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Inducible expression of the human TRP3 cation channel in a prostate cancer cell line

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List of Abbreviations

$[Ca^{2+}]_i$	Concentration of free intracellular calcium ions
CHO	Chinese hamster ovary
CMV	Cytomegalo virus
DAG	Diacylglycerol
DDI- H_2O	Double de-ionised water
ER	Endoplasmic reticulum
EGTA	Ethylene glycerol-bis-(ß-aminoethylether) N, N, N', N'-tetraacetic acid
FCS	Fetal calf serum
Fura-2/AM	Fura-2 acetoxymethyl ester
GFP/EGFP	Green fluorescence protein/ Enhanced GFP
HA	Haemagglutinin
IP_3	D-myo-inositol 1,4,5-trisphosphate
kDa	Kilo Dalton
kb	Kilo bases
lmp agarose gel	Low melting point agarose gel
LNCaP cells	Lymphatic nodule cancer of prostate cells
OAG	1-oleolyl-2-acetyl-sn-glycerol
OPA buffer	One Phor All buffer
PLC	Phospholipase C
RACC	Receptor activated calcium channel
SDS	Sodium dodecyl sulphate

SERCA	Sarcoplasmic endoplasmic reticulum calcium ATP-ase
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SOCC	Store operated calcium channel
TBS-T buffer	Tris-buffered saline with Tween-20
TG	Thapsigargin
TRPC	Mammalian transient receptor potential channel
hTRP	Human transient receptor channel
TRPL	Transient receptor potential like
VOCC	Voltage operated calcium channel

Summary

Prostate cancer is the second leading cause of death from cancer in men and there is currently no curative treatment for the metastatic stage of the disease. Effective treatments are therefore urgently needed. Inducing apoptotic cell death in prostate cancer cells is one possible approach. Calcium has been shown to be involved in apoptosis of prostate cancer cells. Increased calcium levels have been shown to induce cell death in a number of prostate cancer cell lines suggesting that modulation of intracellular calcium may be of therapeutic value in prostate cancer cells. Expression of calcium channels in cancer cell lines is one approach by which intracellular calcium can be increased to induce cell death.

The aims of this thesis were therefore to inducibly express hTRP3 (the human homologue of hTRP3 a member of the TRP calcium channel family) in LNCaP prostate cancer cells and to examine its effects on calcium inflow and cell death. TRP3 was cloned into the pBI-EGFP expression vector that is part of the Tet-on inducible system and transiently co-transfected with the Tet-on vector into LNCaP cells.

Western blot analysis and fluorescence revealed that both EGFP and hTRP3 were inducibly expressed (in the presence of doxycyclin) in LNCaP cells. Calcium measurements in hTRP3 transfected, doxycyclin treated LNCaP cells suggested that hTRP3 was activated by thapsigargin induced store depletion and OAG, but was not constitutively active. Transient transfection of hTRP3 into LNCaP cells also did not induce apoptosis, which was confirmed by Hoechst 33258 staining. This may have been

due to low levels of hTRP3 expression, which resulted in only small increases in stimulated calcium inflow. To determine if hTRP3 can be used to induce cell death in LNCaP cells further experiments optimising expression and activation of hTRP3 need to be carried out.

1. Introduction

1.1 Prostate cancer

Prostate cancer is the second leading cause of death in men after lung cancer. The incidence increases dramatically with age: about 70 % of all men with clinically diagnosed prostate cancer are 65 years or older. The American Cancer Society estimated that 189,000 new cases as well as 30,200 deaths will result from prostate cancer in 2002 (www.cancer.org).

The current therapy for early, organ confined stages of prostate cancer, is surgery. Surgery at these stages of the disease is usually curative. Patients with extra prostatic disease eventually require systemic androgen ablation therapy. Prostate cancer cells are androgen dependent and undergo apoptosis when deprived of androgen by surgical or chemical ablation (Denmeade et al., 1996). However androgen withdrawal is not curative and androgen independent prostate cancer cells commonly arise. Once this stage of the disease has been reached prostate cancer is generally lethal and treatment with androgen ablation is no longer effective. Since less than 5 % of the cancer cells proliferate every day, chemotherapy and current anti-cancer drugs, which have an effect on fast proliferating cancer cells have proven to be of limited success. Therefore effective treatments for the androgen independent stage of prostate cancer are urgently needed.

1.2 Androgen independence in prostate cancer

Several mechanisms have been described of how prostate cancer cells survive androgen withdrawal. Some groups have described up-regulation of the androgen receptor in prostate cancer cells that increases the sensitivity to androgen so that those cells survive with very low androgen levels that are present after androgen ablation (Denmeade et al., 1996, Koivisto et al., 1997). Other groups have described that androgen independence arises and several mechanisms have been reported. Sadi et al reported a greater androgen receptor heterogeneity in androgen independent prostate cancer cells (Sadi and Barrack, 1993) and several mutations of the androgen receptor have been described (Taplin et al., 1995, Elo et al., 1995). Naturally occurring amino acid substitution in the androgen receptor ligand binding domain has been shown to change its ligand specificity and affinity. These changes allow the androgen receptors to bind anti-androgens, adrenal androgens, and other steroids such as estrogen and progesterone allowing the cells to grow in the presence of these hormones (Hakimi et al., 1996, Veldscholte et al., 1990, Berrevoets et al., 1993).

Several molecular changes in androgen independent cancer cells have been described that allow prostate cancer cells to resist apoptosis induced by androgen withdrawal. Mutations in the p53 tumor suppressor gene and up-regulation of the proto-oncogene bcl-2 are two key alterations (Bruckheimer et al., 1999). Bcl-2 has been shown to inhibit apoptosis in androgen independent prostate cancer cells (Furuya et al., 1996). The tumour suppressor p53 induces apoptosis via up-regulation of the cell death effector of the bcl-2 family, bax (Soussi and May, 1996, Korsmeyer, 1995). Up-regulation of bcl-2 and mutations in p53

therefore inhibit apoptosis and are commonly found in androgen independent prostate cancer cells (Apakama et al., 1996).

Three therapeutic approaches are currently being explored in an attempt to effectively treat androgen independent prostate cancer. One approach is to use the host's immune system to destroy tumour cells. The second is to interrupt tumour growth by inhibition of angiogenesis. The last approach is to induce apoptosis in prostate cancer cells (Denmeade and Isaacs, 1997).

1.3 Apoptosis and Calcium

Androgen ablation induces apoptosis in androgen dependent prostate cancer cells. Increased cytosolic calcium is seen in these cells suggesting that it may play a role in androgen ablation induced apoptosis (Colombel and Buttyan, 1995, Isaacs et al., 1992). In further support of this idea calcium channel blockers such as Nifedipine have been shown to antagonise increases in calcium and prevent apoptosis in androgen deprived cells (Furuya et al., 1996).

Treatment of LNCaP prostate cancer cells with thapsigargin, a sesquiterpene lactone has been shown to result in apoptosis. Thapsigargin is a selective inhibitor of the calcium dependent sarco- and endo-plasmic reticulum ATP-ases (SERCA). Inhibition of the SERCA pumps by thapsigargin results in depletion of the ER calcium stores and a 3-4 fold rise in intracellular calcium (Furuya et al., 1994). This result indicates that calcium may regulate thapsigargin induced apoptosis. However the precise mechanism remains

unclear. It has been reported that thapsigargin induces apoptosis due to store depletion independently of an increase in cytosolic calcium (Skryma et al., 2000, He et al., 1997, Bian et al., 1997). However others have suggested that an increase in cytosolic calcium rather than store depletion alone induces apoptosis (Dowd et al., 1992, Wang et al., 1999).

Despite these contradictory results as to the mechanism it appears that modulation of intracellular calcium plays a role in apoptosis in these cells. In further support of a role for calcium in apoptosis, Tapia-Vieyra et al (2001) have demonstrated that an intracellular calcium permeable, non-selective cation channel is activated in LNCaP cells during the onset of apoptosis induced by the withdrawal of serum or ionomycin (Tapia-Vieyra and Mas-Oliva, 2001). Taken together these results demonstrate that modulation of intracellular calcium can mediate apoptosis in prostate cancer cells. Thus calcium is a potential therapeutic target in the treatment of prostate cancer. An alternative way of altering calcium homeostasis is to overexpress calcium channels that may induce apoptosis in prostate cancer cells.

1.4 Calcium signalling and calcium channels

Calcium signalling plays a major role in many cell functions such as fertilisation, contraction, secretion, proliferation and death. Increased levels in cytosolic calcium are mediated by calcium inflow across the membrane or release from internal stores such as the endoplasmic reticulum. Several groups of channels have been described that are activated through different mechanisms. Voltage operated calcium channels (VOCCs)

mediate calcium entry in excitable cells such as neurons whereas receptor activated calcium channels (RACCs) increase intracellular calcium in both excitable and non-excitable cells. RACCs are membrane channels that open after binding of an agonist to its receptor. RACCs can be further divided by their selectivity for calcium into the highly selective calcium channels and the less selective non-selective cation channels. Store operated calcium channels (SOCCS) are subtypes of RACCs that are activated by calcium release from the ER. Calcium release from the ER occurs in response to an agonist binding its receptor which activates production of intracellular second messengers, eg activation of phospholipase C (PLC). This results in the two products D-myo-inositol 1,4,5-trisphosphate (IP_3) and diacyglycerol (DAG). IP_3 binds to the IP_3-receptor on the ER and leads to the release of calcium from this store which in return activates SOCCs on the plasma membrane. In addition to IP_3, the nucleotides cyclic ADP-ribose (cADPR) and nicotinic acid adenine dinucleotide phosphate (NAADP) have been shown to be important regulators of intracellular Ca^{2+} release of a growing number of cell types (Montell et al., 2002, Zitt et al., 2002).

The TRP family named after the Drosophila transient receptor potential mutant is probably the best characterised group of RACCs. TRP channels consist of subunits with six membrane spanning domains and may form tetramers in which the amino acids that link the fifth and sixth transmembrane domain line the pore. TRP channels show a high conductance for cations but at the same time little selectivity. Based on their structure the TRP family has been divided into three groups: short, long and osm-like TRPs. The short TRP subfamily includes Drosophila TRP and TRPL and the mammalian TRPs 1-8

(Harteneck et al., 2000). The mammalian TRPs can be further divided into four subfamilies: TRP1; TRP4, 5; TRP2 and TRP3, 6, 7, 8 due to similar sequence and functional features.

1.5 TRP1

TRP1 expression alone has not been shown to result in a measurable ion current. However TRP1 has been reported to be activated by thapsigargin induced store depletion by one group (Zitt et al., 1996) and to be DAG activated by another (Lintschinger et al., 2000). It has also been shown to co-assemble with other TRP subunits and might be a component of different heteromeric TRP complexes (Strubing et al., 2001).

1.6 TRP2, 4 and 5

TRP4 and TRP5 are activated following stimulation of G_q coupled receptors and by receptor tyrosine kinases. G_q and receptor tyrosine kinase signalling result in PLC activation indicating that a PLC product regulates TRP4 and 5 activation. However the two primary products of PLC activation, IP_3 and DAG have not been shown to activate TRP4 or 5 (Schaefer et al., 2000) and the detailed mechanism of their activation remains unclear. There are few reports examining TRP2 (Vannier et al., 1999, Wissenbach et al., 1998, Liman et al., 1999).

1.7 CaT1 and TRP-p8

The most recently cloned calcium channels that are related to the TRP family are CaT1 and TRP-p8. CaT1 is related to the TRP family and it has been shown that CaT1 mRNA levels are elevated in prostate cancer cells compared to benign hyperplastic cells. Higher expression levels have also been correlated with higher tumour grading. Androgen has been shown to decrease CaT1 mRNA levels whereas a specific androgen receptor antagonist induced CaT1 mRNA. This suggests that androgen down regulates CaT1 expression (Peng et al., 2001). Taken together these results implicate CaT1 in prostate cancer development.

TRP-p8 has been shown to share homology with the TRP family, in particular with the human TRPC7 gene. Tsavaler et al (2001) have shown that trp-p8 mRNA expression in normal human tissue is restricted to prostatic epithelial cells and that it is elevated in prostate cancer. TRP-p8 is also expressed in a number of non-prostatic cancers such as breast, colon, lung and skin (Tsavaler et al., 2001).

1.8 TRP3, 6 and 7

TRP3, 6 and 7 have been shown to be 75 % homologous, to form cationic non-selective channels and to be inwardly and outwardly rectifying. Furthermore it has been reported that they are sensitive to intracellular calcium and are activated by DAG (Hofmann et al., 1999) as well as the DAG analog OAG (Hofmann et al., 1999, Okada et al., 1999). TRP3 was the focus of studies carried out in this thesis and is therefore described in more detail below.

1.9 TRP3 calcium channel

Kiselyov et al (1998) reported that thapsigargin stimulated TRP3 but was not as effective as agonist (carbachol) stimulation. Thapsigargin treated cells could also be further stimulated using the agonist. They therefore suggested that both store depletion and IP_3 receptor bound IP_3 were required for activation (Kiselyov et al., 1998). Zhu et al have shown that TRP3 expressed in HEK-293 cells induced calcium inflow after the activation of phospholipase C but not after store depletion (Zhu et al., 1998). However, there may be a subpopulation that responds to store depletion (Zhu et al., 1998). Consistent with this report McKay et al have also shown that TRP3 transiently transfected into HEK-293 could not be activated by thapsigargin suggesting that it is not store operated (McKay et al., 2000). However calcium inflow occurred after PLC-linked receptor activation or treatment with the DAG analog OAG (McKay et al., 2000). Lintschinger et al (2000) have also shown that treatment of TRP3 expressing HEK-293 cells with OAG results in increased ion conductance. They further reported that co-expression of TRP1 and 3 resulted in a constitutively active conductance that could be further increased by OAG stimulation. No conductance could be detected when HEK-293 cells expressed TRP1 alone and were treated with OAG (Lintschinger et al., 2000).

