is expressed at the plasma membrane of T47D breast cancer cells. Z-stack was performed to obtain three-dimensional images. In figure 9 it is clearly visable that there is co-localization (yellow overlapping). The yello arrow show staining of little “processes” that might have a specific function and have to be further identified.

The biotin-steptavidin staining was used to label the plasma membrane to confirm the co-localization. This method has not been published thus far. It provides a useful tool for labeling the plasma membrane.

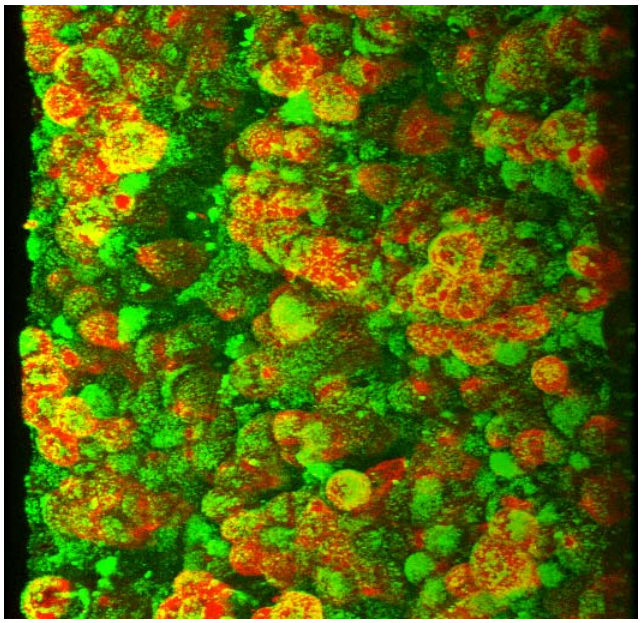


Figure 9: Three-dimensional image, Z-stack of confluent T47D cells stained with biotin-streptavidin (green) and TRPV6 antibody (red)

D) TRPV6 expression in different cell cycle phases in breast cancer cells

I also determined the expression of TRPV6 in different cell cycle phases. To this end I applied different treatments to T47D and MCF-7 breast cancer cells to enrich them in different cell cycle phases. I used normal serum containing medium or serum-free medium to enrich the cells in the G0/G1 phase, and nocodazole treatment to increase the number of cells in the G2/M phase. The effects of these treatments on the cell cycle are shown in figure 10 (MCF-7 cells) and Figure 11 (T47D cells).

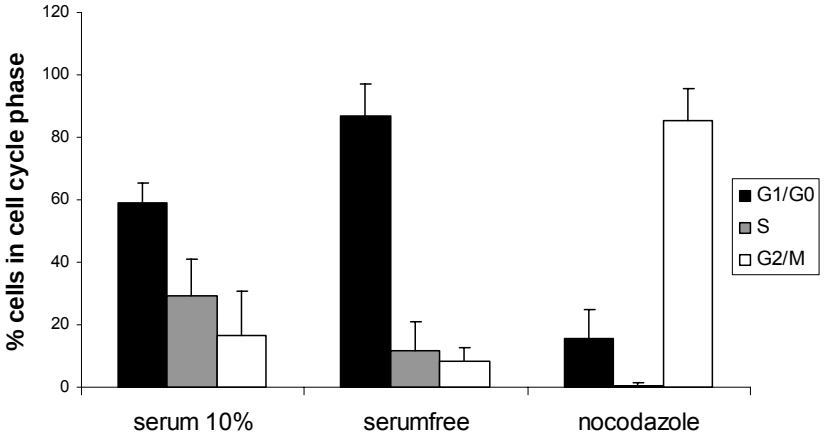


Figure 10 .MCF-7 cells in different cell cycle phases detected with FACS (Dapi staining) after treatment with normal serum medium, serum-free medium or nocodazole

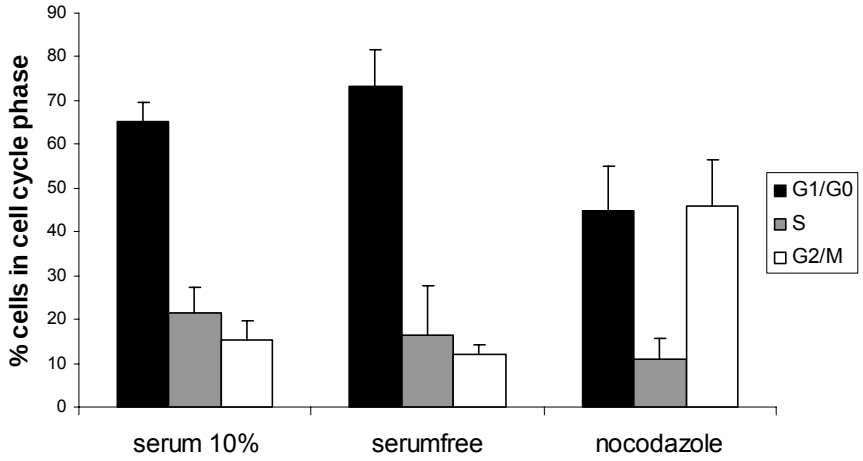


Figure 11 .T47D cells in different cell cycle phases detected with FACS (Dapi staining) after treatment with normal serum medium, serum-free medium and nocodazole

