Use of a TRPC Channel for the Treatment of a Cardiovascular Disease

The invention refers to the use of a TRPC channel, an inactivating mutant thereof, or a nucleotide sequence coding for the TRPC channel or for the inactivating mutant for the production of a medicament for the treatment of a cardiovascular disease and a method of screening a modulator of the TRPC channel or an inactivating mutant thereof.

Atherosclerosis is one of the major causes of cardiovascular diseases in the Western world. In 2001 these diseases accounted for about 1 million deaths in the USA. Moreover, due to live style changes in the developing countries atherosclerosis and related cardiovascular diseases are becoming global epidemics. The WHO reports that cardiovascular diseases will be the leading cause of death in the developing world by 2010.

Thus, there is an immense medical need for new medicines that prevent and treat atherosclerosis. The generation and progression of atherosclerosis is a complex and incompletely understood process that is dependent on a number of epigenetic (e.g. life style, nutrition, exercise) and genetic factors. Numerous clinical observations implicate dysfunction of endothelial cells that line the inner vessel wall in the pathophysiology of atherosclerosis and atherogenesis (Ross, R. N. (1999), Engl. J. Med. 340:115-126).

Therefore, proteins involved in the regulation of endothelial function might be primary targets for anti-atherosclerotic therapies. Ca^{2+}-regulatory proteins seem to be of particular interest as important endothelial functions such as the production of nitric oxide (NO) are controlled by the level of intracellular Ca^{2+}.

TRPC channels, a novel class of ion channel proteins, are Ca^{2+} permeable non-selective cation channels expressed in the cardiovascular and other systems. Based on sequence homology TRPC3, TRPC6 and TRPC7 constitute a distinct TRPC subfamily. In expression systems these channels are activated by G protein coupled

receptors or depletion of intracellular Ca^{2+} stores (Clapham et al. (2001), Nat. Rev. Neurosci. 2:387-396). TRPC3 might also contribute to oxidative stress-activated cation currents in cultured endothelial cells (Balzer et al. (1999) Cardiovasc. Res. 42:543-549).

In order to study the functional role of TRPC1 in smooth muscle cells an antibody against a specific epitope of TRPC1 was used in WO 02/059155. However, this epitope is not found on other TRPC channels. WO 05/049084 discloses functional studies on isolated rat ventricular myocytes using the compound 2-aminoethoxydiphenylborate (2-APB). However, it is known that this compound shows unspecific and questionable effects in particular on TRPC3 (van Rossum et al. (2000), J. Biol. Chem., 275, 28562-28568).

According to the present invention we have suppressed TRPC3, TRPC6 and TRPC7 activity in endothelial cells of atherosclerotic rabbits in vivo using a genetic approach. Surprisingly, we found a dramatic improvement of vascular function and reduction of histological markers of atherosclerosis in vessels treated with a dominant negative TRPC3 gene, which means that the suppression of the activity of TRPC channels, in particular of the channels mentioned above, shows an anti-atherosclerotic effect. These findings establish a novel link between TRPC channels and cardiovascular diseases as atherosclerosis.

Therefore, one subject matter of the present invention is directed to the use of a TRPC channel, an inactivating mutant thereof, or a nucleotide sequence coding for the TRPC channel or for the inactivating mutant for the production of a medicament for the treatment of a cardiovascular disease, in particular atherosclerosis.

Preferred TRPC channels are the TRPC3 channel, TRPC6 channel or TRPC7 channel, in particular the TRPC3 channel or TRPC6 channel, especially the TRPC3 channel.

The corresponding amino acid sequences are SEQ ID NO: 1 (TRPC3), SEQ ID NO: 5 (TRPC6), and SEQ ID NO: 9 (TRPC7), in particular the amino acid sequence SEQ ID NO: 1 coding for the TRPC3 channel. The corresponding nucleotide sequences are SEQ ID NO: 2 (TRPC3), SEQ ID NO: 6 (TRPC6), and SEQ ID NO: 10 (TRPC7), in particular the nucleotide sequence SEQ ID NO: 2 coding for the TRPC3 channel.

According to the present invention the term "inactivating mutant" means a mutant which is functionally inactive as a cation channel but can suppress the channel activity of homologous naturally occurring TRPC channels. Suppression may be accomplished by replacing a homologous TRPC channel subunit in the native multimeric channel assembly with the effect that essentially all naturally occurring TRPC channels in the cell membrane contain mutant channel subunits or are totally replaced by the mutant channel. Mutations that render a channel subunit inactive may be preferably located within the pore regions of TRPC3 (amino acids 603-645 in SEQ ID NO: 1), TRPC6 (amino acids 660-705 in SEQ ID NO: 5) and TRPC7 (amino acids 610-650 in SEQ ID NO: 9). Particular examples are the mutant TRPC3 channel ($TRPC3^{DN}$) with the amino acid sequence of SEQ ID NO: 3, the mutant TRPC6 channel ($TRPC6^{DN}$) with the amino acid sequence of SEQ ID NO: 7, and the mutant TRPC7 channel ($TRPC7^{DN}$) with the amino acid sequence of SEQ ID NO: 11. A nucleotide sequence coding for $TRPC3^{DN}$ is the nucleotide sequence of SEQ ID NO: 4; a nucleotide sequence coding for $TRPC6^{DN}$ is the nucleotide sequence of SEQ ID NO: 8, and a nucleotide sequence coding for $TRPC7^{DN}$ is the nucleotide sequence of SEQ ID NO: 12. A particularly preferred example is the dominant-negative mutant $TRPC3^{DN}$ with the amino acid sequence of SEQ ID NO: 3. A nucleotide sequence coding for $TRPC3^{DN}$ is the nucleotide sequence of SEQ ID NO: 4.