Zitt et al reported that TRP3 cDNA injected into CHO cells produced cation currents that were constitutively active and did not respond to store depletion due to thapsigargin or IP_3. These currents were weakened by strong intracellular calcium buffering and showed little selectivity for Ca^{2+} over Na^{+}. However ionomycin in the presence of extracellular calcium as well as infusion of calcium led to increased currents. These results suggest

that intracellular calcium signals were involved in increased TRP3 activity but not store depletion alone.

Taken together all these results suggest that the mechanism by which hTRP3 is activated varies in different cell lines and that the presence of other TRP channels may effect how it is regulated.

1.10 Calcium channels and cell death

Increased intracellular calcium has shown to be involved in apoptosis. Different methods have been used to achieve a calcium increase and calcium channels may be promising candidates. Indeed Zitt et al (1997) have reported that 2 days after injection of TRP3 cDNA the majority of the cells were dead and that those surviving were those expressing low levels of TRP3. It was suggested that the spontaneous constitutive activity of TRP3 led to a lethal calcium overload of the cells. They also suggested that calcium triggered the activation of TRP3, enhancing the effect of calcium overload (Zitt et al., 1997). In further support of these results, experiments from our laboratory have shown that overexpression of TRPL in LNCaP cells results in a constitutive calcium inflow and induces cell death in 20 % of transfected cells after 72 hrs (Zhang, 2001). Nuclear staining with Hoechst 33258 as well as measurements of caspase activity, tests commonly used to detect apoptosis further revealed that overexpression of TRPL induced apoptosis. These two reports suggest that calcium channels of the TRP family increase intracellular calcium in cells to levels that induce cell death. However TRPL has shown to only kill 20 % (Zhang, 2001) whereas TRP3 has been reported to kill the majority of

cells (Zitt et al., 1997). No further information on the type and percentage of cell death occurring in CHO cells overexpressing TRP3 was given. We therefore decided to examine the effects of hTRP3 (human variant of the mammalian TRP3) expression on calcium inflow and cell death in LNCaP cells. Since hTRP3 expression might have a cytotoxic effect on LNCaP cells we decided to inducibly express hTRP3 in LNCaP cells.

1.11 Inducible hTRP3 expression

Two tetracyclin regulated inducible gene expression systems are currently available from Clontech, the Tet-on and the Tet-off system. Gschwend et al (1997) reported that the Tet-on system used in LNCaP cells produced no background expression and at the same time high inducible expression of the gene of interest (Gschwend et al., 1997). The Tet-on system was currently being used in the laboratory and was therefore chosen in an attempt to inducibly express TRP3. The Tet-on system consists of two plasmids, the Tet-on and the response vector containing a reporter gene (eg EGFP) and a gene of interest (eg hTRP3). The Tet-on regulatory plasmid constitutively produces a transcriptional activator and also contains a Neomycin (G418) resistence gene. The response plasmid (pBI-EGFP) expresses the gene of interest as well as the reporter gene under the control of a bi-directional promoter made up of a single TRE element and two minimal promoters. Binding of the transcriptional activator to the TRE element of pBI-EGFP in a doxycyclin dependent manner induces transcription of both EGFP and the gene of interest.

2. Aims

Intracellular calcium has been implicated in the development of prostate cancer and in the cell death of a number of prostate cancer cell lines (eg LNCaP, PC3). Results from our laboratory have also shown that expression of TRPL, a member of the TRP family that is constitutively active, increases calcium inflow and induces apoptosis in LNCaP cells (Zhang, 2001). These results suggest that modulation of intracellular calcium may be a potential therapeutic target for prostate cancer. The relatively small induction of apoptosis clearly limits the use of TRPL as a potential treatment in prostate cancer. We therefore decided to explore the effects of another member of the TRP family on a prostate cancer cell line. hTRP3, has been shown to be constitutively active in some cell lines and its overexpression in CHO cells has been shown to kill the majority of transfected cells. The aims of this thesis were therefore to inducibly express hTRP3 in LNCaP cells using the Tet-on inducible expression system. Further to examine whether hTRP3 could alter calcium inflow in LNCaP cells in a constitutively active, store depleted or OAG regulated manner. And finally to examine the effects of hTRP3 expression on cell death.

3. Materials and methods

3.1 Materials

Cell culture: LNCaP cells were kindly provided by Professor Wayne Tilley, Department of Surgery (Flinders University, Adelaide, SA, Australia). RPMI media 1640 (RPMI), trypsin, penicillin, streptomycin and fetal calf serum (FCS) were obtained from Life

Technologies (Melbourne, VIC, Australia). Ethylenediaminetetra-acetic acid di-sodium salt (EDTA.Na_2H_2O) came from AJAX laboratory Chemicals (NSW, Australia). Dimethylsulphoxide (DMSO) and Hepes were from BDH Chemicals Australia (Kilayth, VIC, Australia).

Plasmid DNA: The pcDNA3 containing the hTRP3 DNA was a kind gift from Professor Dr. T. Gudermann (Berlin, Germany). The pGL3-Basic vector was from Promega Corporation (Madison, USA) whereas the pBI-EGFP vector was supplied by Clontech Laboratories (Palo Alto, USA).

Molecular biology: DNA molecular weight marker (SPP-1bacteriophage DNA restricted with Eco RI) were from Bresatec (Thebarton, SA, Australia) whereas T4 DNA ligase, T4 ligase buffer, adenosine triphosphate (ATP), restriction enzymes, restriction enzyme buffers were from Amersham Pharmacia Biotech (unit 38, Castle Hill, Sydney, NSW, Australia).

Western blot analysis: Sodium dodecylsulphate (SDS) were from Roche (Castle Hill, NSW, Australia), leupeptin and pepstatin A were obtained from Auspep Pty.Ltd. (Parkville, Australia). Phenylmethylsulfonylfluoride (PMSF), glycine, _-mercaptoethanol, acrylamide, N'N'bis-methyleneacrylamide, bovine serum albumin (BSA), paraformaldehyde, ethyleneglycol-bis (_-aminoethyl ether)-N,N,N'N'-tetraacetic (EGTA), sodium pyruvate, dithiothreitol (DTT), bromophenol blue, ammonium persulfate (AP), N,N,N',N'- tetramethylethylenediamine (TEMED), 5-bromo-4-chloro-3-

indolyl phosphate p (BCIP), Tris base, Triton X-100, Tween 20, coomassie brilliant blue G-250 and Hoechst 33258 were from Sigma-Aldrich (Castle Hill, NSW). Molecular weight protein markers (seeBlue TM pre-stained standard) were obtained from Novex (San Diego, CA 92121, U.S.A) whereas glycerol was obtained from Difco Laboratories (Detroit, Michigan 48515, U.S.A.). Non fat milk powder was obtained from Bonlac Foods Ltd (VIC, Australia).

hTRP3 antibodies: The 12.5 AP anti TRP3 rabbit antibody was raised by Helen Brereton and Lyn Harland in our laboratory against the 15 amino acid synthetic peptide (FTYARDKWLPSDPQC) corresponding to a sequence within the S_3 –S_4 loop of rat TRP3. The anti TRP3 rabbit antibody was a gift from Craig Montell, Baltimore, USA.

Calcium measurements: Fura-2 acetoxymethylester (Fura-2/AM) and Pluronic F127 was from Molecular Probes (Eugene, OR, USA). Digitonin was from Sigma-Aldrich (Castle Hill, NSW).

Other chemicals: glycerol, 85 % (w/v) phosphoric acid, and calcium chloride ($CaCl_2$) came from AJAX laboratory Chemicals (NSW, Australia). Methanol and ethanol were obtained from Merck Pty. Ltd. (Kilsyth, VIC, Australia). All other chemicals and materials were of the highest grade commercially available.

3.2 Cloning of the pBI-EGFP/hTRP3 vector

3.2.1 Generation of electro competent bacteria

A single colony of the bacterial strain XLI Blue was inoculated into LB (Luria Bertani, 10 ml) media and incubated over night at 37 °C. The overnight culture was then inoculated into LB (500 ml) and incubated for a further 3 hrs. When the optical density (660 nm) of the culture was greater than 0.6 it was centrifuged (2500 g, 15 min, 4 °C). The supernatant was removed and the bacterial pellet was washed twice with sterile water. The cells were then resuspended in 10 % glycerol (10 ml) at 4 °C and recentrifuged (2500 g, 15 min, 4 °C). The bacterial pellet was resuspended in 10 % glycerol (0.5 ml). The competent cells were then aliquoted (45 µl) and stored at -80 °C.

3.2.2 Transformation of bacteria

The DNA (100 ng in H_2O) to be electroporated was added to one aliquot of XLI-Blue (45 µl) bacteria and incubated for 2 min on ice. The bacteria were then transferred into an electroporation cuvette (Bio-Rad). The cells were electroporated using the Bio-Rad gene pulser (18 kV/cm). SOC media (1 ml) was added immediately to the bacteria, the mixture was transferred into an Eppendorf tube and incubated for 1 hr at 37 °C. It was then plated out on agar plates containing ampicillin (50 µg/ml) and incubated for 16 hrs at 37 °C.

3.2.3 Plasmid extraction

Plasmid DNA was extracted using a Qiagen Midi prep kit according to manufacturer's instructions.

3.2.4 Restriction enzyme digests

For restriction enzyme digests a total volume of 10 µl containing restriction enzyme buffer (OPA) (1 µl), restriction enzyme (1 µl), DNA (250 ng) and H_2O to make up to 10 µl was prepared on ice and then incubated at 37 °C for 1 hr. Sample (5 µl) and 6 x loading buffer (1 µl) were combined and 5 µl of the mixture was loaded onto a 1 % agarose gel. The gel was run (110 V, 20 min) until the first dye front had migrated 3/4 of the length of the gel. The DNA was stained using ethidiumbromide (0.5 µg/ml), visualised under UV light and photographed.

3.2.5 Gel purification of DNA fragments

Enzyme digests with a total volume of 40 µl were set up, 5 µl of the digested sample was run on a 1 % agarose gel, stained with ethidiumbromide and visualised under UV light to determine whether the digestion was complete. To the remaining 35 µl of the sample ethidiumbromide (1 µl) and 6 x loading buffer (5 µl) were added, all of the sample was loaded on a 1 % low melting point (lmp) agarose gel and electrophoresed for 30 min (140 V). The fragment of interest was cut out of the lmp gel under UV light and purified using hot phenol extraction. The slug containing the band of interest was weighed, made up to 300 µg with H_2O, NaCl (30 µl of a 5 M solution) was added and the mixture was vortexed and heated (70 °C, 15 min) to dissolve the agarose. An equal volume of tris saturated phenol (pH 8.0) was added and the sample was centrifuged (12000 g, 15 min). The aqueous top layer was transferred into a new tube, chloroform (200 µl) was added

and the sample was centrifuged (1 min at 12000 g). The aqueous top layer was transferred into a new tube and the DNA was precipitated using pre-cooled (-20 °C) 100 % ethanol (750 µl) over night at -20 °C. The samples were centrifuged (12000 g, 15 min, 4 °C), wash precipitated with cold 70 % ethanol (900 µl) and centrifuged (12000 g, 5 min, 4 °C). The pellet was vacuum dried in the Speedi-Vac for 5 min and finally dissolved in H_2O (5 µl).

3.2.6 Ligation reactions

A cocktail was set up containing T_4-ligase (1 µl), 10 x OPA buffer (1 µl), 10 mM ATP (1 µl), purified vector and insert (1 µl) and H_2O (5 µl) to make a total volume of 10 µl and incubated over night at 10 °C. H_2O (190 µl), glycogen (1 µl of a 1 mg/ml solution), tris buffered phenol (200 µl, pH 8.0) and chloroform in iso-amyl-alcohol ($CHCl_2$: IAA 24:1, 200 µl) were added to the samples. The samples were centrifuged (12000 g, 2 min), the upper phase transferred into a new tube and the chloroform extraction repeated. The upper phase was transferred into a new tube and sodium acetate (20 µl of a 3 mM solution, pH 5.2) and ice cold ethanol (500 µl) were added. The DNA was precipitated (30 min, -20 °C) and centrifuged (12000 g, 5 min, 4 °C). The pellet was rinsed with 70 % ethanol, centrifuged (12000 g, 5 min, 4 °C), vacuum dried for 5 min and dissolved in H_2O (5 µl).

3.2.7 Sequencing of the pBI-EGFP/hTRP3

A reverse primer of 19 bp (GGT CAT TGA ACA TGA AGG C) was constructed from the coding region of hTRP3 (499bp-518bp, gene bank Y13758, C. Montell). DNA sequencing was carried out by the DNA Sequencing Core, Flinders University of South Australia.

3.3 Cell culture of LNCaP prostate cancer cell line

3.3.1 Cell culture

LNCaP cells were routinely cultured in RPMI media supplemented with 10 % fetal calf serum, penicillin (100 units/ml) and streptomycin (100 μg/ml) at 37 ˚C in a 5 % CO_2 humidified atmosphere. LNCaP cells were passaged once a week. Media was removed and the cells were incubated for 10 min with 0.2 % (w/v) EDTA (0.2 ml) and 0.1 % trypsin (2 ml) to detach the cells. To neutralise the cell suspension RPMI (5 ml) was added. The cells were centrifuged (300 g, 5 min), resuspended in RPMI media and diluted 1:5.