The effect of this on TRPV6 mRNA expression is shown in figure 12. The real-time PCR data demonstrated that there is no significant change in expression of TRPV6 mRNA in MCF-7 cells cpompared to T47D cells. In contrast, the expression level of TRPV6 mRNA was significantly lower in T47D cells after treatment with nocodazole compared to normal serum and serum-free

conditions (the higher the delta delta CT value, the lower is the expression level).

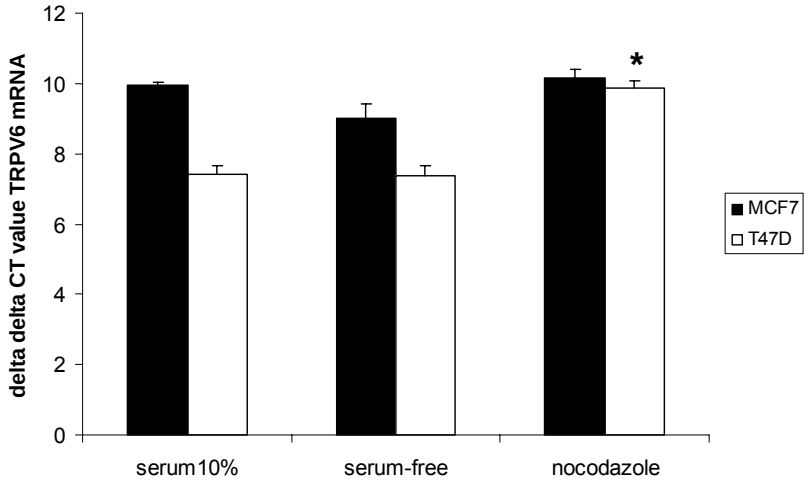


Figure 12: TRPV6 mRNA expression detected using real time PCR in MCF-7 and T47D cells after different treatments to enrich them in certain cell cycle phases (*=p<0.05)

With the different treatments that were applied, I could not enrich enough cells in the different cell cycle phases and therefore this initial set of results was not yet sufficiently reliable. In the future, more experiments should be done and the conditions and treatments should be optimized. Of interest would be to analyze, using FACS, how MCF-7 cells stably expressing TRPV6 change their cell cycle or how a stable knockdown of TRPV6 in T47D cells affects the cell cycle phases.

E) Role of TRPV6 in cell adhesion and migration

Another aspect that I wanted to investigate is the function of TRPV6 in breast cancer cell adhesion and its implication in cancer cell migration. Therefore, I developed a new protocol for a cell adhesion assay and tested a phagokinetic migration assay and a transwell assay. Thus far, the experiments were only performed with MCF-7 cells and T47D cells and they need to be repeated with stably TRPV6 expressing MCF-7 cells to be able to identify TRPV6 mediated effects. Initial results of the cell adhesion assay show that MCF-7 cells are more dependent on components from the serum for their adhesion (figure 13). It appears that T47D cells can adhere better in serum-free medium.