Moreover, "inactivating mutants" may consist of any part of TRPC3, TRPC6 or TRPC7 that retain the ability to interact with naturally occurring TRPC channels at any step of protein synthesis or transport to the plasmamembrane and thereby suppress the function of the naturally occurring TRPC channels. Such part may be for example amino acids 0-302 of TRPC3 (SEQ ID NO: 1) (see Balzer et al. (1999) Cardiovasc. Res. 42:543-549).

Inactivating mutants may be detected and/or analyzed using the whole cell patch clamp method as exemplarily described in the Examples.

Another subject matter of the present invention is directed to the use of a TRPC channel, an inactivating mutant thereof, or a nucleotide sequence coding for the TRPC channel or for the inactivating mutant for the discovery of a TRPC channel modulator, in particular an inhibitor, as a medicament for the treatment of a cardiovascular disease, in particular atherosclerosis.

Preferred TRPC channels are the TRPC3 channel, TRPC6 channel or TRPC7 channel, in particular the TRPC3 channel or TRPC6 channel, especially the TRPC3 channel, as described above in detail.

In general, the TRPC channel or an inactivating mutant thereof, or a nucleotide sequence coding for the TRPC channel or for the inactivating mutant thereof is brought into contact with a test compound and the influence of the test compound on the TRPC channel, an inactivating mutant thereof, or a nucleotide sequence coding for the TRPC channel or for the inactivating mutant is measured or detected.

According to the present invention the term "TRPC channel modulator" means a modulating molecule ("modulator") of the TRPC channel, in particular an inhibitory or activating molecule ("inhibitor" or "activator"), especially an inhibitor of the TRPC channel identifiable according to the assay of the present invention. An inhibitors is generally a compound that, e.g. bind to, partially or totally block activity, decrease, prevent, delay activation, inactivate, desensitize, or down-regulate the activity or expression of at least one of the TRPC channels as preferably described above in detail. An activator is generally a compound that, e.g. increase, open, activate, facilitate, enhance activation, sensitize, agonize, or up-regulate the activity or expression of at least one of the TRPC channels as preferably described above in detail. Such modulators include genetically modified versions of the TRPC channels, preferably an inactivating mutant of the TRPC channels, such as $TRPC3^{DM}$, as well as

naturally occurring or synthetic ligands, antagonists, agonists, peptides, cyclic peptides, nucleic acids, antibodies, antisense molecules, ribozymes, small organic molecules and the like.

An example for the measurement of the TRPC channel activity is an assay comprising the steps of:

(a) contacting a fluorescent cell expressing a TRPC channel,

(b) stimulating Ca^{2+} influx by a channel activator before, simultaneously or after contacting the fluorescent cell with the modulator or test compound, and

(c) measuring or detecting a change in fluorescence.

Further details and alternative or preferred embodiments of that assay are described below and in the Examples.

Therefore, another subject matter of the present invention is directed to a method of screening a modulator of TRPC or an inactivating mutant thereof, or a nucleotide sequence coding for TRPC or for the inactivating mutant, wherein the method comprises the steps of:

(a) contacting a cell expressing a TRPC channel or an inactivating mutant thereof,

(b) stimulating Ca^{2+} influx by a channel activator before, simultaneously or after contacting the cell with a test compound, and

(c) measuring or detecting a change of the TRPC channel activity.

In a preferred embodiment the method further comprises the step of:

(d) selecting a test compound with an activity against a cardiovascular disease by comparing the changes of the TRPC channel activity in the absence of the test compound.

In another preferred embodiment the expression of the TRPC channel or an inactivating mutant thereof in the cell is controlled by an inducible promoter, preferably by a promoter which is selected from a tetracycline inducible promoter.

In a particular preferred embodiment the cell is a fluorescent cell as e.g. further described below.

Preferred cells or cell lines according to the present invention are MDCK, HEK 293, HEK 293 T, BHK, COS, NIH3T3, Swiss3T3 or CHO cells, in particular a HEK 293 cell line.