3.3.2 Seeding of LNCaP cells for transfection

LNCaP cells were seeded at 100.000 cells per well in a six well plate containing RPMI media (2 ml) per well. Cells were grown for two days before reaching a confluence of 60-70 % required for transfection.

3.3.3 Growing cells on glass coverslips for calcium measurements

Round glass coverslips were sterilised with 100 % alcohol and then flamed afterwards. The coverslips were further placed in petri dishes (35 mm) and coated with a 0.01 % poly-L-lysin solution. The coated coverslips were dried over night in the laminar flow hood under UV light. The coverslips were stored at room temperature in a dry environment. For calcium measurements LNCaP cells were seeded at a density of 50.000 cells per petri dish containing RPMI media (2 ml). Cells were grown for another two days to obtain optimal adherence and 60-70 % confluence.

3.4 Transfection of LNCaP cells

3.4.1 Transient transfection of LNCaP cells

Cells were seeded at a density of 1 x 10^5 cells per well in a 6 well plate or 50 000 cells per glass coverslip and incubated for a further 48 hrs. Cells were 60-70 % confluent at that stage. 12 hrs before transfection the media was replaced with antibiotic free RPMI media since antibiotics have been shown to interfere with the transfection reagent Lipofectimine 2000. For the transfection of one well in a 6 well plate, Lipofectimine 2000 (3 µl) was added to the transfection media Optimem (200 µl) and incubated at room temperature for 5 min. Tet-on vector (1 µg) and pBI-EGFP or pBI-EGFP/hTRP3 vector (1 µg) were added to Optimem (200 µl) and then combined with the Optimem/Lipofectimine 2000 mixture. The mixture was incubated for another 30 min at room temperature. Finally the transfection mixture was added to one well in a 6 well

plate. After 6 hrs the media was changed to complete RPMI containing doxycyclin (1 µg/ml). Experiments were performed after a further 24 to 96 hrs.

3.4.2 Proliferation assay for G418 and hygromycin B

LNCaP cells were seeded into 96 well plates at a density of 5 000 cell/well in RPMI media (100 µl) and incubated for 48 hrs. The cells were then treated with different amounts of G418 or hygromycin B (100-800 µg/ml). After a further 3, 6, 9 and 12 days the cells were washed in 1 x PBS (3 x) and fixed in formal saline (100 µl of 10 % (w/v) p-formaldehyde in PBS buffer). The plates were wrapped in alfoil and stored at 4 ˚C until all time points had been collected. The fixative was shaken off the plates and the cells were stained with methylene blue (100 µl of 1 % (w/v) methylene blue in 0.01 % borate buffer, pH 8.5). The plates were incubated at room temperature for 30 min, the methylene blue solution was shaken off and the plates were washed by serial dipping in 4 tanks of borate buffer (0.01 M, pH 8.5) with the buffer shaken off after each tank. The methylene blue was then eluted by the addition of ethanol and 0.1 M HCL (100 µl of 1:1 (v/v)) to each well. The absorbence at 620 nm was read on a microplate reader (Titertek, Multiscan, MCC, Australia).

3.4.3 Stable transfection of LNCaP cells with the Tet-on plasmid

LNCaP cells were grown in a 6 well plate (0.5×10^5 cells per well) for 48 hrs and transfected with the Tet-on plasmid (2 µg) as described above. After a further 48 hrs G418 (700 µg/ml) in RPMI (2 ml) was added, selection was carried out over a 4 week

period. During the selection period the media supplemented with G418 was changed every 4 days. Cells that were not Tet-on positive died within this period of time whereas G418 resistant clones formed colonies. These colonies were detached using 0.2 % EDTA and 0.1 % trypsin and grown for use in subsequent experiments. It was then tested whether the clones were Tet-on positive by transfection with the pBI-EGFP vector (2 µg) and addition of doxycyclin (1 µg/ml). GFP positive cells were detected after a further 24 hrs. The Tet-on positive clones were used for experiments.

3.4.4 Preparation of cell extracts for western blot analysis

LNCaP cells were grown in serum containing media in 6 well plates for 48 hrs and then transfected using Lipofectimine 2000. After a further 48 hrs the cells were harvested using 0.1 % trypsin and 0.2 % EDTA. Cells were centrifuged (300 g, 5 min), washed twice with ice cold PBS and recentrifuged (500 g, 2 min). Lysis buffer (5 mM Tris-HCL, pH 7.4, 5 mM EDTA, 1 % Triton X-100, 0.1 % SDS, 20 µM leupeptin and 20 µM pepstatin A, 100 µM PMSF and 0.1 % _-mercaptoethanol) was then added using 10 µl/4 x 10^5 and cells were incubated for 30 min on ice. Finally the samples were centrifuged (1000 g, 5 min, 4 ˚C), the cell debris was removed and the supernatant was stored at -20 ˚C until used.

3.4.5 Protein estimation

Protein concentrations were determined using the Bradford method (Bradford 1976) using BSA standards (0-45 µg) and samples (1 µl) dissolved in 100 µl DDI H_2O. 1 ml of

Bradford reagent was then added and the absorbence (595 nm) was measured in a spectrophotometer (Shimadzu) after an incubation time of 5 min at room temperature. The amount of protein in each sample was determined using the standard curve determined from the BSA samples.

3.5 Western blot analysis

3.5.1 Gel electrophoresis

The samples were prepared (40 µg protein, 25 % of 4 x sample buffer (62.5 mM Tris-HCl, pH 6.8, 10 % glycerol, 2 % SDS (w/v), 5 % ß-mercaptoethanol 0.00125 % bromophenol blue (w/v) containing 5 mg DDT/ 333 µl of sample buffer) and H_2O to make up to a total volume of 20 µl), heated for 2 min in a boiling water bath and put on ice for further 1 min before centrifuging at maximum speed for 2 min. They were then loaded on a 4 % stacking gel, run on a 9 % SDS-Page separating gel and electrophoresed (200 V, 45 min).

3.5.2 Protein transfer and hTRP3 detection

16 pieces of Whatman paper were wet in transfer buffer (39 mM glycine, 48 mM Tris base pH 8.3, 0.037 % SDS (w/v), 20 % methanol (v/v)), eight pieces were placed on top of each other on the semi dry blotter (Hariz Blot, Atto Corporation, Tokyo, Japan), carefully avoiding any bubbles followed by a piece of (gel sized) Hybond-C membrane. The gel was placed on the Hybond membrane, another 8 pieces of Whatman paper were added and it was assured that paper and semi dry blotter were wet. The transfer was then

performed (60 mA for 2 gels, 35 mA for 1 gel, 1.5 hrs). Afterwards the membrane was stained in 1 % Ponceau (w/v) to check the protein load was the same in each lane and subsequently rinsed in TBS-T buffer (20 mM Tris-HCl pH 7.5, 500 mM NaCl, 0.1 % Tween 20 (v/v)) until the membrane was clear. The membrane was incubated in 5 % milk powder in TBS-T to block non-specific protein binding for 2 hrs and afterwards washed (3 x 5 min) in TBS-T. It was then incubated with the primary antibody (Harland/Brereton 12.5 anti-hTRP3 rabbit antibody 1/1000 dilution, Craig Montell rabbit anti-hTRP3 antibody (John Hopkins University, Baltimore, Maryland) 1/100 dilution) over night with gentle rocking at 4 ˚ C. The membrane was again washed (1 x 15 min and 3 x 5min) and then incubated with the secondary antibody (peroxidase conjugated mouse anti-rabbit antibody, 1/1000 dilution for the membrane incubated with the Harland/Brereton hTRP3 antibody and 1/2000 dilution for the Montell antibody incubated membrane) for 2 hrs at room temperature. The membrane was washed as before and developed using ECL according to the manufacturer's instructions. The gel was stained using 100 % Comassi blue for 1 hr and destained using destain over night. It was further vacuum dried in a cellophane membrane for 2 hrs.

3.6 Calcium measurements

Fura-2/AM is a dye that has the ability to enter the cytosol of the cell where it is cleaved to Fura-2/free acid. Fura-2/free acid binds calcium. When calcium has bound to Fura-2/free acid the peak of emitted fluorescence shifts from 380 nm (free Fura-2/free acid) to 340 nm (bound Fura-2/free acid). The ratio of 340/380 nm over time can be used to

measure the amount of calcium bound to Fura-2/free acid and therefore the free cytosolic and nuclear calcium concentration.

3.6.1 Loading and location of Fura-2/AM in LNCaP cells

For calcium measurements LNCaP/Tet-on cells were grown on coverslips as previously described (section 2.3.3). The cells were washed three times with RPMI (37 °C) and then immersed in the loading mixture (10 μM Fura-2/AM, 0.0075 % Pluronic acid (w/v) in 1 ml RPMI (5 % foetal calf serum)) for 45 min. LNCaP cells were washed in RPMI (3 x) and in calcium free modified Hanks (3 x) to remove excess Fura-2/AM and then placed in the perfusion chamber. Single cells were selected in a field using an Axon imaging program, the program was started and fluorescence was measured (360 nm) as a function of time. After 2 min 40 _M digitonin was added causing a decrease in fluorescence. When the response reached a plateau 0.1 % Triton-X 100 was added resulting in a further decrease in fluorescence. The decrease in fluorescence induced by digitonin over the total decrease in fluorescence due to digitonin and Triton-X 100 allowed determination of the percentage of Fura-2/free acid in the cytosol. The second decrease due to Triton-X 100 over the total amount of dye released by both reagents allowed determination of the percentage of Fura-2/ free acid in the organelles.

3.6.2 Basal and agonist activated calcium inflow

LNCaP cells were loaded with Fura-2/AM, washed (RPMI and modified Hanks) and placed in the perfusion chamber as described above. Single, GFP positive cells in a field

were selected at 490 nm using an Axon imaging program. The program was started and fluorescence at 340 nm and 380 nm as well as the ratio 340/380 were measured over time. After 2 min the agonist (OAG (100 _M) or thapsigargin (100 nM)) was added and calcium was added back after the response reached a plateau to ensure a final concentration of 2 mM. To determine the basal calcium inflow, calcium (2 mM) was added 2 min after the run had started and no agonist was added. To determine the loading condition ionomycin and EGTA were added. Ionomycin porates the cell membrane and allows calcium to enter the cells. The Fura-2/free acid in the cytosol binds the calcium and results in an increase in fluorescence that correlates to the amount of dye in the cell. EGTA is a chelator that chelates all calcium that then cannot be bound to Fura-2/free acid and therefore results in a decrease in fluorescence to auto-fluorescence levels. An increase in fluorescence of at least three fold over starting levels (0.1 fluorescence units) is an indicator that Fura-2/AM has been effectively loaded into cells. After the agonist response reached a plateau, ionomycin (25 _M) was added, resulting in an increase in fluorescence.

3.7 Viability assay

To determine the effect of hTRP3 transfection on cell viability and cell death LNCaP cells were observed over time under phase contrast and when stained with Hoechst 33258. Hoechst 33258 is a dye that stains the nuclei of alive and dead cells and allows detection of changes in morphology of the nuclei such as condensation and fragmentation.

3.7.1 Hoechst 33258 staining

LNCaP/Tet-on cells were grown on glass coverslips for 48 hrs, transiently transfected with the pBI-EGFP or pBI-EGFP/hTRP3 plasmid (2 _g) and stained with Hoechst 33258 (10 _g/ml) 24, 48 and 72 hrs after transfection for 30 min at 37 ˚C. The coverslips were washed in PBS buffer (3 x) and transferred to a slide where the cells were fixed in glycerol (5 _l). The GFP positive cells were selected, changes of the nucleus judged under the microscope (Olympus, BX 50, magnification: 400 x) using UV light (360 nm) and photomicrographs were taken (Photometrix, Cool Snap fx).

4. Results

The Tet-on expression system was chosen to inducibly express TRP3 so that gene expression could be tightly regulated. The Tet-on system consists of two plasmids, the Tet-on and the pBI-EGFP plasmid containing EGFP and a gene of interest. The Tet-on plasmid constitutively produces a transcriptional activator, which binds to the TRE element within the minimal CMV promotor of pBI-EGFP in a doxycyclin dependent manner and induces transcription of both EGFP and the gene of interest. Initially hTRP3 was therefore cloned into the pBI-EGFP vector.

4.1 Cloning of hTRP3 into the pBI-EGFP vector

The pcDNA3 plasmid containing hTRP3 was obtained from Professor T. Gudermann (Berlin, Germany). An enzyme digest was initially performed using NcoI and XbaI to ensure that we had obtained the correct plasmid and that it had not been altered during

transformation or extraction from bacteria. The fragment sizes obtained were 4.3 kb for the hTRP3 insert and 3.3, 1.0, 0.7, 0.3 kb for the pcDNA3 vector as predicted (Figure 1 A) and confirmed that the pcDNA3 was correct and contained the hTRP3 insert.