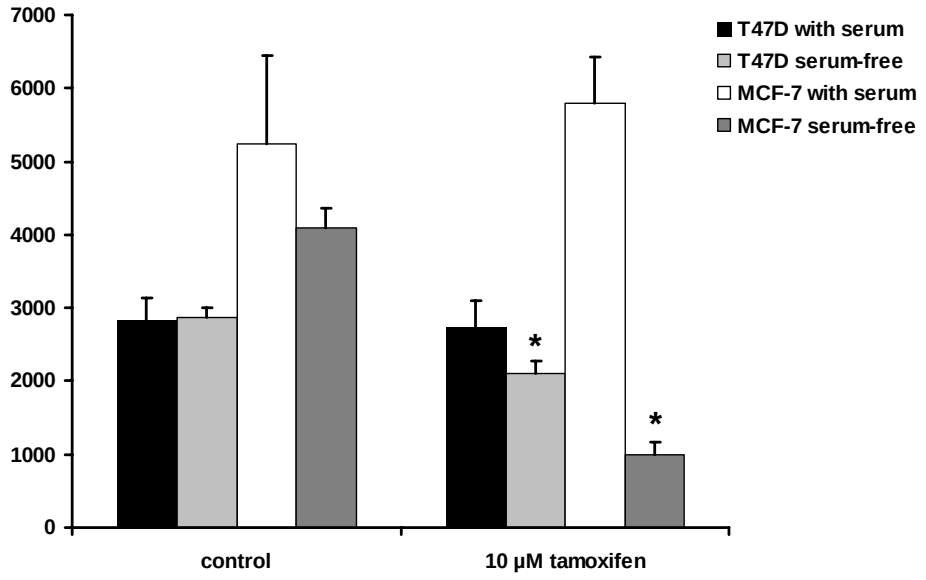


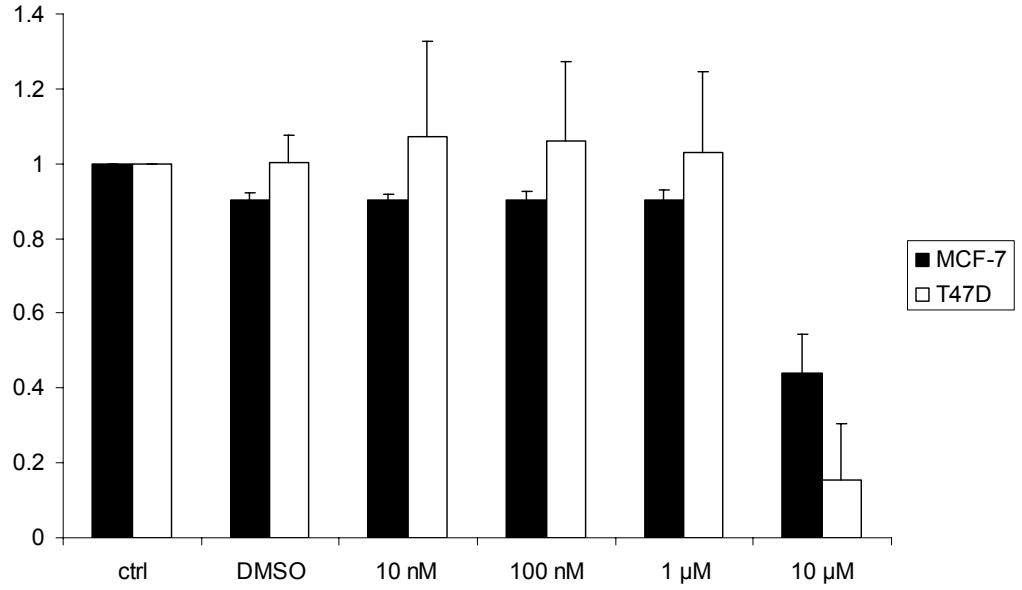
Figure:13 . Preliminary results : Adhesion assay with T47D and MCF-7 cells in serum containing and serum-free medium

The migration and pharmacokinetic assays still need to be optimized to improve the quality of the data. Using these assays, the effect of tamoxifen could be further more investigated.

F) Effect of tamoxifen on viability of breast cancer cells

Tamoxifen treatment was additionally investigated using a viability assay in MCF-7 and T47D cells. A time and dose response of T47D cell viability after tamoxifen treatment (dose response, after 24h) shows that T47D cells are more susceptible to tamoxifen compared to MCF-7 cells (figure 14).

A.



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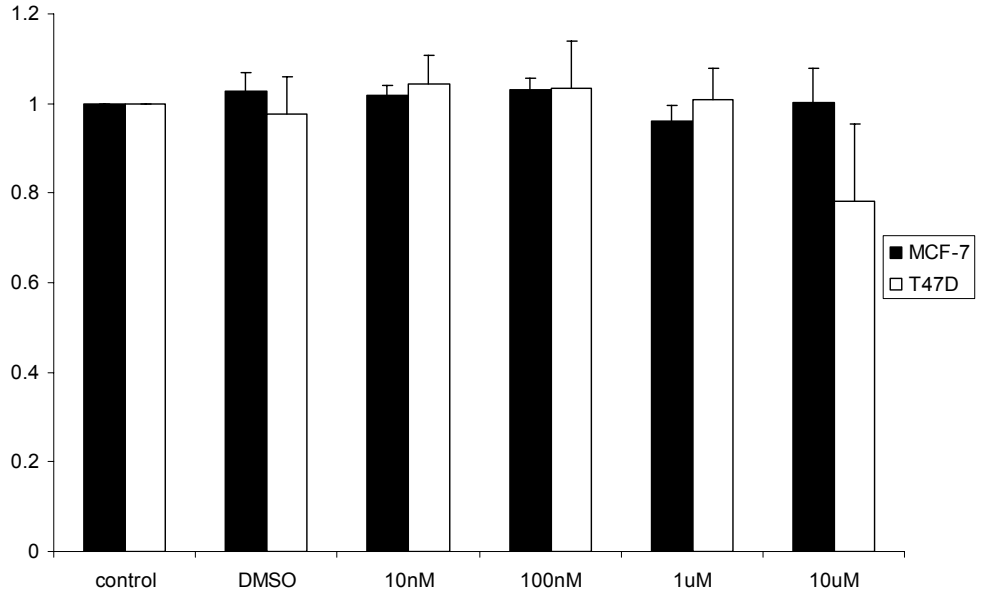

figure14: Cell viability (XTT assay) of MCF-7 and T47D cells in serumfree (A) and serum containing (B) medium treated with different doses of tamoxifen for 24h

For this experimental series, a stable TRPV6 expressing MCF-7 cell line would be useful to determine weather tamoxifen is more effective in cells expressing high levels of TRPV6.