TRPC channel activity can be measured or detected by measuring or detecting a change in ion fluxes, in particular Ca^{2+} fluxes, by e.g. patch clamp techniques, whole cell currents, radiolabeled ion fluxes, or in particular fluorescence e.g. using voltage-sensitive dyes or ion-sensitive dyes (Vestergarrd-Bogind et al. (1988), J. Membrane Biol., 88:67-75; Daniel et al. (1991) J. Pharmacol. Meth. 25:185-193; Hoevinsky et al. (1994) J. Membrane Biol., 137:59-70; Ackerman et al. (1997), New Engl. J. Med., 336:1575-1595; Hamil et al. (1981), Pflugers. Archiv., 391:185).

Examples of such dyes are Di-4-ANEPPS (pyridinium 4-(2(6-(dibutylamino)-2-naphthalenyl)ethenyl)-1-(3-sulfopropyl) hydroxide), CC-2-DMPE (1,2-ditetradecanoyl-sn-glycero-3-phosphoethanolamine triethylammonium), DiSBAC2 (bis-(1,2-dibarbituric acid)-trimethine oxanol), DisBAC3 ((bis-(1,3-dibarbituric acid)-trimethine oxanol), SBFI-AM (1,3-benzenedicarboxylic acid,4,4'-[1,4,10-trioxa-7,13-diazacyclopentadecane-7,13-diylbis(5-methoxy-6,12-benzofurandiyl)]bis-(tetrakis-[(acetyloxy)methyl]ester)), fluo3am (1-[2-Amino-5-(2,7-dichloro-6-hydroxy-3-oxy-9-xanthenyl)phenoxy]-2-(2'-amino-5'- methylphenoxy)ethane- N,N,N',N'-tetraacetic), fluo4am (1-[2-Amino-5-(2,7-dichloro-6-hydroxy-4-oxy-9-xanthenyl) phenoxy]-2-(2'-amino-5'-methylphenoxy)ethane- N,N,N',N'-tetraacetic) or fura2am (1-[2-(5'-carboxyoxazol-2'-yl)-6-aminobenzofuran-5-oxy]-2-(2'-amino-5'-methyl-phenoxy)-ethane-N,N,N',N'-tetraacetic).

Examples of the channel activators are diacylglycerols, in particular 1-Oleyl-2acetyl-sn-glycerol (OAG), G_q-coupled receptor agonists, such as phenylephrine and in particular trypsin, an agonist that stimulates receptor tyrosine kinases such as epidermal growth

factor (EGF) or diacylglycerol generating enzymes such as phospholipases or activators thereof.

The channel activators, in particular OAG, can be used for the direct stimulation of the TRPC channels which is an additional advantage of the assay of the present invention compared to the indirect, receptor-mediated activation of the channels because, for example, in the present assay the rate of false-positive results are substantially reduced.

In general, a cell is provided which expresses a TRPC channel or an inactivating mutant thereof under an inducible promoter, as e.g. described above. Such cell can be produced using genetic methods known to a person skilled in the art and as described in the Examples. After having induced the expression of the TRPC channel or an inactivating mutant thereof the cells are usually plated into e.g. microtiter plates and grown. Usually the cells grow at the bottom of multiwell plates and are fixed. Thereafter, the cells are generally washed and loaded with a dye in a suitable loading buffer, preferably with a fluorescent dye such as fluo4am. After having removed the loading buffer, the cells are incubated with the test compound or modulator, in particular with a biochemical or chemical test compound as described above, e.g. in the form of a chemical compound library. Ca^{2+} measurements can be carried out using e.g. a Fluorescense Imaging Plate Reader (FLIPR). To stimulate Ca^{2+} influx through the TRPC channel a channel activator such as OAG should generally be applied.

As an alternative mode of TRPC channel activation trypsin as a G_q-coupled receptor agonist can be applied.

The expected effects of inhibitors would be a reduction of e.g. the fluorescence increase. Activators would lead to a further increase of e.g. an activator-evoked fluorescence or induce e.g. an activator-independent fluorescence increase.

Thereafter, suitable modulators, in particular inhibitors can be analyzed and/or isolated. For the screening of chemical compound libraries, the use of high-throughput

assays are preferred which are known to the skilled person or which are commercially available.

According to the present invention the term "chemical compound library" means a plurality of chemical compounds that have been assembled from any of multiple sources, including chemically synthesized molecules and natural products or combinatorial chemical libraries.

Advantageously the method of the present invention is carried out on an array and/or in a robotics system e.g. including robotic plating and a robotic liquid transfer system, e.g. using microfluidics, i.e. channelled structured.

In another embodiment of the present invention, the method is carried out in form of a high-through put screening system. In such a system advantageously the screening method is automated and miniaturized, in particular it uses miniaturized wells and microfluidics controlled by a roboter.

In a particularly preferred embodiment the assay/method of the present invention is carried out in a cell line containing a gene of a TRPC channel under the control of an inducible promoter, as detailed above, wherein the channel activator is solubilised. For example, preferably the activator OAG is solubilised in the presence of a serum albumin, e.g. bovine serum albumin, or plutonic acid.