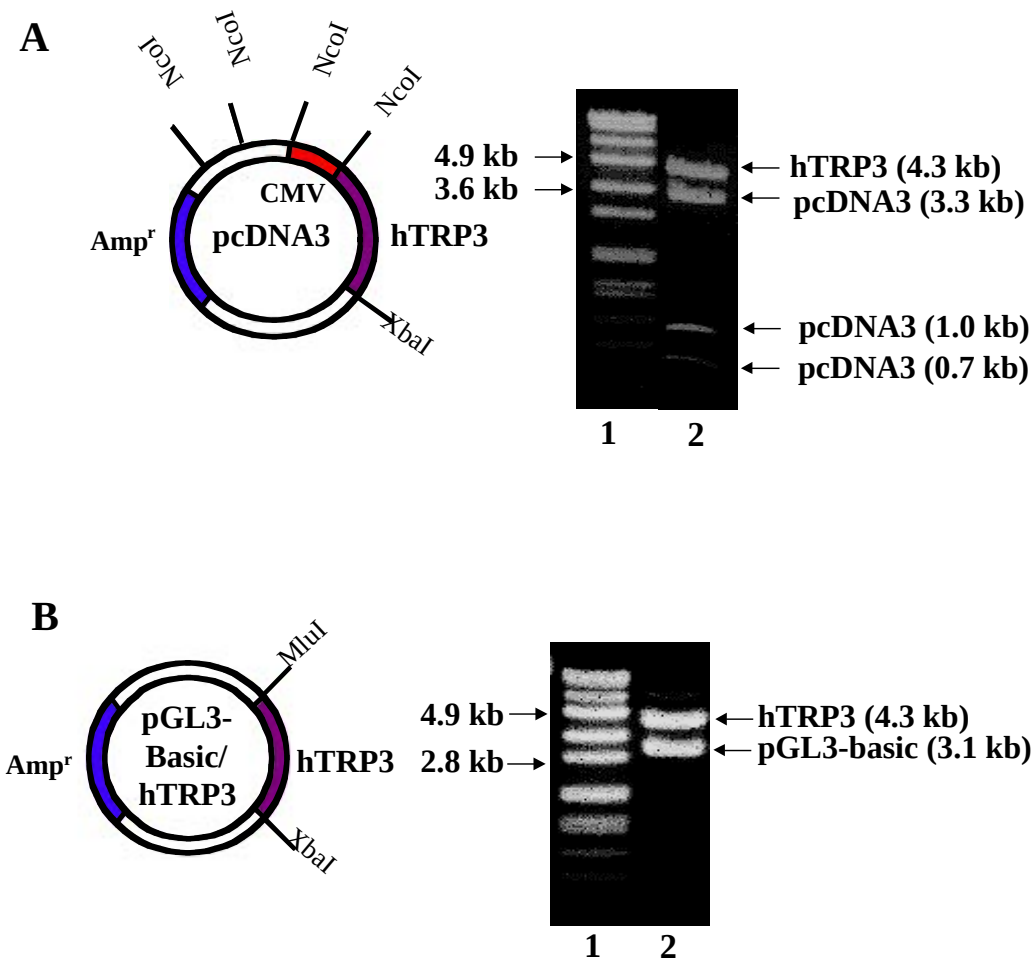


Figure 1: Restriction enzyme digests of pcDNA3/hTRP3 and pGL3-Basic/hTRP3. pcDNA3/hTRP3 was digested with NcoI and XbaI. Lane 1 SPP1/EcoRI DNA molecular weight markers. Lane 2 pcDNA3/hTRP3 cut with NcoI and XbaI (A). pGL3-Basic/hTRP3 was digested with MluI and XbaI. Lane 1 SPP1/EcoRI DNA molecular weight markers. Lane 2 pGL3-Basic/hTRP3 cut with MluI and XbaI (B).

The pBI-EGFP expression vector contained two enzyme sites in the multiple cloning site (MluI and NheI) that were unique and would not cut within hTRP3. However hTRP3 could not be excised from pcDNA3 using these two enzymes. The hTRP3 fragment was therefore initially cloned into the pGL3-Basic vector using NcoI and XbaI. The pGL3-Basic/hTRP3 construct was then cut with MluI (upstream of NcoI) and XbaI to ensure that hTRP3 was correctly inserted into pGL3-Basic. A 4.3 kb band corresponding to hTRP3 was excised (Figure 1 B). The TRP3 fragment was then cloned into the pBI-EGFP vector using MluI and XbaI. The XbaI site could be used because NheI and XbaI are isoschizomeres. The final construct was digested with NcoI to ensure that hTRP3 was correctly inserted into the pBI-EGFP vector (Figure 2). The fragment sizes were 5.0 kb and 4.4 kb as predicted and indicated that hTRP3 had been successfully cloned into pBI-EGFP.

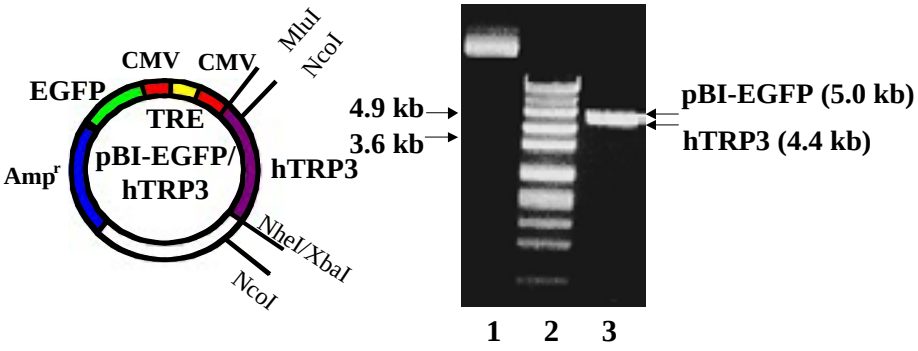


Figure 2: Restriction enzyme digest of pBI-EGFP/hTRP3. pBI-EGFP/hTRP3 was digested using NcoI. Lane 1 undigested pBI-EGFP/hTRP3. Lane 2 SPP1/EcoRI DNA molecular weight markers. Lane 3 pBI-EGFP/hTRP3 digested with NcoI. Fragments: pBI-EGFP (5.0 kb), hTRP3 insert (4.4 kb).

4.2 Sequencing of the pBI-EGFP/hTRP3 construct

pBI-EGFP/hTRP3 was sequenced to confirm that hTRP3 had been inserted correctly and that the start codon was still intact. A reverse primer of 19 base pairs from within hTRP3 had to be designed since a forward primer within the CMV promotor would bind to both CMV promotors and no sequence would have been obtained. This primer was then used to sequence across the cloning site . The 5' to 3' sequence obtained is shown in Figure 3.A. The pBI-EGFP sequence, the pGL3-Basic sequence containing the MluI restriction enzyme site, the pcDNA3 sequence containing the NcoI site and the hTRP3 sequence containing the start codon are shown in the 3' to 5' sequence (Figure 3 B) and further confirmed that hTRP3 had been inserted correctly into pBI-EGFP.

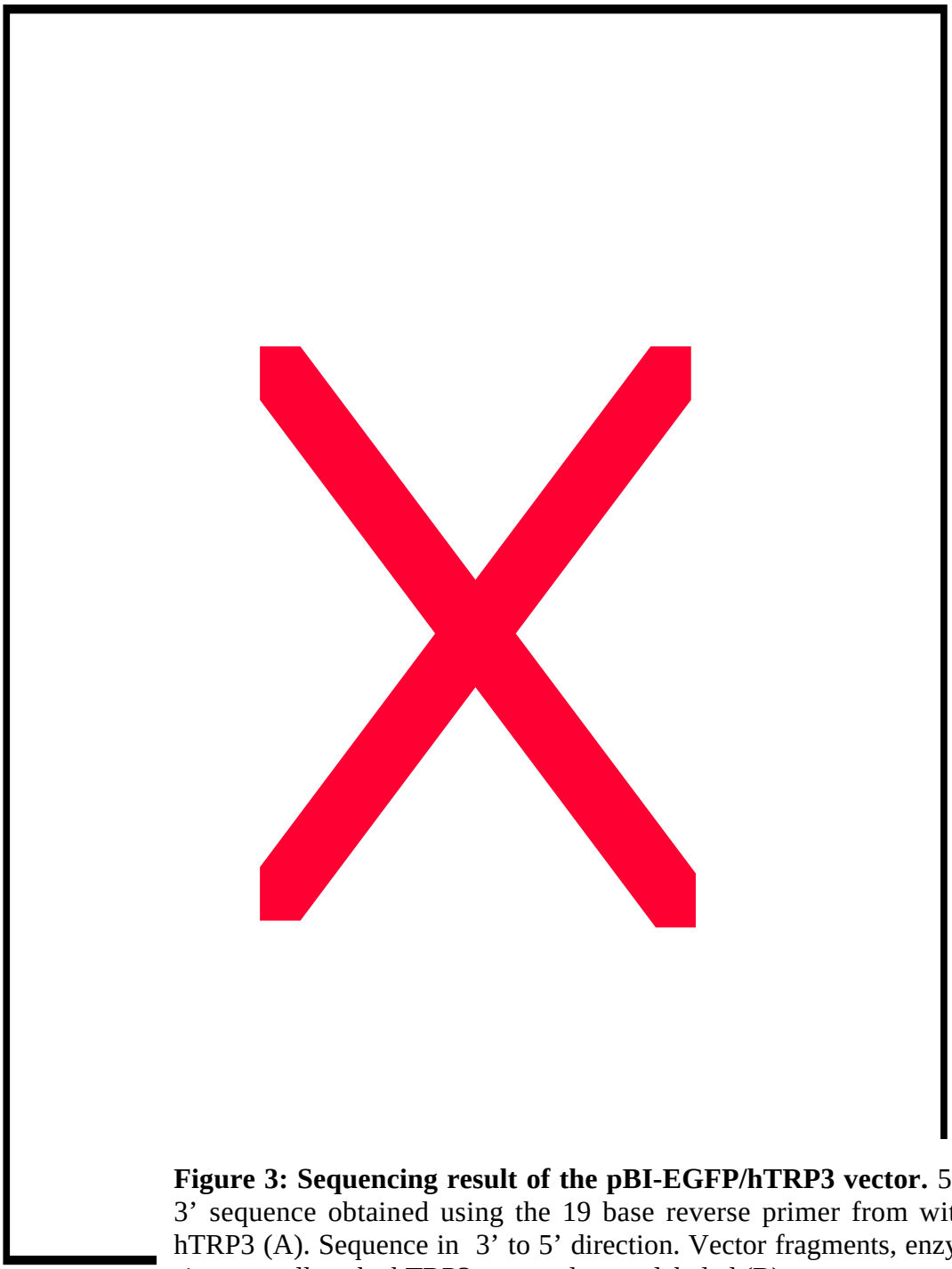


Figure 3: Sequencing result of the pBI-EGFP/hTRP3 vector. 5’ to 3’ sequence obtained using the 19 base reverse primer from within hTRP3 (A). Sequence in 3’ to 5’ direction. Vector fragments, enzyme sites as well as the hTRP3 start codon are labeled (B).

4.3 Transient transfection of LNCaP cells

Initial experiments were carried out to optimise transfection efficiency and gene expression. The pBI-EGFP/hTRP3 construct had not been finished at this stage therefore pBI-EGFP was used in these initial experiments.

To determine the conditions for maximal transfection efficiency LNCaP cells were transiently co-transfected using increasing amounts of the pBI-EGFP (0.25 μg, 0.5 μg, 1 μg) and Tet-on vector (0.25 μg, 0.5 μg, 1 μg). The experiment was repeated 4 times. 6 hrs after transfection doxycyclin (1 μg per ml) was added to induce gene expression. The number of GFP positive cells as a percentage of the total number of cells was determined for each transfection after a further 24 and 48 hrs. The transfection efficiencies after 24 hrs were similar to those after 48 hrs and therefore only the 48 hr data are shown (Figure 4).

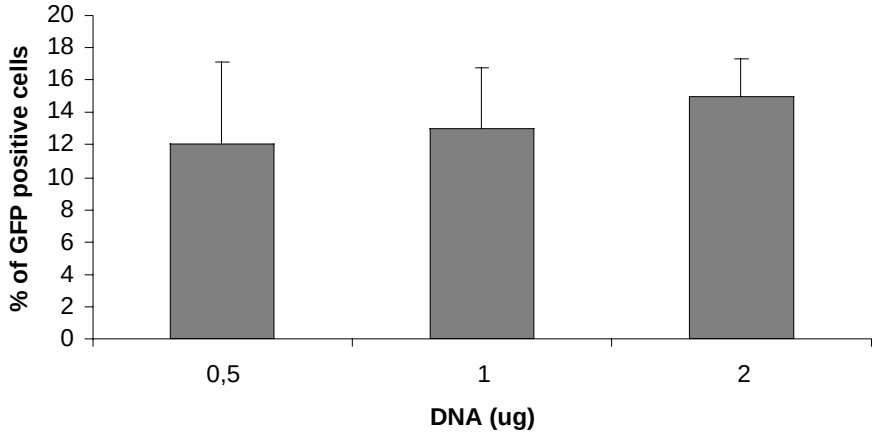


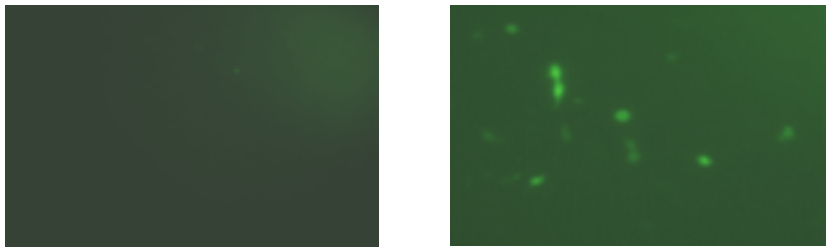
Figure 4: Transfection efficiency using different amounts of pBI-EGFP vector transiently co-transfected into LNCaP cells. LNCaP cells were transiently co-transfected with 0.5, 1 and 2 µg of the pBI-EGFP and of the Tet-on vector. The number of EGFP positive cells as a percentage of the total amount of cells was determined after 48 hrs. Results are mean values (+/- SEM) of 3 experiments.

The transfection efficiency increased slightly from 12 % when 0.5 µg/ml to 15 % when 2 µg/ml of DNA were used but was highly variable. Lipofectimine 2000 was a major source of the variability observed. The transfection efficiency was generally higher when a new batch was used and generally decreased with the number of times Lipofectimine

2000 had been used. 2 μg of total DNA resulted in the highest transfection efficiency of 15 % after 48 hrs and was therefore used in further transfections.