G) Endocytosis in not involved in the effect of tamoxifne on TRPV6

The results of my second paper show that TRPV6-mediated calcium uptake is inhibited by tamoxifen. To ensure that this effect is not due to endocytosis, I performed membrane biotinylation and selection of the membrane fraction with streptavidin beads of MCF-7 cells transiently transfected with an EYPF-TRPV6 constuct. Subsequently, the Western blot using a GFP-antibody showed no difference in the amount of TRPV6-EYPF on the membrane (figure 15). The importance of this experiment is ensure that the observed inhibitory effect of tamoxifen on TRPV6-mediated calcium influx has nothing to do with endocytosis of TRPV6 on the membrane. There is no difference in detected protein with or without tamoxifen and therefore, no endocytosis involved in this effect.

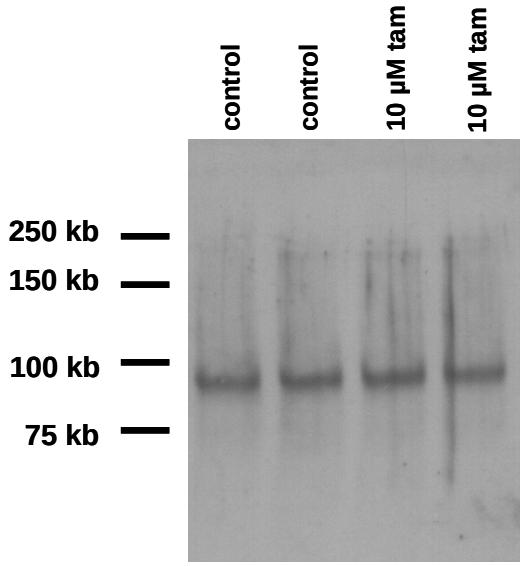

Figure 15: Membrane biotinylation of MCF-7 cells transiently transfected with a TRPV6-EYFP construct and treated for 10 min with 10 µM tamoxifen, separation with streptavidin beads and Western blot.
primary.antibody: anti-GFP antibody (ab13970) 1:2000, ON, 4°C;
sec. antibody: HRP-rabbit anti-chicken/turkey IgG 1:8000, 2h, RT

H) Detection of TRPV6 knockdown using ion imaging

Recently in collaboration with Gergely G. Kovaces, I could also succeed in finding a model to demonstrate a knock-down of TRPV6 with ionic imaging. The KD-value of Fura-2 for cadmium is much lower than for calcium and it is known that TRPV6 can transport cadmium as well. Therefore, we tried to use cadmium influx to monitor small differences in TRPV6 expression after transfecting MCF-7 cells with a TRPV6shRNA-GFP contruct. The obtained results show a significant decrease in cadmium influx in the transfected cells. Therefore, this assay might be useful to evaluate the effects of the TRPV6 knockdown in breast cancer cells (figure 16).

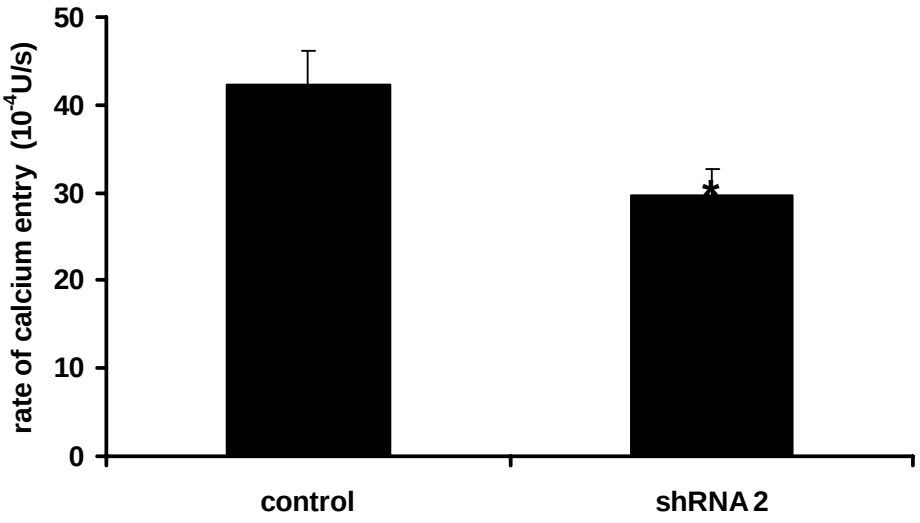

Figure 16: T47D cells, effect of with a Sure Silencing™ shRNA plamid of TRPV6 (2, Part # 1019A) (pGeneClip™ hMGFP Vector) from SABiosciences, MD, USA