Another subject matter of the present invention is directed to a method for producing a medicament for the treatment of atherosclerosis, wherein the method comprises the steps of:

(a) carrying out the method as described above,

(b) isolating a detected test compound suitable for the treatment of a cardiovascular disease, in particular atherosclerosis, and

(c) formulating the detected test compound with one or more pharmaceutically acceptable carriers or auxiliary substances.

Pharmaceutically acceptable carriers or auxiliary substances are for example a physiological buffer solution, e.g. sodium chloride solution, demineralised water, stabilizers, such as protease or nuclease inhibitors, or sequestering agents, such as EDTA.

The following Figures, Sequences and Examples shall explain the present invention without limiting the scope of the invention.

Description of the Figures

Figures 1A and 1B show that $TRPC3^{DN}$ does not carry functional ion currents

Figures 2A and B show the dominant-negative effect of $TRPC3^{DN}$ on TRPC3 and TRPC6

Figure 3 shows the effect of $TRPC3^{DN}$ on acetylcholine-induced vasoreactivity in carotid arteries of atherosclerotic rabbits

Figure 4 shows a sonographic assessment of flow-induced vasoreactivity in $TRPC3^{DN}$ expressing carotid artery segments

Figure 5 shows the size of atherosclerotic plaques in $TRPC3^{DN}$ expressing carotid artery segments

Figure 6 shows the effect of $TRPC3^{DN}$ expression on mean macrophage density in atherosclerotic carotid arteries

Figures 7A and 7B show the detection of OAG-activated Ca^{2+} signals in inducible TRPC3 and TRPC6 cell lines using FLIPR technology

Figures 8A and 8B illustrate the IC_{50} for TRPC3 and TRPC6 inhibition by SKF 96365 in doxycycline-induced cell lines by a FLIPR assay

Description of the Sequences

SEQ ID NO: 1 shows the amino acid sequence of TRPC3

SEQ ID NO: 2 shows a nucleotide sequence coding for TRPC3

SEQ ID NO: 3 shows the complete amino acid sequence of the dominant-negative TRPC3 channel ($TRPC3^{DN}$), (mutated amino acids are in bold)

SEQ ID NO: 4 shows the complete nucleotide sequence of $TRPC3^{DN}$; (mutated nucleotides are in bold)

SEQ ID NO: 5 shows the amino acid sequence of TRPC6

SEQ ID NO: 6 shows a nucleotide sequence coding for TRPC6

SEQ ID NO: 7 shows the amino acid sequence of a dominant-negative TRPC6 channel ($TRPC6^{DN}$), (mutated amino acids are in bold)

SEQ ID NO: 8 shows the complete nucleotide sequence of $TRPC6^{DN}$; (mutated nucleotides are in bold)

SEQ ID NO: 9 shows the amino acid sequence of TRPC7

SEQ ID NO: 10 shows a nucleotide sequence coding for TRPC7

SEQ ID NO: 11 shows the amino acid sequence of a dominant-negative TRPC7 channel ($TRPC7^{DN}$), (mutated amino acids are in bold)

SEQ ID NO: 12 shows the complete nucleotide sequence of $TRPC7^{DN}$; (mutated nucleotides are in bold)

Examples

1. Construction and functional properties of $TRPC3^{DN}$

To study TRPC channel function in vitro and in vivo we used a dominant-negative channel mutant to modulate native TRPC channel activity. The applicability of this approach for TRPC channels had been previously demonstrated (Hofmann et al. (2002) Proc. Natl. Acad. Sci. U.S.A., 99, 7461-7466). We generated a dominant-negative TRPC3 channel ($TRPC3^{DN}$) by exchanging amino acids 621-623 of wild type human TRPC3 (NP_003296) for alanines by site directed mutagenesis. The insert was cloned into a modified pcDNA3 vector backbone using gateway technology (Invitrogen, Karlsruhe, Germany).

A HEK 293 line stably expressing the muscarinic M_1–receptor (HM1 cells) was used in this study (Peralta et al. (1988) Nature, 334, 434-437). Cells were grown at 37°C in DMEM/F12 (1:1) medium supplemented with 10% fetal calf serum, 2 mM glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin (Invitrogen, Karlsruhe, Germany) in 5.5% CO_2. G418 (0.5 mg/ml) was added to the growth medium. The cDNAs for hTRPC3 (U47050), hTRPC6 (AF080394), $TRPC3^{DN}$, and eGFP (pEGFP-N1, BD Biosciences, Palo Alto, CA) or YFP-tagged versions of the channel proteins were transfected using LipofectAMINE 2000 (Invitrogen, Karlsruhe, Germany) according to the manufacturers' instructions

For electrophysiological experiments cells were transfected with the indicated amounts of cDNA in 35 mm dishes and plated onto coverslips 12 – 24 hrs after transfection. Cells were used 24-48 hrs after plating. If not indicated otherwise 0.4 µg eGFP was co-transfected as expression marker for patch-clamp experiments.