To determine the conditions for maximal gene expression different concentrations of doxycylin (0, 10, 50, 200 ng and 1 μg/ml) were added to pBI-EGFP and Tet-on transiently co-transfected LNCaP cells. After 48 hrs photomicrographs were obtained of cells treated with 0 and 1 μg/ml of doxycyclin. These showed that no GFP positive cells were present in the absence of doxycyclin compared to the presence of GFP positive cells when cells were treated with 1 μg/ml doxycyclin (Figure 5 A).

A



- Dox **+ Dox**

B

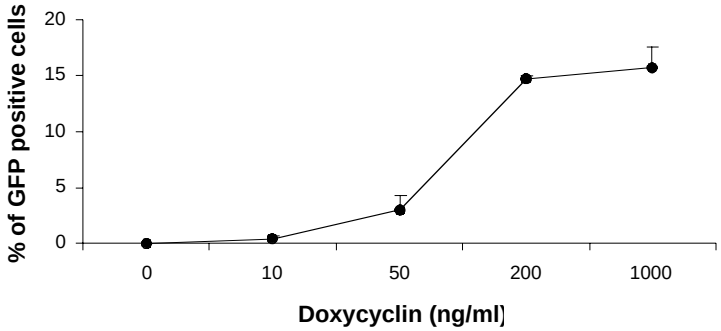


Figure 5: Doxycyclin dependent induction of EGFP after 48 hrs. Photomicrographs of pBI-EGFP and Tet-on co-transfected cells in the presence or absence of doxycyclin after 48 hrs are shown (A). pBI-EGFP and Tet-on co-transfected cells were treated with 0, 10, 50, 200, 1000 ng of doxycyclin for 48 hrs. The percentage of GFP positive cells was determined (B). Results are mean values (+/- SEM) of 3 experiments.

To quantify these results the number of GFP positive cells as a percentage of the total population was determined for each concentration of doxycyclin after 24 and 48 hrs. The data for 24 hrs differed only slightly from the data obtained for 48 hrs and was therefore not shown. The percentage of GFP positive cells increased from 0 % in the absence of doxycyclin to 15.7 % in the presence of 1000 ng/ml doxycyclin (Figure 5 B). 5 μg/ml of doxycyclin was also used, however this appeared to have a toxic effect on LNCaP cells. They stopped proliferating after 48 hrs and began to round up and detach from the culture dish (data not shown).

Since 1 μg/ml doxycyclin resulted in the greatest level of GFP expression it was used in subsequent experiments. The findings shown in figure 5 A and B suggest that background expression of GFP was very low in the absence of doxycyclin and GFP could be inducibly expressed in response to doxycyclin.

4.4 Stable transfection of LNCaP cells

The transfection efficiency for LNCaP cells transiently co-transfected with the pBI-EGFP and Tet-on vector was only 15 %. Low transfection efficiencies can be difficult to work with particularly if the effects of the treatments on the cells are only small. To overcome this we attempted to produce a double stable cell line containing the Tet-on and pBI-EGFP or pBI-EGFP/hTRP3 plasmid. The first step in producing the Tet-on/pBI-EGFP stable LNCaP cell line was to set up a stable cell line containing the Tet-on vector.

The Tet-on vector contains a gene that codes for G418 resistance and therefore allows selection for Tet-on stable clones. A proliferation assay for G418 and hygromycin B was

set up to determine the amount of G418 and hygromycin B that inhibited growth and killed LNCaP cells and could be used in the selection of stable cell lines.

G418 inhibited proliferation in a dose dependent manner. 700 μg/ml and higher doses killed all cells by 9 days (Figure 6 A) and 700 μg was therefore used to select stably transfected clones containing G418 resistance. It was not clear why the cell number was reduced after 9 days in cells not treated with hygromycin B. However 100 and 200 μg of hygromycin B stopped proliferation and induced death in LNCaP cells within 9 days and 100 μg/ml was therefore chosen for the selection of cells containing hygromycin B resistance (Figure 6 B).

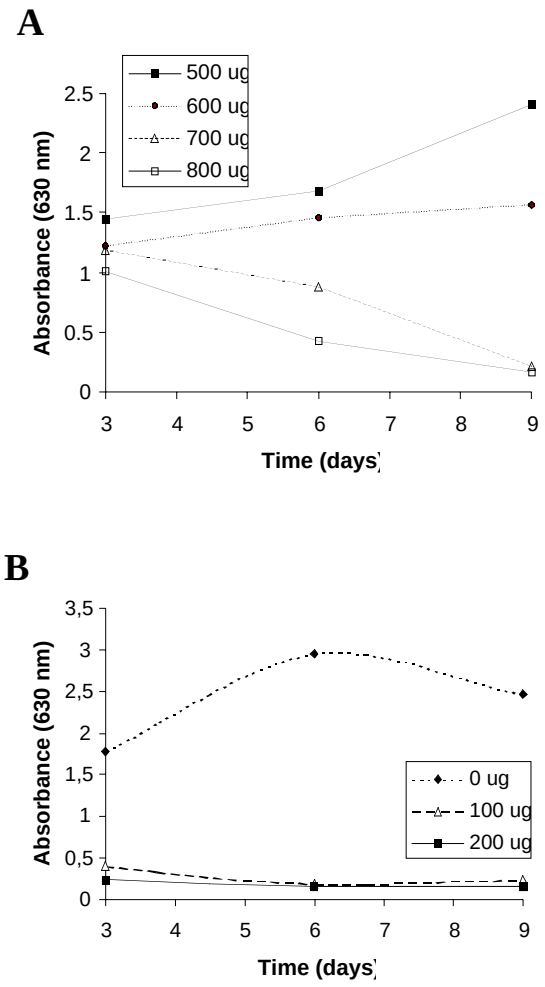


Figure 6: Proliferation assay for G418 and hygromycin B. Proliferation assays were carried out on LNCaP cells (as described in Materials and Methods section 2.4.2) treated with different concentrations of G418 (A) and hygromycin B (B) over a period of 9 days.

To produce a stable Tet-on cell line LNCaP cells were transfected with the Tet-on vector and G418 was added 48 hrs after transfection (700 µg/ml). Selection was carried out over

the next 4 weeks. Six G418 resistant clones were then isolated, grown up and transfected with pBI-EGFP. Only those clones containing the Tet-on plasmid should express GFP when transfected with pBI-EGFP alone. This fact allowed selection of those clones stably transfected with Tet-on. All of the 6 clones isolated were GFP positive and showed a similar transfection efficiency of around 15 % as seen in co-transfections (data not shown).

To obtain a double stable cell line containing Tet-on as well as pBI-EGFP or pBI-EGFP/hTRP3 the Tet-on stables were co-transfected with the pTK-Hyg and the pBI-EGFP or pBI-EGFP/hTRP3. The pTK-Hyg plasmid contains a gene coding for hygromycin B resistance and therefore allows selection of hygromycin B resistant cells. The transfection was performed using a 1:10 ratio of pTK-Hyg to PBI-EGFP or pBI-EGFP/hTRP3 to increase the chances that hygromycin B resistant cells were also positive for pBI-EGFP or pBI-EGFP/hTRP3. Although the attempt to obtain a double stable was repeated three times and the amount of hygromycin B used was reduced to 50 µg/ml in the final attempt the few surviving cells did not proliferate and died after 6-8 weeks. A stable clone containing both plasmids (Tet-on and pBI-EGFP or pBI-EGFP/hTRP3) was therefore not obtained.

4.5 Transfection with the pBI-EGFP/hTRP3 plasmid

The Tet-on stables described in the previous section were used for transient transfections with pBI-EGFP/hTRP3. The Tet-on stables were seeded and transfected with either the pBI-EGFP or the pBI-EGFP/hTRP3 plasmid. The transfection efficiency for both

plasmids was determined as the number of GFP positive cells as a percentage of the total number of cells. The average transfection efficiency for the pBI-EGFP plasmid was 18.5 % similar to that seen in previous experiments. However the average transfection efficiency for the pBI-EGFP/hTRP3 plasmid was 5.5 % (Figure 7).

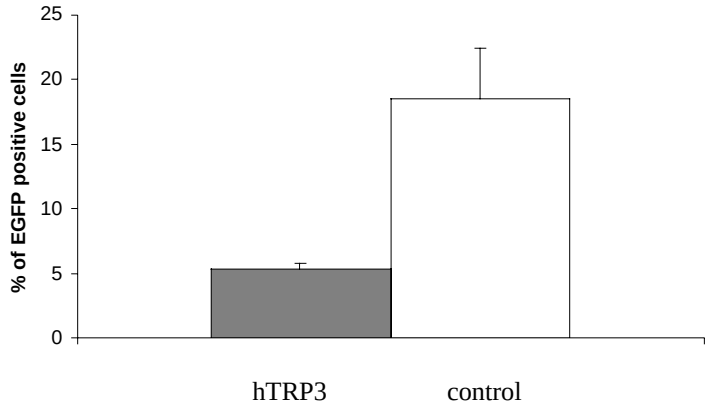


Figure 7: Transfection efficiency for LNCaP/Tet-on cells transiently transfected with PBI-EGFP/hTRP3 or pBI-EGFP. Cells were transfected using 2 ug of vector DNA and the numbers of GFP positive cells as a percentage of the total amount of cells was determined after 48 hrs. Results are mean values (+/- SEM) of 2 experiments.

4.6 Detection of hTRP3

To determine whether hTRP3 was being inducibly expressed LNCaP/Tet-on cells were transiently transfected with pBI-EGFP/hTRP3 and incubated in the presence or absence of doxycyclin (1 μg/ml) for 48 hrs. Non-transfected cells were used as an additional negative control. Cell lysates were prepared, fractionated using PAGE and analysed by western blot. To detect hTRP3 two different antibodies were used. The first one was prepared in our laboratory (Harland/Brereton) and the second one was obtained from C. Montell (John Hopkins University, Baltimore, Maryland).

Examination of the western blot obtained using the Harland/Brereton antibody revealed the presence of two bands (96 and 190 kD) in hTRP3 transfected, doxycyclin treated cells that were not present in hTRP3 transfected cells not treated with doxycylin and non transfected control cells (Figure 8 A).

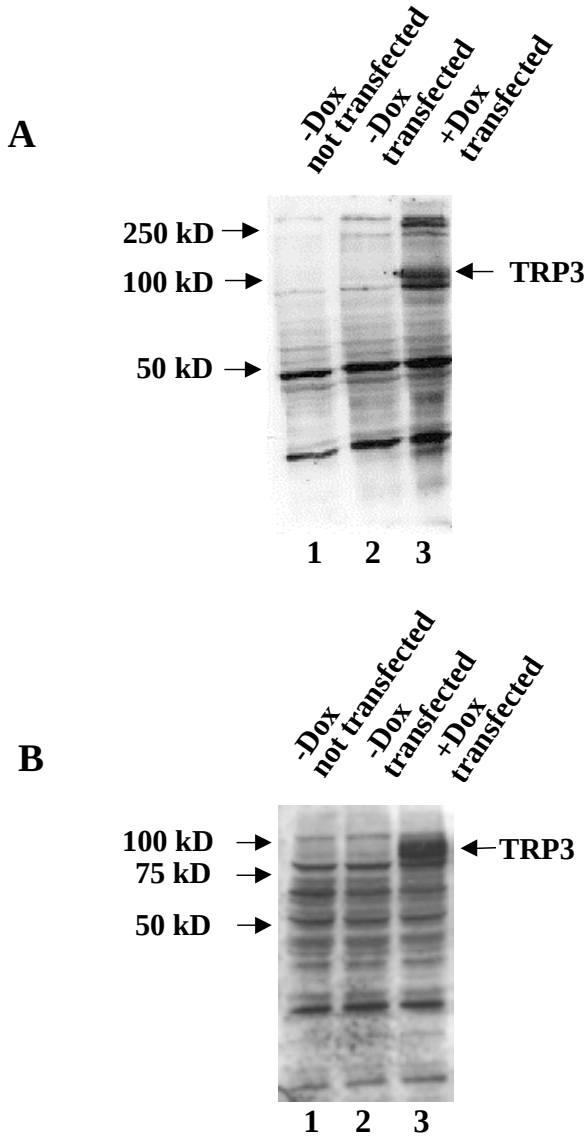


Figure 8: Western blot analysis of hTRP3 expressed in LNCaP/Tet-on cells. Samples were obtained from non transfected LNCaP cells and cells transfected with pBI-EFGP/hTRP3 in the absence or presence of doxycyclin (Lanes 1-3). Western blot analysis was performed using the Harland/Brereton (A) and the Montell antibody (B).

The molecular weight of the smaller band is close to the reported molecular weight of hTRP3 (98 kD) suggesting that the antibody produced in our laboratory could detect hTRP3. The larger band was calculated to be 190 kD which is close to twice the reported size for hTRP3. The size of the bands (96 and 190 kD) and the fact that they were only detected in samples from doxycyclin treated cells suggest that hTRP3 was being inducibly expressed in LNCaP cells. A number of bands of various sizes appeared in each lane and may be due to non-specific antibody binding. This indicates that the antibody does not bind hTRP3 with high specificity. Examination of a number of these bands found in all lanes, also indicated that the protein loads in each lane may be different. This is further complicated by the fact that the three lanes differ in width. From this western blot we therefore cannot discount the possibility that these two bands (96 and 190 kD) are visible due to increased levels of protein in samples from doxycyclin treated cells. The bands corresponding to the molecular weight of hTRP3 described above could not be detected in three subsequent experiments. Similar difficulties using this antibody to detect overexpressed hTRP3 in HII4E cells have been encountered by other members of our laboratory.