I) Resveratrol might inhibit TRPV6-mediated calcium uptake

Similar to tamoxifen a study using resveratrol was performed. Resveratrol is a plant polyphenol and exhibits a similar structural characteristic as tamoxifen. Resveratrol can reduce cell viability in MCF-7 and T47D cells (data not shown). Therefore, we tested its inhibitory potential and we could detect that resveratrol inhibits TRPV6 mediated Ca^{2+} uptake in TRPV6 expressing

xenopus ooctes dose dependently (figure 17).

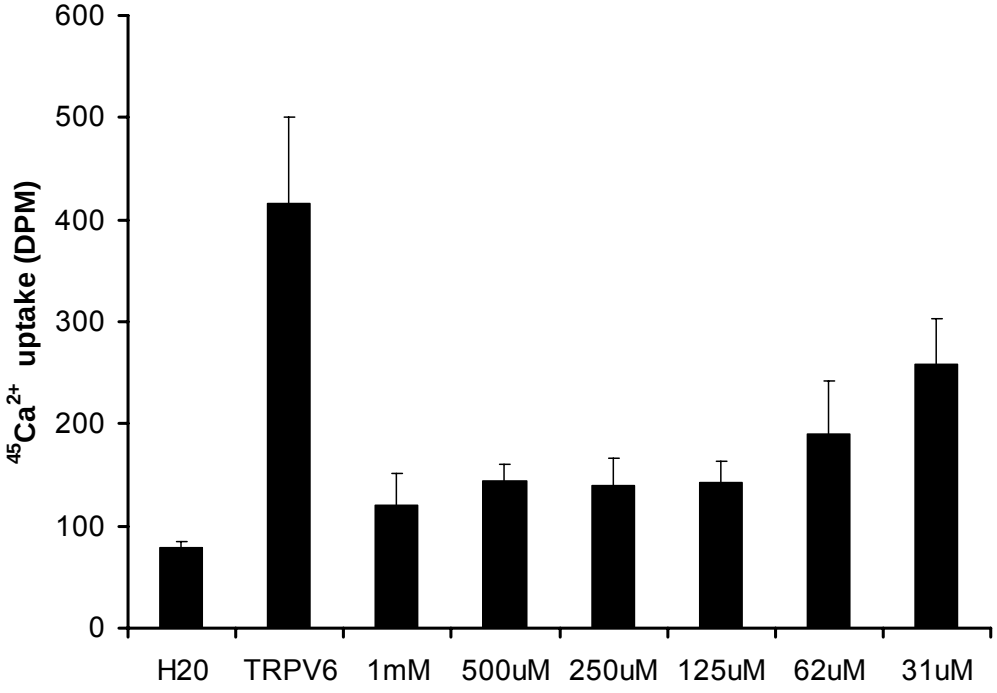

Figure 17. Resveratrol inhibits TRPV6 calcium transport activity of $^{45}Ca^{2+}$ into Xenopus oocytes expressing TRPV6. Dilutions of resveratrol were used to inhibit TRPV6 mediated calcium uptake into oocytes. Columns, mean; bars, SD; N=6.

The cell viabitlity of T47D and MCF-7 cells after 24h of resveratrol treatment with different doses in serum-free medium is shown in figure 18 and figure 19.

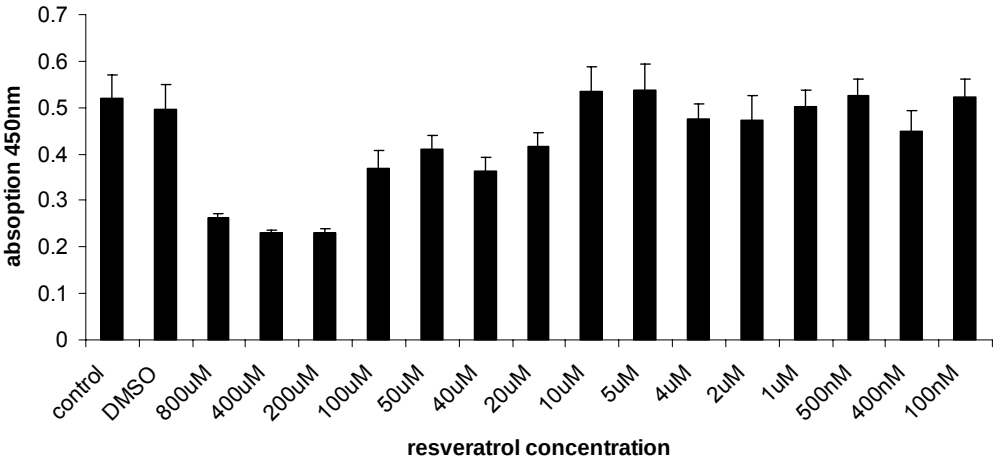

Figure 18: T47D cell viability (XTT assay) after 24h resveratrol treatment with different doses and without serum