Whole-cell currents were recorded from fluorescent cells at room temperature with a HEKA EPC 10 patch clamp amplifier and PULSE software (HEKA, Lambrecht, Germany). Patch pipettes with resistances of 2 - 5 MΩ in standard extracellular buffer were pulled from borosilicate glass. The holding potential was set to –70 mV and currents during 160 ms voltage ramps from –100 to +60 mV were sampled with 6.6 kHz. All recordings were filtered at 2 kHz.

Standard external buffer contained (in mM): NaCl 140, KCl 5.4, $CaCl_2$ 2, $MgCl_2$ 1, glucose 10, HEPES 10, and pH was adjusted to 7.4 with NaOH. The standard pipette buffer contained (in mM): CsOH 120, gluconic acid 120, $MgCl_2$ 2, $CaCl_2$ 3, Cs_4-BAPTA 5, HEPES 10; pH was adjusted to 7.3 with gluconic acid. Free [Ca^{2+}] was calculated to be ~200 nM using the CaBuf program (G. Droogmans, KU Leuven). Receptor-activated currents were elicited in HM1 cells by application of 10 µM carbachol.

All statistical data is expressed as means ± SEM. Statistical analysis was performed using SigmaStat (SPSS, Chicago, IL). Results were pooled and analyzed using the Mann-Whitney rank sum test. The significance level was set to $p < 0.05$.

1.1 $TRPC3^{DN}$ does not carry functional ion currents

To establish that $TRPC3^{DN}$ acts as a negative TRPC channel modulator the functional properties of the channel mutant were investigated by whole cell patch clamp.

Representative current recordings from HM1 cells transfected with 3 µg TRPC3 or $TRPC3^{DN}$ are shown in Figures 1A and 1B. TRPC currents were elicited by the acetylcholine receptor agonist carbachol (10 µM). Currents were recorded before (con) and in the presence of carbachol (carb).

On average the current density of $TRPC3^{DN}$ expressing cells (1,3 pA/pF, n = 11) was not different from cells transfected with LacZ as a negative control (1,2 pA/pF, n = 10).

In accordance with the recordings shown in Figures 1A and 1B cells transfected with TRPC3 as a positive control displayed significantly greater current densities (11,2 pA/pF, n = 11; p < 0,001). Thus, $TRPC3^{DN}$ did not carry notable ion currents.

Previous studies suggest that TRPC3 directly interacts with itself as well as TRPC6 and TRPC7 (Hofmann et al. (2002) Proc. Natl. Acad. Sci. U.S.A., 99, 7461-7466). Therefore, the modulatory effects of $TRPC3^{DN}$ were tested by co-expression with wild type TRPC3 and TRPC6 channels.

1.2 Dominant-negative effect of $TRPC3^{DN}$ on TRPC3 and TRPC6

According to Figure 2A HM1 cells were co-transfected with 7 µg TRPC3-YFP and 7 µg LacZ as control (open bars) or 7 µg $TRPC3^{DN}$ (gray bars). According to Figure 2B HM1 cells were co-transfected with 7 µg TRPC6-YFP and LacZ or $TRPC3^{DN}$ as in Figure 2A.

Carbachol-induced currents were measured at –70 mV and normalized to the cell capacitance. $TRPC3^{DN}$ significantly ($^{***}p<0.001$) suppressed currents through both TRPC3 and TRPC6.

2. Anti-atherosclerotic effects of $TRPC3^{DN}$ in vivo

As shown in Figures 2A and 2B $TRPC3^{DN}$ conferred a dominant-negative effect on TRPC3 and TRPC6 channels. Based on sequence homology and previous reports (Hofmann et al. (2002), supra) it can be assumed that also the homologous TRPC7 channel is suppressed by $TRPC3^{DN}$. To investigate the effects of $TRPC3^{DN}$ expression and, thus, inhibition of TRPC3, TRPC6, and TRPC7 on atherosclerosis viral gene transfer of $TRPC3^{DN}$ to vessels of atherosclerotic rabbits were used.

Carotid arteries were infected with viruses harbouring $TRPC3^{DN}$ or eGFP as a control. 8 weeks after gene transfer the disease state was evaluated by functional and histological parameters.

2.1 Virus generation and animal studies

Adenoviruses encoding the dominant negative mutant of TPRC3 were generated, amplified and purified at large scale. Subsequently, the correctness of the sequence was checked by DNA sequencing (Medigenomix, Martinsried, Germany) and specific expression of the proteins was confirmed by Western Blotting.

White New Zealand rabbits, 20 weeks of age, were fed on a diet with 1% cholesterol + 5% corn oil. After 1 week of feeding, transgene expression was induced by catheter-based viral gene transfer (see below). Cholesterol feeding was continued for the whole course of the experiment. At least 8 animals were independently investigated in each group.

Serum cholesterol levels were assessed before the initiation of feeding, directly before gene transfer, and 2, 4 and 8 weeks after gene transfer. The measurements were carried out in a validated laboratory specialized on veterinarian serum determinations (Synlab, Augsburg, Germany). Also the LDL and HDL subfractions were determined with standard techniques. No significant differences were observed for serum cholesterol, LDL- or HDL cholesterol in control versus $TRPC3^{DN}$ receiving animals ($p > 0.05$) for all time points measured ($n = 8$ each group).