Due to the problems encountered with the Harland/Brereton antibody we obtained an alternate hTRP3 antibody from C. Montell (John Hopkins University, Baltimore, Maryland). Western blot analysis of pBI-EGFP/hTRP3 transfected LNCaP/Tet-on cells, using the Montell antibody revealed two bands of 89 kD and 96 kD that are close to the reported size of hTRP3 (98 kD) (Figure 8 B). These two bands only appeared in transfected cells in the presence of doxycyclin suggesting that they were induced by

doxycyclin and may be hTRP3. The presence of two bands may be due to a modified (phosphorylated or glycosylated) form of hTRP3. Similar to the results obtained using the Harland/Brereton anti-body a number of bands appeared in every lane that may have been due to non-specific binding of the antibody. Protein estimation had been performed before loading and examination of a number of bands present in all lanes confirmed that the protein loads for each lane were identical. Therefore it is unlikely that these bands are due to unequal protein loads. In contrast to the Harland/Brereton antibody the C. Montell antibody detected the 89 and 96 kD bands in 3 subsequent experiments.

In both western blots (C. Montell and Harland/Brereton antibody) a band of 96 kD was detected which is close to the reported size of hTRP3 (98 kD). Moreover in both western blots the bands thought to be hTRP3 were only detected in samples of hTRP3 transfected cells in the presence of doxycyclin suggesting that hTRP3 was inducibly expressed.

4.7 Peak of hTRP3 expression

To determine the time at which hTRP3 was maximally expressed LNCaP/Tet-on cells were transfected with the pBI-EGFP/hTRP3 vector and incubated in the presence or absence of doxycylin for 96 hrs. Cell lysates were prepared, fractionated using PAGE and analysed by western blot analysis using the C. Montell anti-body. Examination of the western blot obtained revealed the presence of two bands (89 and 96 kD) in only those lanes containing samples of cells transfected with the pBI-EGFP/hTRP3 plasmid treated with doxycylin (lanes 1, 3, 5, 7) as seen in earlier experiments (Figure 9).

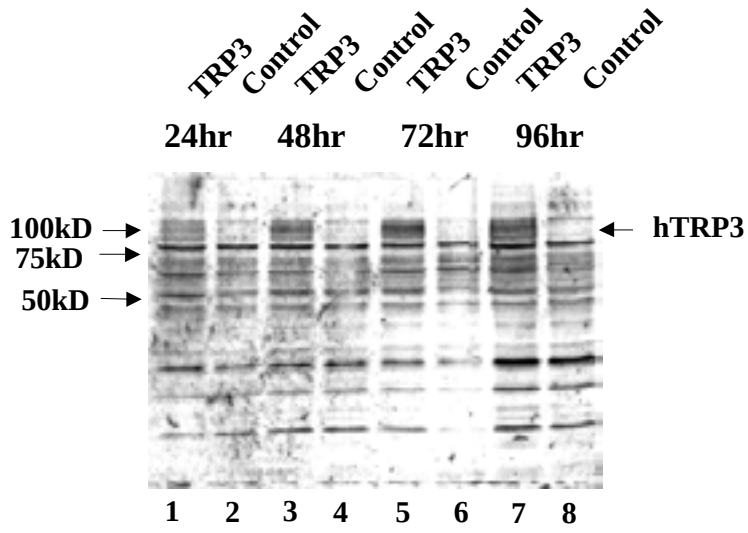


Figure 9: Time course of hTRP3 expression. LNCaP/Tet-on cells were transfected with pBI-EGFP/hTRP3 and incubated in the presence or absence of doxycyclin. Samples were prepared from cells incubated in the presence or absence of doxycyclin after 24, 48, 72, 96 hrs, fractionated and analysed via Western blot analysis using the Montell antibody.

As before a number of bands of various sizes appeared in each lane. Examination of several bands that are present in all lanes indicate that protein loads are even in most

lanes but are decreased in lane 6 (control, 72 hrs) and increased in lane 7 and 8 (hTRP3, 96 hrs) which complicates the comparison between the amounts of hTRP3 after 96 hrs and the other time points. However comparison of the bands thought to be hTRP3 in lanes 1, 3 and 5 (24, 48 and 72hrs) indicate that hTRP3 expression is increased after 48 and 72 hrs compared to 24 hrs in 2 subsequent experiments. An increase between 48 and 72 hrs could be detected in the blot shown but not in 2 subsequent blots. The cells were also confluent after 48 hrs and this time point was therefore chosen to perform experiments.

4.8 Localisation of Fura-2/AM

Fura-2/AM has the ability to enter mammalian cells and is frequently used to measure changes in intracellular calcium. When Fura-2/free acid binds to calcium the peak of emitted fluorescence changes from 380 nm to 340 nm and by determining the ratio of the emitted fluorescence at 340/380 nm the cytosolic and nuclear calcium concentration entering the cell can be obtained. Since changes in cytosolic calcium concentrations of hTRP3 transfected LNCaP cells were going to be measured using Fura-2/free acid it was important to assure that the majority of the signal was due to Fura-2/free acid in the cytosol and not in cell organelles. Cells were therefore loaded with Fura-2/AM and treated with digitonin to release Ca^{2+} from the cytosol and subsequently with Triton-X 100 to release Ca^{2+} from the organelles. Digitonin is a detergent that destroys cell membranes, but not the membranes of inner cell organelles. The addition of digitonin therefore results in a decrease of the fluorescence signal due to the release of dye from the cytosol. Triton-X 100 is a detergent that destroys all membranes and therefore results

in a further decrease of the fluorescence signal due to the release of dye from the cell organelles. It is therefore possible to determine the percentage of dye in the cytosol and the organelles. After Fura-2/AM loading the addition of digitonin resulted in a decrease in fluorescence. The addition of Triton-X 100 resulted in a further decrease in fluorescence due to the release of Fura-2/AM or Fura-2/free acid from the organelles (Figure 10).

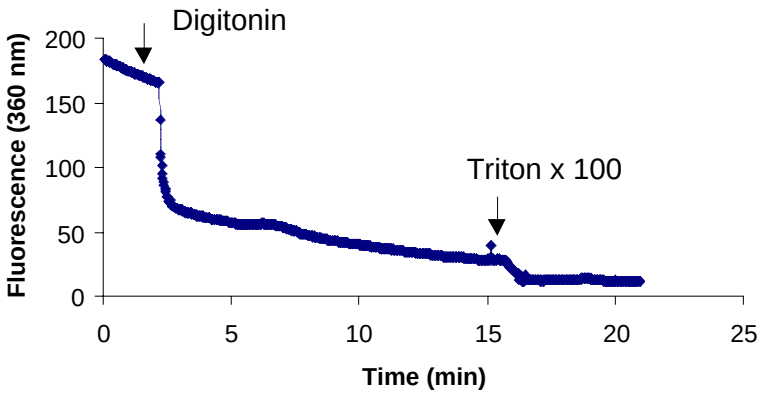


Figure 10: Localisation of Fura-2-AM in LNCaP/Tet-on cells. LNCaP/Tet-on cells were transfected with pBI-EGFP and treated with doxycyclin. After 48 hrs cells were loaded with Fura-2/AM and treated with digitonin and Triton X 100. Changes in fluorescence (360 nm) were measured over time. A representative trace out of 11 experiments is shown.

The percentage of dye in organelles compared to cytosol was assessed. The percentage of dye in the cytosol was determined by dividing the decrease in fluorescence due to

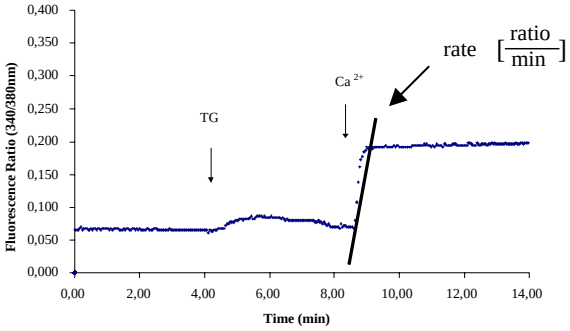
digitonin by the total decrease in fluorescence after the addition of Triton-X 100. The cytosolic fraction was determined to be 90 % indicating that most of the Fura-2/AM and Fura-2/ free acid was in the cytosol. This shows that the majority of the signal in subsequent calcium measurements is due to changes in cytosolic calcium which is important in experiments measuring calcium inflow. Generally the cytosolic fraction of Fura-2/ free acid should be higher than 80 %.

4.9 Thapsigargin stimulated calcium inflow

Thapsigargin has been shown to inhibit the SERCAs and to result in capacitative calcium inflow into the cytoplasm secondary to calcium release from internal stores. hTRP3 appears to behave differently in a variety of cell lines. In some cell lines hTRP3 appears to be activated by thapsigargin (Kiselyov et al., 1998) but not in others (McKay et al., 2000, Zhu et al., 1998). We therefore examined the effect of thapsigargin on calcium inflow in our system.

LNCaP/Tet-on cells were transiently transfected with pBI-EGFP or pBI-EGFP/hTRP3, treated with doxycyclin (1 μg/ml) and 48 hrs after transfection loaded with Fura-2/AM in calcium free medium. The fluorescence ratio of bound and free Fura-2/AM (340/380 nm) was then measured as a function of time in single cells after the addition of thapsigargin and calcium (Figure 11).

A



B

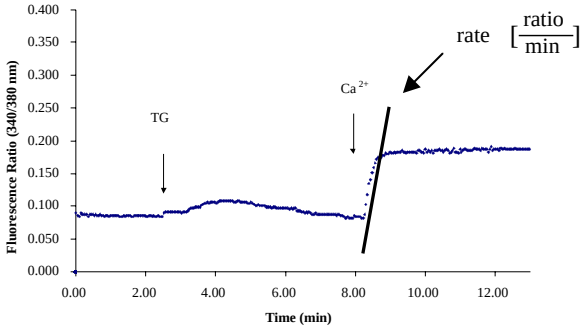


Figure 11: Calcium inflow in LNCaP/Tet-on cells transfected with either pBI-EGFP/hTRP3 or pBI-EGFP. LNCaP/Tet-on cells were transfected with pBI-EGFP/hTRP3 (A) or pBI-EGFP (B), loaded with Fura-2/AM, treated with thapsigargin and then with calcium (see arrows). The fluorescence ratio of Fura-2/AM (340/380 nm) was measured over time and was used as a measure of cytosolic calcium. Representative traces are shown from experiments with 16 hTRP3 transfected and 23 control transfected cells.

The addition of thapsigargin alone resulted in a very small increase in fluorescence in hTRP3 as well as in control transfected cells that was thought to be due to ER store depletion. When calcium was added back a substantial increase in fluorescence was observed. This was considered to be due to calcium inflow across the membrane due to activation of store operated calcium channels. The rates of calcium inflow after the addition of calcium were calculated by dividing the increase in fluorescence (during the slope rise) by the time (Figure 12 A).

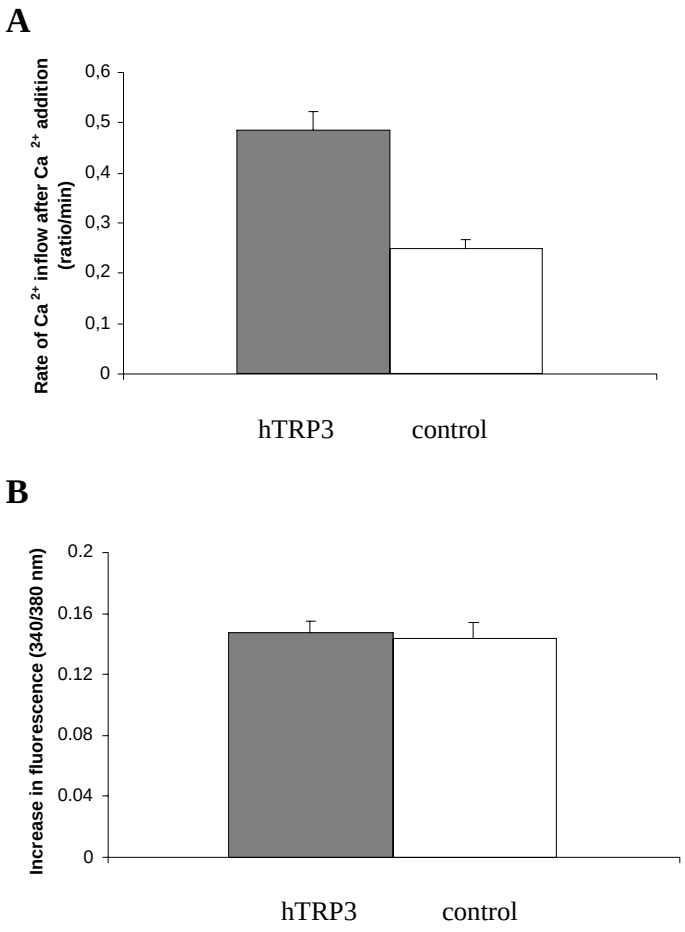


Figure 12: Thapsigargin stimulated calcium inflow in LNCaP/Tet-on cells. The rate (A) as well as the total increase (B) of calcium inflow were determined (from traces shown in the previous figure) after the addition of calcium in pBI-EGFP/hTRP3 and pBI-EGFP transfected cells. Results are mean values (+/- SEM) of experiments with 16 hTRP3 transfected and 23 control transfected cells.