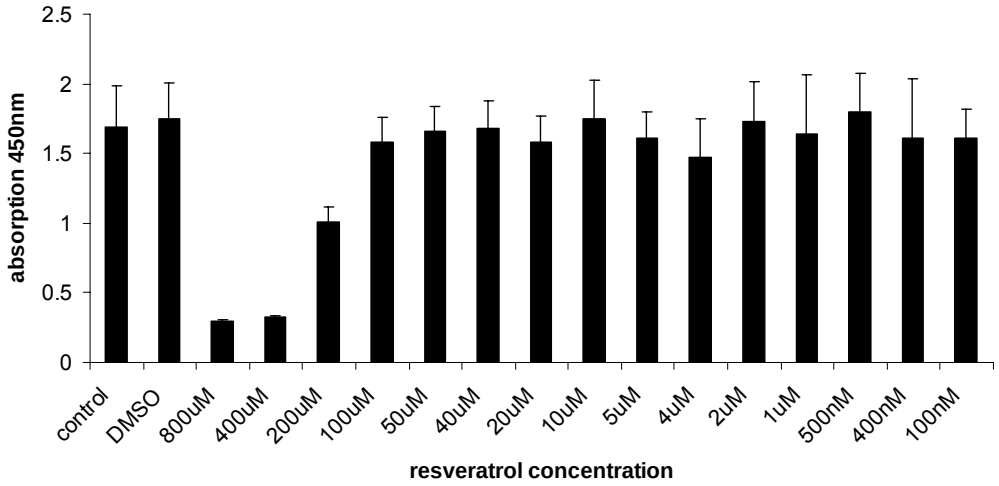

Figure 19: . MCF-7 cell viability after resveratrol treatment for 24h with different doses without serum

It was clearly demonstrated that resveratrol reduces cell viability of T47D and MCF-7 breast cancer cells in a dose-dependent manner. For this project, it would be interesting to see how potent resveratrol is in inhibiting Ca^{2+} uptake in TRPV6 expressing MCF-7 cells. This would be possible to test using Ca^{2+} imaging. Resveratrol is also known to inhibit PKC function and might be therefore a promising compound for conducting studies in regard to previous results (218).

5. Overall discussion and outlook

This PhD thesis addresses the role of TRPV6 in the pathogenesis of adenocarcinomas. The first part of the project focused on the role of TRPV6 in breast cancer progression. We started by confirming the upregulation of TRPV6 in breast cancer tissues and then demonstrated that TRPV6 is important for the proliferation of breast cancer cells *in vitro*. A knockdown of TRPV6 using the siRNA approach led to a significant decrease in cell viability in T47D breast cancer cells compared to untreated cells. Calcium and calcium influx directly controls life and death decisions in cells. Calcium signaling is required for cell proliferation and changes in intracellular calcium levels can influence the cell cycle. Therefore, the concentration of calcium is highly regulated within the cellular compartments. The G1 phase, the G1/S, and the G2/M transition require special calcium signaling pathways, e.g. for the expression of immediate-early genes, such as FOS, JUN and MYC (219). In this context it is interesting that TRPC6 and TRPV6 have both been shown to modulate NFAT-dependent gene transcription in prostate cancer cell lines (220;221). It was also found that oncogenes interact with the intracellular calcium homeostasis. On the one hand, calcium signaling can be influenced by oncogenes. The proto-oncogene Ras can bind to PLCε and activate it and subsequently increase the generation of IP_3 (222). On the other hand, calcium can modulate oncogene signaling. For example, the Ras pathway and the ERK1 and ERK2 pathways are affected by calcium (223-225).

Calcium release from the ER and resulting calcium influx into the mitochondria is required for apoptosis (226). In tumor cells, the calcium release from the ER can be decreased by BCL2 that inhibits the calcium pump SERCA2 (227;228). This might be one mechanism how tumor cells increase their chances of survival. If the calcium flux to the mitochondria is reduced, apoptosis is probably reduced. Roderick *et al.* suggests in a review article that control of cancer cell proliferation by inhibitors of plasma membrane calcium channels has received much attention and remains a potential strategy for tumor medication (229).