2.2 Endothelial gene transfer to the carotid artery

For gene transfer to the carotid artery a cervical midline incision was made and the left common artery was exposed. A segment of 4 cm was isolated with two small atraumatic clips (BIEMER vessel clips, FD 561 R). Approximately 0.2 ml $TRPC3^{DN}$ or

eGFP virus solution (titer 10^{10} pfu) was injected by a small needle (0.4x20) into the isolated segment. The incubation time was 20 min. Then, the clips were removed and the blood circulation was restored. The cervical wound was sutured and the animal was allowed to recover. The rabbits obtained analgesia (Temgesic®, Buprenorphin 0.01 mg/kg sc. every 12 h) for 72 hours post operation.

2.3 Measurement of Endothelial Vasoreactivity

At the end of the experiment, eight weeks after gene transfer, basal vessel diameter and acetylcholine-induced vasoreactivity were determined by high definition ultrasound of the carotid artery.

In vivo measurements of luminal vessel diameter was performed on carotid artery segments of atherosclerotic rabbits (Figure 3) transduced with $TRPC3^{DN}$ (hatched bars) or eGFP (open bars) and in healthy (non-atherosclerotic) rabbits (filled bars). Acetylcholine-induced vasoreactivity was measured 4 times every minute after the injection of the given doses of acetylcholine. $TRPC3^{DN}$ expression significantly improved acetylcholine-induced vasoreactivity compared to eGFP expressing segments (*** $p<0.001$). A decrease of vasoreactivity was observed in eGFP expressing segments vs. healthy controls (# $p<0.01$).

In a second set of experiments, flow-induced vasoreactivity in response to administration of sodium chloride solution was tested (Figure 4).

Luminal vessel diameter of carotid artery segments was measured in atherosclerotic rabbits transduced with $TRPC3^{DN}$ (hatched bars) or eGFP (open bars) and in healthy (non-atherosclerotic) rabbits (filled bars). Flow-induced vasoreactivity was tested during administration of 100 ml sodium chloride solution (applied over 5 min). Measurements taken after 80 ml of infusion and 1-2 min after application of the total volume are shown. $TRPC3^{DN}$ expression significantly improved flow-induced vasoreactivity compared to eGFP expressing segments (** $p<0.01$).

2.4 Determination of histological markers of atherosclerosis

In order to assess disease progression histologically animals were injected with heparin and sacrificed. Carotid arteries were dissected proximally 1 cm from the sternum, distally at the epiglottis. The vessels were flushed with saline, dried carefully and shock frozen. A piece of artery from the central area was excised for investigation of GFP expression, HE and van Gieson staining and immunohistochemistry.

The rest of the artery was used for Sudan macrostaining to detect lipid plaques. For this purpose, sudan red staining was directly induced after perfusing the vessels with saline for 2 minutes and cutting longitudinally in the middle. Thereafter, the vessels were transferred to Sudan stain solution (Sudan III dissolved in alcohol and acetone).

Relative plaque size was determined by Sudan red staining followed by histological image analysis of carotid artery segments trancduced with $TRPC3^{DN}$ or eGFP (Figure 5). The right carotid arteries from rabbits that received $TRPC3^{DN}$ (to the left arteries) were evaluated as untreated controls. $TRPC3^{DN}$ decreased plaque size vs. both eGFP (**$p < 0.01$) and untreated (*$p < 0.05$) controls.

As another marker of atherosclerosis progression macrophage infiltration of the vessel intima was studied by immunocytochemisty. For histological staining, 6 µm slices were cut from the middle of all vessels which had been prepared by removing fat. The slices were fixed with acetone for 10 minutes at room temperature and air dried. They were then permeabilized with 0.6% H_2O_2 in 100% methanol for 5 minutes, washed with PBS three times and incubated with 20% rabbit serum. Next the slices were incubated with an anti-macrophage monoclonal antibody (1:100, clone RAM-11, DAKO, Hamburg, Germany) at 20°C for 20 - 30 minutes and washed three times with PBS. They were then incubated with the appropriate biotin-labelled secondary antibodies for 30 minutes at 20°C, and again washed three times with PBS. Subsequently slices were incubated with ABC reagent for 30 minutes, washed with PBS, and stained with

diaminobenzidine and H_2O_2 for 10 minutes. Counterstaining with Harris/hematoxylin/eosin was carried out thereafter. Analysis was performed by directly counting single positive cells in the vessel intima in each slide at high magnification (typically 600-fold) by a standardized counting procedure (Figure 6).

Macrophages were identified by immunocytochemistry in the intimal layer of carotid arteries expressing $TRPC3^{DN}$ or eGFP. $TRPC3^{DN}$ caused a significant reduction of macrophage infiltration compared to eGFP expressing vessels ($^*p < 0.05$).