The rates of calcium inflow for hTRP3 transfected LNCaP cells were calculated to be 0.48 fluorescence units/min and for control transfected cells 0.25 fluorescence units/min. This result was shown to be significant ($p < 0.01$). This increase in the rate may be due to the activation of expressed hTRP3 channels on the plasma membrane. The total increase in fluorescence was determined after the addition of calcium for hTRP3 and control transfected cells. However no differences in the total increase could be detected between hTRP3 and control cells (Figure 12 B). This may be due to refilling of the ER stores after depletion. These results indicate that expressed hTRP3 calcium channels were activated by store depletion induced by thapsigargin.

These experiments were also performed the other way round, adding calcium first and thapsigargin second. The resulting traces were the same as when thapsigargin was added first. The first response was always small whereas the second showed a larger inflow. There was no difference in the first response whether thapsigargin or calcium was added first between hTRP3 transfected and control transfected cells (data not shown). If hTRP3 was constitutively active in this system the addition of calcium would have increased the response to calcium when it was added first.

4.10 OAG activated calcium inflow

It was next determined whether hTRP3 expressed in LNCaP cells functioned as an OAG (1-oleoyl-2-acetyl-sn-glycerol) sensitive calcium channel. OAG is a membrane permeable diacylglycerol analogue that has been shown to directly stimulate hTRP3

gated calcium inflow, independent of protein kinase C activation (McKay et al., 2000). LNCaP/Tet-on cells were transiently transfected with the pBI-EGFP or pBI-EGFP/hTRP3 plasmid and treated with doxycyclin (1 _g/ml). 48 hrs later cells were loaded with Fura-2/AM and the changes in cytosolic calcium measured in selected GFP positive cells after the addition of OAG (100 _M) and then calcium (2 mM) as shown in Figure 13.

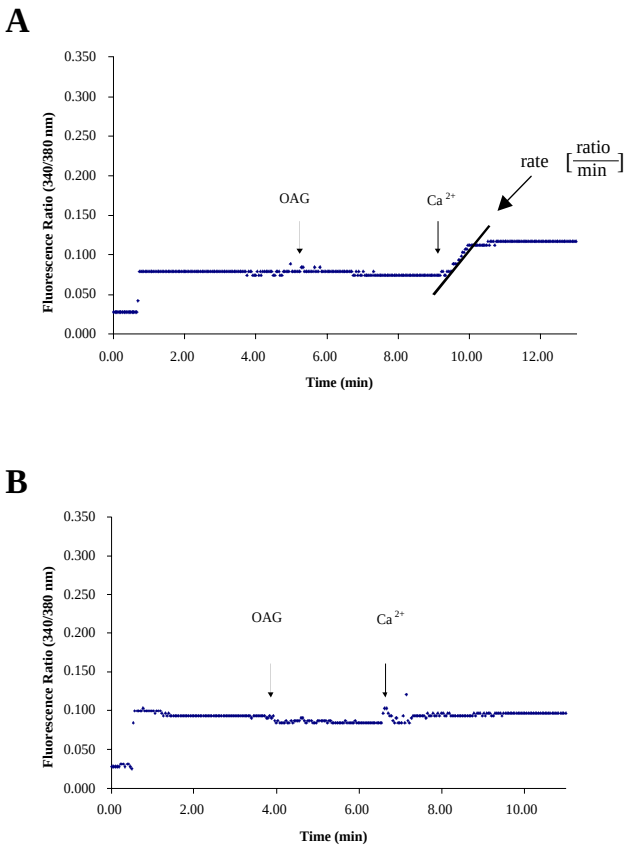


Figure 13: Calcium inflow in LNCaP/Tet-on cells transiently transfected with either pBI-EGFP/hTRP3 or pBI-EGFP. LNCaP/Tet-on cells were transfected with pBI-EGFP/hTRP3 (A) or pBI-EGFP (B), loaded with Fura-2/AM, treated with OAG and calcium (see arrows). The fluorescence ratio (340/380 nm) of Fura-2/AM was measured over time and used as a measure of cytosolic calcium. Representative traces are shown from experiments with 42 hTRP3 transfected and 27 control transfected cells.

When calcium was added to OAG treated, hTRP3 transfected cells an increase in the fluorescence ratio was detected in 62 % of the hTRP3 transfected LNCaP/Tet-on cells compared to 7 % of the control cells (Figure 14).

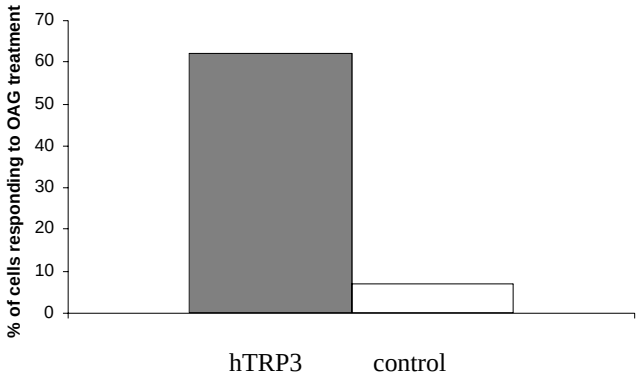


Figure 14: Percentage of cells responding to OAG. The numbers of cells responding to OAG treatment were determined as a percentage for pBI-EGFP/hTRP3 and pBI-EGFP control transfected cells.

Changes in cytosolic calcium were measured as a function of time during these treatments in 42 individual LNCaP cells transfected with hTRP3 and in 27 control transfected cells. hTRP3 and control cells showed no response when OAG was added. The average increase in fluorescence was calculated in both groups in only those cells that responded. The increase for hTRP3 transfected cells was 0.044 and 0.019 for control cells (Figure 15 A).

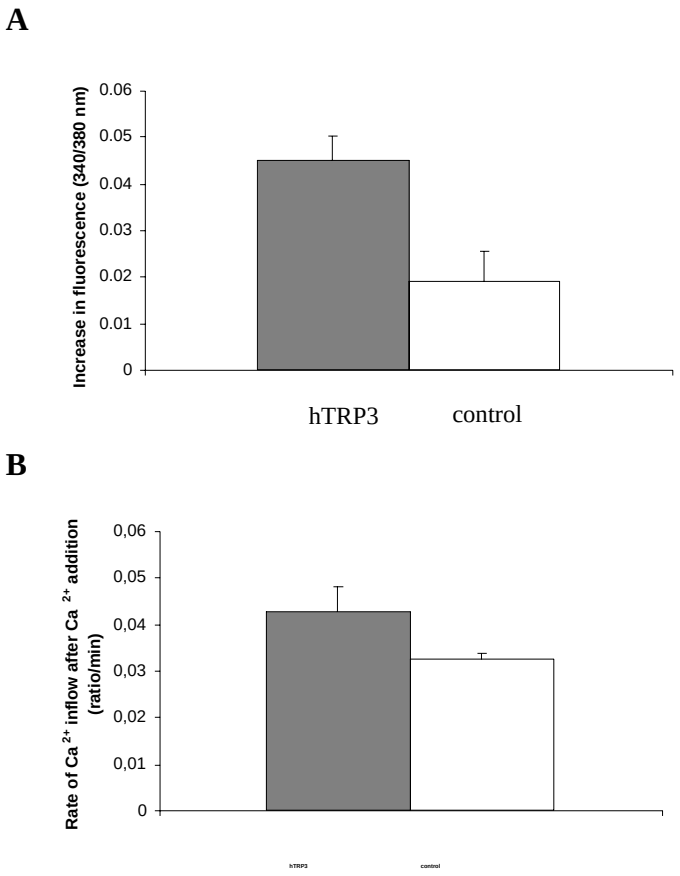


Figure 15: OAG stimulated calcium inflow in LNCaP/Tet-on cells. The total increase (A) as well as the rate (B) of calcium inflow were determined (from traces shown in figure 4.13) after the addition of calcium in pBI-EGFP/hTRP3 and pBI-EGFP transfected cells. The total increase and the rate were determined in single cells and only those cells responding to OAG. Results are mean values (+/- SEM) of experiments with 42 hTRP3 transfected cells and 27 control transfected cells.

This suggests that hTRP3 transfection results in higher levels of cytosolic calcium when activated with OAG. The average rate of calcium inflow after the addition of calcium was also determined for hTRP3 and control transfected cells in only those cells responding to

OAG. The average rate of calcium inflow for hTRP3 transfected cells was 0.043 fluorescence units/min compared to 0.033 fluorescence units/min for control transfected cells (Figure 15 B). This increase suggests that hTRP3 transfection results in an increase in the rate of calcium inflow. However these were not significantly different ($P > 0.01$). Taken together these results suggest that hTRP3 can be activated by OAG in our system.

OAG experiments were also performed by adding calcium first and OAG second. Again traces appeared to be the same. There was no increase when calcium was added and the response of hTRP3 (6 experiments) and control (5 experiments) transfected cells to OAG treatment was variable. However in the thapsigargin experiments that were performed earlier than the OAG experiments, cells seemed to respond to calcium when it was added first. It was therefore expected to be the same in the OAG experiments. Possible causes as to this response appear to have changed with time are described in the discussion.

4.11 Effect of hTRP3 on cell viability and cell death

As shown in the previous section hTRP3 was expressed in LNCaP cells and measurements of the changes in cytosolic calcium using OAG suggested that it might be OAG activated. In other studies the overexpression of hTRP3 has also been shown to enhance cytosolic calcium and induce cell death. The effect of hTRP3 on viability and cell death in LNCaP cells was therefore examined. Hoechst 33258 isa nuclear stain that is commonly used to investigate nuclear condensation and fragmentation, which are associated with cells undergoing apoptosis. Hoechst 33258 was used to investigate the effects of hTRP3 on cell viability and cell death. LNCaP cells were transiently

transfected with pBI-EGFP/hTRP3 or pBI-EGFP and treated with doxycyclin (1µg/ml). The cells were stained with Hoechst 33258, GFP positive cells were selected and photomicrographs of GFP and Hoechst stained nuclei at 24, 48 and 72 hrs (Figure 16) were obtained.

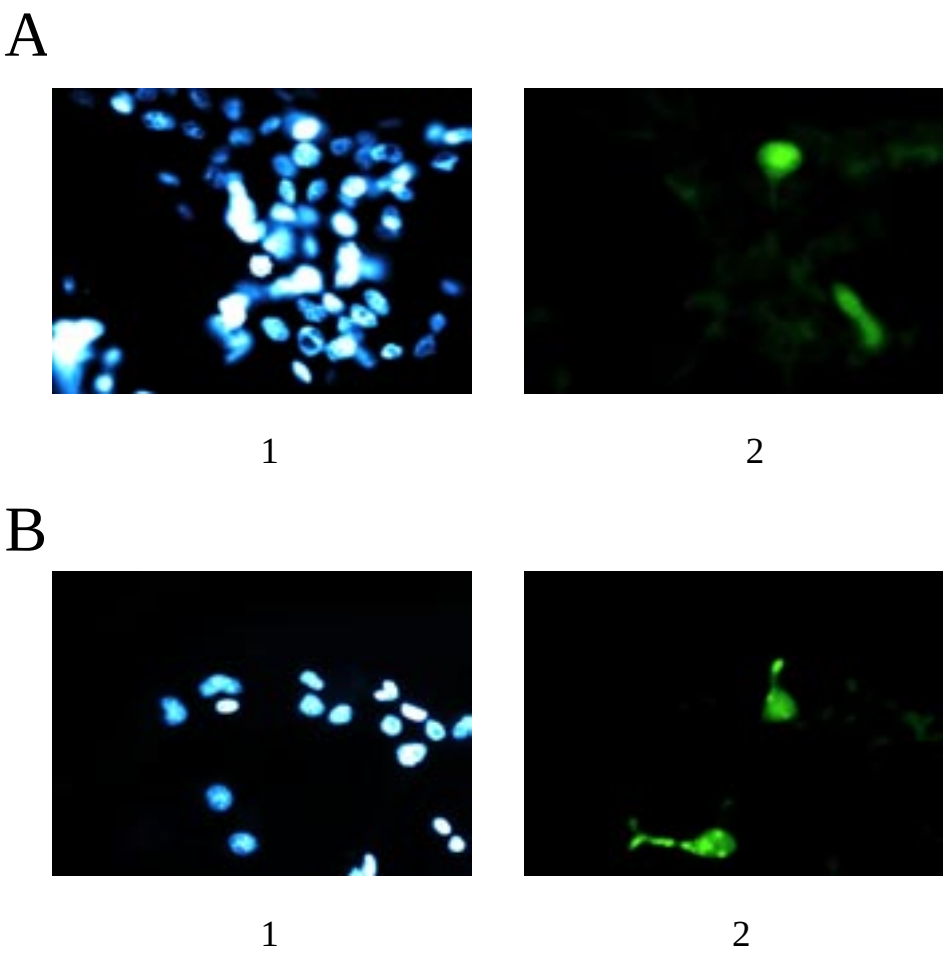


Figure 16: Examination of the nuclear morphology of LNCaP cells transiently transfected with pBI-EGFP/hTRP3 or pBI-EGFP. LNCaP cells were transfected with pBI-EGFP/hTRP3 (A) or pBI-EGFP (B), stained with Hoechst 33258 (A/B1) after 24, 48 and 72 hrs, examined for nuclear condensation and fragmentation and photomicrographs were taken. The photomicrographs of Hoechst 33258 (A/B 1) and EGFP (A/B 2) shown in this panel were taken after 72 hrs.

Three fields with 60-100 cells/field were analysed for each time point and hTRP3 or control transfected cells. Condensed and fragmented nuclei could not be detected in GFP positive hTRP3 or control cells. This suggests that hTRP3 transfection did not result in cell death in LNCaP cells.

Since OAG was shown to increase calcium inflow in our system we also treated pBI-EGFP/hTRP3 and pBI-EGFP transfected cells with OAG (100 _M) and observed whether this would have an effect on cell death. However cells remained adhered to the culture dish and proliferated. No cell death could be detected in hTRP3 or control transfected cells within 96 hrs after the addition of OAG. Cells were observed by microscopy but not stained with Hoechst 33258 (data not shown).