TRPV6 that is expressed at considerably higher levels in cancer tissue compared to normal tissue would therefore be a promising therapeutic target that could be blocked. No specific TRPV6 inhibitors are known so far, although there are numerous reports on the role of TRPV6 in specific cancer types. Other known TRP channels that are highly expressed in cancer cells are TRPM8 and TRPM1. Their protein expression is changed from normal to tumorigenic stage. The TRPC1, TRPC6, TRPM5, and TRPV1 are also increased in cancer tissues (230-234). Certainly, some of these channels might also be therapeutic targets or diagnostic markers.

Our hypothesis that TRPV6 may serve as s therapeutic target for breast cancer is supported by the outcome of the second part of this project. Given the chemical structure of tamoxifen, which resembles that of known calcium channel blockers, we tested its inhibitory potential on TRPV6 mediated calcium influx. Indeed, we demonstrated that tamoxifen citrate inhibits TRPV6 activity. This effect does not work via the estrogen receptor, but the inhibition involves PKC modulation, as demonstrated via activation of PKC which reduced the inhibitory effect of tamoxifen. Interestingly, calphostin C, a known inhibitor of PKC, had no additive effect on the tamoxifen induced calcium influx inhibition and it inhibited calcium influx independent of tamoxifen. Treatment of MCF-7 breast tumor cells with calphostin C resulted in cell death (235). Calphostin C is a potent inhibitor of PKC that operates through a novel mechanism, involving binding to a calcium-induced hydrophobic site on the PKC regulatory domain and preventing activation by DAG and phorbol esters (236;237).

Therefore, we conclude that the inhibitory effect of tamoxifen on calcium influx via TRPV6 is mediated by PKC. It would also be important to identify the amino acid residue where PKC phosphorylates TRPV6 in order to establish the link between PKC and TRPV6.

The exact role of PKC in tamoxifen-induced apoptosis is not clear and there are some contradictions as to whether tamoxifen activates or inhibits PKC (238). In a partially purified rat brain PKC preparation, tamoxifen inhibits PKC activity with an IC_{50} of 25μM (239). Tamoxifen induces membrane translocation and down-regulation of PKC following transient activation and

this may be mediated through release of arachidonic acid and generation of oxidative stress (240). Since PKC activity is greater in neoplastic breast tissue as compared to normal breast tissues, tamoxifen is likely to contribute *in viv*o to the inhibition of proliferation by virtue of its PKC-inhibitory action (241).

The PKC family comprises 12 serine threonine kinases that are divided into 3 groups, the classical isozymes (cPKC):α, β1, β2, γ, the novel isozymes (nPKC): δ, ε, η, θ and the atypical isozymes (aPKC): ξ, 1/λ. PKC activation in general starts with stimulation of tyrosine-kinase receptors and G-protein-coupled receptors that activate phospholipase C. Activation of phospholipasse C increases the plasma membrane level of diacylglycerines and results in re-localization and activation of PKC isozymes. Next, PKC binds to the plasma membrane which induces conformational changes that expose the binding sites of the kinase domain and the MEK-ERK (242;243) and PI3K-Akt (244) pathways get activated. PKC is involved in cell proliferation, differentiation, apoptosis, and angiogenesis and the different PKC isozymes have tissue specific roles (245). *In vitro* studies suggest a positive correlation between elevated PKC levels and both the invasive and chemotactic potential of human breast cancer cell lines (246). It was shown that PKC-α is over-expressed in breast cancer (247). Stable transfection of T47D cells with PKC-α renders T47D human breast cancer cells hormone-independent *in vitro* and *in vivo*. Also some results in this study suggest that PKC-α over-expression may predict resistance to tamoxifen (248). Estrogen receptor positive breast cancer cell lines express a considerable amount of PKC-δ, whereas estrogen receptor negative breast cancer cell lines express very little PKC-δ (249). PKC-δ is likely to play a major role in anti-estrogen resistance in breast cancer cells and has been linked with acquired resistance to tamoxifen in breast cancer patients (250). Our results demonstrate that tamoxifen has a similar effect on TRPV6 mediated calcium uptake and therefore suggest that PKC-δ is not involved in this effect. PKC-ε expression and activation have been reported to be decreased in malignant breast cancer as compared to adjacent normal tissue (251). The different expression levels of PKC-isozymes make it more challenging to target the right PKC isozyme form in breast cancer. One study was designed to investigate the role of the PKC-β specific inhibitor LY379196. The study revealed that this inhibitor and found

significantly reduced growth of the breast cancer cells MCF-7, MDA-MB-231, and BT474 (252). In the future, one could identify the specific isozyme responsible for the tamoxifen-induced inhibition of TRPV6. The problem of the application of PKC inhibitors in cancer therapy is that there are many isoforms of PKC expressed in different tissues with different functions. Therefore, the probability of serious side-effects for PKC inhibitors is very high. In summary, our data strongly suggest that the mechanism of action of tamoxifen involves PKC mediated reduction of calcium influx via TRPV6 in breast cancer cells. This could explain the beneficial effect of tamoxifen on estrogen receptor negative breast carcinomas. Therefore, TRPV6 could serve as an interesting new target for the treatment of certain cancer types that highly express TRPV6.