3. Assay for high throughput identification of TRPC3, TRPC6 and TRPC7 modulators

To develop an assay for the identification of TRPC channel modulators, recombinant HEK 293 cell lines were produced using the Flp-In T-REx system (Invitrogen, Karsruhe, Germany) that express TRPC3 (accession # U47050) or TRPC6 (accession # AF080394) cDNAs under the control of an inducible promoter. Channel cDNAs were amplified by standard techniques and cloned into pcDNA5/FRT/TO (Invitrogen, Karlsruhe, Germany). After transfection of Flp-In T-Rex 293 cells with the channel constructs stable cell lines were selected with hygromycin. Cells were maintained according to the manufactures instructions and channel expression of was induced by addition of 1 µg / ml doxycycline for 20 - 30 h. Cells were plated into 96 well poly-d-lysine coated black walled clear bottom microtiter plates (BD Biosciences, Bedford, MA) at a density of 40000-50000 cells/well 20 – 30 h before experiments. Cell were then washed and loaded with 2 µM fluo4am (Molecular Probes, Eugene, OR) for 30 - 45 min at room temperature in a buffer containing 1x HBSS (#14065-049, Invitrogen), 1 mM $CaCl_2$, 20 mM HEPES, 0.02 % Pluronic F-127 (Molecular Probes), and 0.05% bovine serum albumin (pH = 7.4). Loading buffer was removed and cells incubated with test compounds for 10 min at room temperature. Ca^{2+} measurements were performed using a 96 well Fluorescence Imaging Plate Reader (FLIPR), (Molecular Devices Corporation, Sunnyvale, CA). To stimulate Ca^{2+} influx through TRPC3 or TRPC6 we applied the channel activator 1-oleyl-2-acetyl-sn-glycerol (OAG) (Hofmann et al., 1999), at a final concentration of 30 – 50 µM. OAG was dissolved in a buffer

containing 1x HBSS (#14065-049, Invitrogen), 1 mM $CaCl_2$, 20 mM HEPES, 0.02 % Pluronic F-127 (Molecular Probes) (pH = 7.4) and added to the cells. Alternatively, OAG could be dissolved in a buffer containing 1x HBSS (#14065-049, Invitrogen), 1 mM $CaCl_2$, 20 mM HEPES, and 0.1 % bovine serum albumin (pH = 7.4).

Figures 7A and 7B show that responses to OAG were observed only in doxycyclin-treated but not in non-induced cells. Thus, this result demonstrates that the OAG-activated Ca^{2+} signal is specifically mediated by TRPC3 or TRPC6 respectively.

Fluorescence changes in fluo-4 loaded cells were measured using FLIPR II. The bar indicates application of 50 µM OAG. Shown are mean fluorescence values ± SEM from 9 wells each. Fluorescence values were normalized to the mean baseline fluorescence (F_0).

To identify modulators of TRPC3 or TRPC6 with the assays described above induced cells were used and test compounds are added to the wells before or after application of OAG. The expected effect of inhibitors would be a reduction of the fluorescence increase. Channel activators would lead to a further increase of the OAG-evoked signal or induce an OAG-independent fluorescence increase. SKF 96365 (1-(ß-[3-(4-methoxyphenyl)propoxy]-4-methoxyphenethyl)-1H-imidazole-HCl) has been described as an inhibitor of nonselective cation channels including TRPC3 and TRPC6 (Boulay et al. (1997), J. Biol. Chem., 272, 29672-29680; Zhu et al. (1998), J. Biol. Chem., 273, 133-142).

As shown in Figures 8A and 8B SKF 96365 inhibited OAG-activated fluorescence responses in TRPC3 expressing cells with an IC_{50} of 1,8 ± 0,12 µM (n = 8) and in TRPC6 expressing cells with an IC_{50} of 5.04 ± 0.17 µM (n = 8). Thus, the given examples demonstrate the ability of the assays to identify TRPC3 and TRPC6 modulators.

Fluorescence changes induced by 30 µM OAG in TRPC3- and TRPC6 expressing cells were measured in the presence of the given concentrations of SKF 96365. Mean

inhibition values ± SEM are shown. Inhibition is expressed as % of control fluorescence in the absence of SKF 96365. Dose-response curves were fitted to the general dose-response equation. The spline curves represent the best fits to the composite data.

As an alternative mode of TRPC3 and TRPC6 channel activation the protease-activated receptor agonist trypsin (200 nM) was applied to induced Flp-In T-REx-TRPC3 and Flp-In T-REx-TRPC6 cells. This treatment exemplifies the use of G_q-coupled receptor agonists for stimulation of TRPC3 or TRPC6. At t = 60 sec after application trypsin induced significantly greater increases in fluorescence in induced Flp-In T-REx-TRPC3 and Flp-In T-REx-TRPC6 cells compared to non-induced controls (Table 1). Hence, G_q-coupled receptor agonists, e.g. trypsin, may also be used in the assay to stimulate TRPC3 and TRPC6 responses.