5. Discussion

Prostate cancer is the second leading cause of death from cancer in men. There is currently no effective treatment for the aggressive androgen independent state of the disease. One of the approaches being investigated to treat this stage of the disease is the induction of apoptosis in these cancer cells. Increased cytosolic calcium induced by thapsigargin has been shown to result in apoptosis in a prostate cancer cell line suggesting that calcium can regulate apoptosis and may be a potential therapeutic target in prostate cancer cells. Increased intracellular calcium can also be induced by the overexpression of TRPL and TRP membrane calcium channels in some cell lines. Results from our laboratory have shown that expression of TRPL in LNCaP cells not only

increased intracellular calcium but also induced apoptosis (Zhang, 2001). However, the level of TRPL induced apoptosis was only 20 % of transfected cells. It was therefore the aim of studies carried out in this thesis to examine the effects of hTRP3 expression (another TRP channel) on cell viability and cell death in LNCaP cells.

hTRP3 was successfully cloned into the pBI-EGFP vector and was used with the Tet-on plasmid to inducibly express hTRP3 in LNCaP prostate cancer cells.

The transfection efficiency of LNCaP cells using Lipofectimine 2000 was 18.5 % for control (pBI-EGFP) transfected cells. The transfection efficiency as measured by GFP expression in hTRP3 transfected cells in the absence of doxycyclin was 0 % compared to 5.5 % in the presence of doxycyclin. The difference between hTRP3 transfected cells in the absence and presence of doxycyclin suggests that EGFP was inducibly expressed. The three fold difference in transfection efficiency between hTRP3 and control transfected cells may be explained by the fact that the pBI-EGFP/hTRP3 vector is bigger (9.4 kb) than the pBI-EGFP vector (5.1 kb). 2 _g of DNA used for transfection therefore contained only half as much pBI-EGFP/hTRP3 as pBI-EGFP. The positively charged headgroup of the lipid Lipofectimine 2000 coats the negatively charged DNA with a cationic lipid layer that allows fusion with the plasma membrane of the cell. The DNA then gets internalised into the cytosol. The difference in size of the two plasmids might also make internalisation of the DNA from the DNA-lipid complex into the cell less efficient.

Alternatively LNCaP cells transiently transfected with the pBI-EGFP/hTRP3 vector may be killed by hTRP3 expression. We therefore transiently transfected LNCaP cells using pBI-EGFP and pBI-EGFP/hTRP3. However, no apoptotic cells were detected after 24, 48 and 72 hrs as determined by Hoechst 33258 staining.

The ratio of the transfection efficiency of hTRP3 compared to control transfected cells remained constant between experiments. However the transfection efficiency was highly variable between experiments and depended on the age of Lipofectimine 2000, the age of the cells and the age of the Optimem media used in transfections. To avoid the low transfection efficiency that was found in the transient transfection system we attempted to obtain LNCaP cell lines stably expressing Tet-on and pBI-EGFP or pBI-EGFP/hTRP3. As a first step Tet-on was transfected into LNCaP cells and Tet-on positive clones selected using G418. Four clones were raised from single cells, transiently transfected with pBI-EGFP and treated with doxycyclin to determine whether they contained the Tet-on plasmid. One clone stably expressing Tet-on was chosen and co-transfected with pTK-Hyg and pBI-EGFP or pBI-EGFP/hTRP3. The transfected cells were treated with hygromycin B but cell lines stably expressing Tet-on, pTK-Hyg and pBI-EGFP or pBI-EGFP/hTRP3 were not obtained. In three attempts to produce double stable cell lines all cells died after a period of 8 weeks even when smaller amounts of hygromycin B were used. It was unclear why no double stable cell lines were produced since others have stably and inducibly expressed a luciferase reporter in LNCaP cells using a similar system (Gschwend et al., 1997) and hTRP3 has also been stably expressed in other cell lines (Kiselyov et al., 1998, Zhu et al., 1998). This might be due to high toxic

concentrations of hygromycin B even though the dosage had been reduced or due to LNCaP cells loosing the pTK-Hyg plasmid that contains the hygromycin B resistance gene. Despite our inability to produce a double stable cell line the Tet-on stable cell line produced was used for further experiments.

To confirm hTRP3 was being expressed in our system western blot analysis with two anti-hTRP3 antibodies (Harland/Brereton and Montell) was performed. The Harland/Brereton anti-hTRP3 antibody, raised in our laboratory, detected two bands (96 and 180 kD) in one out of four experiments. Problems in detecting TRP3 using this antibody have been encountered also by other members of our laboratory (Lyn Harland, personal communication). One member of our laboratory was able to detect a 98 kD band in non-transfected PC-12 cells with the TRP3 antibody that was thought to be endogenous TRP3 indicating that the antibody worked in the rat system (Yordanos Tesfai, personal communication). The western blot also showed many bands that appeared in the negative control similar to those seen in LNCaP cells transfected with hTRP3 and were indicative of non-specific binding. Another member of our laboratory could not detect overexpressed hTRP3 in H24E cells using this antibody (Helen Brereton, personal communication). Due to this problem a second rabbit anti-hTRP3 antibody was obtained from C. Montell (John Hopkins University, Baltimore, Maryland). In western blots using this antibody two bands were detected (89 and 96 kD). Both close to the size of the smaller band obtained with the Harland/Brereton antibody (96 kD) and the reported size (97 kD) (Montell et al., 1999, Zhu et al., 1998). These bands were detected in two subsequent experiments and only in those samples from cells transfected with pBI-

EGFP/hTRP3 and incubated in the presence of doxycyclin. This suggested that hTRP3 was inducibly expressed. The appearance of two bands differing in size may be due to modifications of the hTRP3 protein such as glycosylation or phosphorylation. It is unlikely that one of the bands is not related to hTRP3 since both only appeared in cells that were transfected with hTRP3 and treated with doxycyclin.

Montells group has developed two anti-hTRP3 antibodies, a rabbit antibody as well as a chicken anti-hTRP3 antibody. The rabbit antibody has been reported to detect only overexpressed hTRP3 whereas the chicken antibody has been reported to detect endogenous hTRP3 as well. The reported size of the band obtained with either antibody was 97 kD (Montell et al., 1999). Another group has detected hTRP3 expression using a HA-antibody to detect HA-tagged hTRP3 (Zhu et al., 1998). The reported size was 97 kD. However the commercially available rabbit anti-TRP3 antibody (Chemicon International, Inc., Temecula, CA) has been reported to detect a band of more than 100 kD (as shown in manufacturers manual).

In western blots using either antibody many bands were detected in all lanes and indicated a high level of non-specific binding of the antibody. The intensity of the 96 kD band considered to be hTRP3 obtained by both antibodies was lower than the intensity of some of the other bands considered to be due to non specific antibody binding and suggested that hTRP3 was expressed at low levels. One possible reason for low levels of hTRP3 expression was the low transfection efficiency of 5.5 % in pBI-EGFP/hTRP3 transfected cells. It was also observed that the fluorescence of hTRP3 transfected cells

was decreased compared to control transfected cells indicating that GFP expression was lower. Two genes under the control of one promoter may reduce individual expression of each. This idea is supported by the reduced expression of EGFP.

Calcium measurements were performed using thapsigargin to deplete internal calcium stores, which has been reported to activate hTRP3 (Kiselyov et al., 1998). Cells were treated with thapsigargin and calcium was added. In our system the calculated rates of initial calcium inflow were significantly higher (0.48 fluorescence units/ min) in hTRP3 transfected and doxycyclin treated cells than in control transfected cells, treated with doxycyclin (0.25 fluorescence units/ min). However the total increase in cytosolic calcium was not different in hTRP3 transfected, doxycyclin treated cells from the increase in control transfected, doxycyclin treated cells. This result suggests that only the rate but not the total increase are different in LNCaP cells. The increased rate of calcium inflow may be due to the activation of hTRP3 channels on the plasma membrane. The fact that no difference in the total increase was determined suggests that hTRP3 is activated by store depletion but due to activation of other endogenous CRAC channels the total $[Ca^{2+}]_i$ was not different between hTRP3 tranfectants and controls. However Zhu et al (1998) have shown that not only the rate but also the total increase are increased in hTRP3 transfected cells.

The calcium measurements were also performed adding calcium first and thapsigargin afterwards. However the traces did not show any difference. The first response was always small and the second response large. The addition of calcium alone did not result

in an increased $[Ca^{2+}]_i$ in hTRP3 transfected, doxycyclin treated cells suggesting that hTRP3 was not constitutively active in these cells.

Calcium measurements using OAG as an activator of hTRP3 were also performed. In 62 % of hTRP3 transfected cells an increase in cytosolic calcium due to OAG stimulation could be detected. However 7 % of control transfected cells also showed a response. The average rate of $[Ca^{2+}]_i$ was 0.043 fluorescence units/min for hTRP3 compared to 0.033 fluorescence units/min for control transfected cells and suggest that OAG increased the open probability of hTRP3. The total increase in cytosolic calcium was determined as well. It was higher in hTRP3 transfected cells (0.045 fluorescence units) than in control transfected cells (0.019 fluorescence units). When statistical analysis of the total increase and the rate of $[Ca^{2+}]_i$ was performed, the difference was not significant ($p > 0.01$). However in our system even in cells that were prepared, loaded and in which calcium was measured at the same time the OAG response was inconsistent. The observation that not all cells transfected with hTRP3 responded to OAG activation may be due to variable hTRP3 expression from cell to cell. The small response of hTRP3 transfected cells to OAG may be due to low hTRP3 expression. However we cannot explain why a small percentage of control cells responded to OAG and there are no other reports of OAG activated calcium channels in LNCaP cells.

This experiment was also performed adding calcium first and OAG second. These traces did not show any difference. When calcium was added no response was seen and when OAG was added mostly hTRP3 transfected cells responded. However we would have expected to see a small increase in fluorescence when calcium was added as seen in

thapsigargin treated cells. This was not the case. Since these experiments were performed later than the thapsigargin experiments it may be due to the LNCaP cells. Other members from our laboratory have reported a high heterogeneity in LNCaP cells, too (Lei Zangh, personal communication).

Our result suggesting that hTRP3 is activated by OAG in LNCaP cells are supported by results from other groups. Store depletion independent hTRP3 activation by OAG in CHO cells microinjected with hTRP3 has been reported by one group (Hofmann et al., 1999). Another group performed calcium measurements in HEK 293 cells transiently transfected with hTRP3 using barium as a surrogate ion for calcium (McKay et al., 2000). Barium is readily passed by calcium channels but is a poor substrate for membrane and endoplasmic reticulum calcium ATP-ases and therefore rules out their influences on calcium inflow into the cytosol. However McKay et al reported an increase in barium inflow mediated by OAG activated hTRP3 in HEK293 cells compared to control cells.

Taken together the results presented alone suggest that hTRP3 is activated by both store depletion and OAG in LNCaP cells.

The effect of hTRP3 expression in LNCaP on cell viability and cell death was also examined. LNCaP/Tet-on cells were transiently transfected with pBI-EGFP or pBI-EGFP/hTRP3, stained with Hoechst 33258 and the nucleus of the GFP positive cells examined for condensation and fragmentation after 24, 48 and 72 hrs. No fragmented nuclei could be detected and the cells remained adherent, appeared viable and

proliferated normally in comparison to control cells. In unstimulated hTRP3 expressing LNCaP cells no increase in $[Ca^{2+}]_i$ occurred unless stimulated by agonists. We therefore treated hTRP3 transfected LNCaP cells with OAG to increase calcium and induce cell death. However no increase in cell death could be detected. This was judged over a period of 96 hrs by observing cell morphology using a light microscope. These results suggested that hTRP3 did not induce apoptosis in LNCaP cells. In CHO cells where hTRP3 has been reported to induce cell death after 48 hrs it has also been reported to be constitutively active (Zitt et al., 1997). TRPL has also shown to be constitutively active in LNCaP cells when calcium measurements were performed and to induce apoptosis in our laboratory 48 hrs after transfection. Other groups (Hofmann et al., 1999, McKay et al., 2000) have shown that hTRP3 was OAG regulated, not constitutively active and did not induce cell death. These results suggest that constitutive activity may be necessary to induce cell death.

Taken together the low intensity of the hTRP3 band in western blot analysis and the small changes in cytosolic calcium stimulated by OAG may explain why apoptosis was not induced in LNCaP cells.

6. Conclusion

hTRP3 has been successfully cloned into the pBI-EGFP vector and has been used to inducibly express GFP and hTRP3 in LNCaP cells as shown by fluorescence and western blot analysis.

Calcium measurements were performed to measure calcium inflow and have shown that hTRP3 is not constitutively active in our system. Calcium measurements using thapsigargin to deplete internal calcium stores showed that the rate of $[Ca^{2+}]_i$ inflow but not the total increase in calcium was enhanced in hTRP3 transfected cells. Calcium measurements using OAG to activate hTRP3 also showed that more hTRP3 transfected cells responded to OAG than control transfected cells and that the rate as well as the total increase in calcium were higher in hTRP3 transfected cells. The above results suggested that hTRP3 was activated by store depletion as well as by OAG .

Studies examining cell viability and cell death revealed that hTRP3 expression in LNCaP cells did not induce apoptosis. However western blot analysis and GFP fluorescence suggest that expression levels might have been too low to induce apoptosis. Furthermore hTRP3 was agonist activated in LNCaP cells. To determine the effects of hTRP3 on cell viability further experiments designed to improve expression of hTRP3 and optimise its activation would have to be carried out.

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