A fellow of our group is also involved in developing specific inhibitors against TRPV6. The original series of inhibitors that were designed by Christopher Landowski and Matthias Hediger were tested for their ability to block TRPV5 and TRPV6 mediated calcium uptake using radiotracer uptake studies in TRPV6 or TRPV5 expressing Xenopus oocytes. We performed frog surgery to get the oocytes, injected cRNA of TRPV6 and TRPV5 into oocytes, and performed radioactive uptake studies. A paper with the outcome of this project is in preparation:
Christopher P. Landowski, Katrin A. Bolanz, Yoshiro Suzuki, and Matthias A. Hediger. “Chemical inhibitors for the Ca^{2+} entry channel TRPV6.”
To validate the potential of TRPV6 as therapeutic target, *in vivo* experiments will need to be performed. The xenograft approach would be useful to test the inhibitors in a mouse model. Yet, a better approach would be to use the MMTV-neu mice as breast cancer model. These transgenic mice carry a rat *Erbb2*/HER-2/*neu* oncogene tagged with ovalbumin epitopes OT-I and OT-II, which are recognized by T-cell receptors, under the control of the MMTV promoter (termed fusion protein *neuOT-I/OT-II*). In addition, this strain carries a mouse Trp53 mini-gene, harboring a G to A point mutation in codon 172 (changing Arg to His; R172H) driven by the rat whey acidic protein promoter. Approximately 85% of compound mutant females develop focal mammary tumors at 6-10 months of age. Both virgin and breeder mice develop tumors.

Approximately 37% of tumor-bearing mice develop metastatic disease in the lung. High expression of *neu* is detected in tumor tissue while very low levels are found in lung and ovary. Female mice carrying only the *neuOT-I/OT-II* mutation develop focal mammary tumors at approximately 18 months of age (253). In preliminary studies, we tested paraffin sections of these mice, but we could not yet localize TRPV6 successfully with our antibodies (Figure 20).

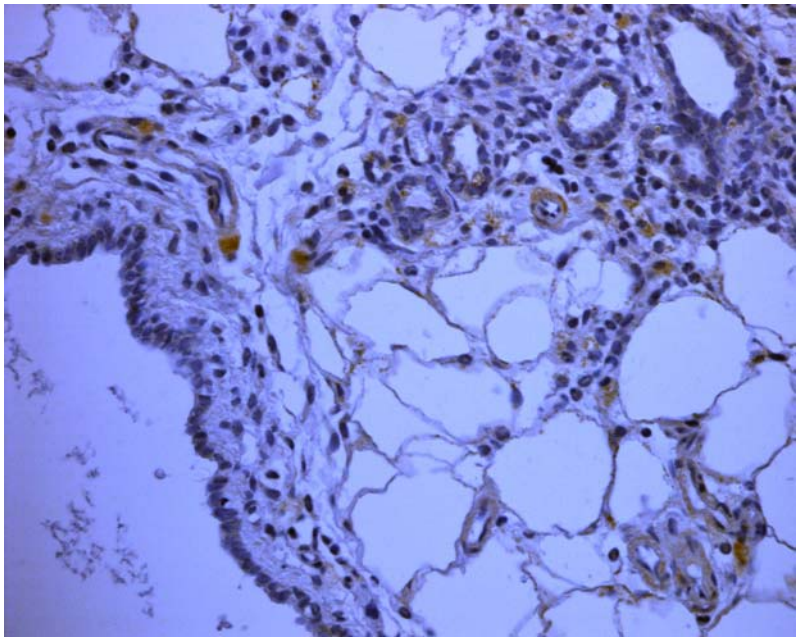

Figure 20. Preliminary data: Immunohistochmical staining of a paraffin section of MMTV-neu mouse cancer tissue with anti-TRPV6 antibody.

The TRPV6 expression in these mice could be also analyzed using real-time quantitative PCR to investigate weather TRPV6 mRNA expression increases with increasing tumor stages. The MMTV-neu mice could serve as an excellent animal model to investigate the efficiency of TRPV6 inhibitors *in vivo.* Taken together, the results of this PhD-study highlight the potential of TRPV6 as a therapeutic target for cancer treatment and thus shed light on the important role of calcium channels in the pathogenesis of adenocarcinomas in general.

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Declaration of Originality

Last name, first name: **Bolanz, Katrin Annika**

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I hereby declare that this thesis represents my original work and that I have used no other sources except as noted by citations.
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I am aware that in case of non-compliance, the Senate is entitled to divest me of the doctorate degree awarded to me on the basis of the present thesis, in accordance with the “Statut der Universität Bern (Universitätsstatut; UniSt)”, Art. 20, of 17 December 1997.

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