Table 1: Fluorescence changes (F/F_0) in induced and non-induced Flp-In T-REx-TRPC3 and Flp-In T-REx-TRPC6 cells in response to 200 nM trypsin

	Flp-In T-REx-TRPC3	Flp-In T-REx-TRPC6
induced	2.71 ± 0.022 (n = 9)	2.26 ± 0.05 (n = 12)
non-induced	1.65 ± 0,034 (n = 9)	0,88 ± 0.009 (n = 12)

Values are given as means ± SEM. Induced and non-induced groups were significantly different ($p < 0.001$, t-test).

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SEQ ID NO: 2

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SEQ ID NO: 4

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SEQ ID NO: 8

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SEQ ID NO: 9

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SEQ ID NO: 10

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SEQ ID NO: 11

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SEQ ID NO: 12

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Claims

1. Use of a TRPC channel, an inactivating mutant thereof, or a nucleotide sequence coding for the TRPC channel or for the inactivating mutant for the production of a medicament for the treatment of a cardiovascular disease.

2. The use according to claim 1, wherein the TRPC channel is selected from the TRPC3 channel, TRPC6 channel and/or TRPC7 channel.

3. The use according to claim 2, wherein the amino acid sequence of TRPC3 is the amino acid sequence of SEQ ID NO: 2, the amino acid sequence of TRPC6 is the amino acid sequence of SEQ ID NO: 5, and the amino acid sequence of TRPC7 is the amino acid sequence of SEQ ID NO: 9.

4. The use according to claim 2, wherein the inactivating mutant is $TRPC3^{DN}$ with the amino acid sequence of SEQ ID NO: 3, $TRPC6^{DN}$ with the amino acid sequence of SEQ ID NO: 7 or $TRPC7^{DN}$ with the amino acid sequence of SEQ ID NO: 11.

5. The use according to claim 2, wherein the nucleotide sequence coding for TRPC3 is the nucleotide sequence of SEQ ID NO: 2, the nucleotide sequence of TRPC6 is the nucleotide sequence of SEQ ID NO: 6, and the nucleotide sequence of TRPC7 is the nucleotide sequence of SEQ ID NO: 10.

6. The use according to claim 4, wherein the nucleotide sequence coding for $TRPC3^{DN}$ is the nucleotide sequence of SEQ ID NO: 4, the nucleotide sequence coding for $TRPC6^{DN}$ is the nucleotide sequence of SEQ ID NO: 8, and the nucleotide sequence coding for $TRPC7^{DN}$ is the nucleotide sequence of SEQ ID NO: 12.

7. Use of a TRPC channel, an inactivating mutant thereof, or a nucleotide sequence coding for the TRPC channel or for the inactivating mutant for the discovery of a

TRPC channel modulator as a medicament for the treatment of a cardiovascular disease.

8. The use according to claim 7, wherein the TRPC channel is selected from the TRPC3 channel, TRPC6 channel and/or TRPC7 channel.

9. The use according to claim 8, wherein the amino acid sequence of TRPC3 is the amino acid sequence of SEQ ID NO: 1, the amino acid sequence of TRPC6 is the amino acid sequence of SEQ ID NO: 5, and the amino acid sequence of TRPC7 is the amino acid sequence of SEQ ID NO: 9.

10. The use according to claim 8, wherein the inactivating mutant is $TRPC3^{DN}$ with the amino acid sequence of SEQ ID NO: 3, $TRPC6^{DN}$ with the amino acid sequence of SEQ ID NO: 7, or $TRPC7^{DN}$ with the amino acid sequence of SEQ ID NO: 11.

11. The use according to claim 8, wherein the nucleotide sequence coding for TRPC3 is the nucleotide sequence of SEQ ID NO: 2, the nucleotide sequence of TRPC6 is the nucleotide sequence of SEQ ID NO: 6, and the nucleotide sequence of TRPC7 is the nucleotide sequence of SEQ ID NO: 10.

12. The use according to claim 10, wherein the nucleotide sequence coding for $TRPC3^{DN}$ is the nucleotide sequence of SEQ ID NO: 4, the nucleotide sequence coding for $TRPC6^{DN}$ is the nucleotide sequence of SEQ ID NO: 8, and the nucleotide sequence coding for $TRPC7^{DN}$ is the nucleotide sequence of SEQ ID NO: 12.

13. The use according to any of the claims 5-8, wherein the TRPC channel, an inactivating mutant thereof, or a nucleotide sequence coding for the TRPC channel or for the inactivating mutant is brought into contact with a test compound and the influence of the test compound on the TRPC channel, an inactivating

mutant thereof, or a nucleotide sequence coding for the TRPC channel or for the inactivating mutant is measured or detected.

14. The use according to any of the claims 1-13, wherein the cardiovascular disease is atherosclerosis.

15. A method of screening a modulator of the TRPC channel or an inactivating mutant thereof, or a nucleotide sequence coding for the TRPC channel or for the inactivating mutant, wherein the method comprises the steps of:
    (a) contacting a cell expressing a TRPC or an inactivating mutant thereof,
    (b) stimulating Ca^{2+} influx by a channel activator before, simultaneously or after contacting the cell with a test compound, and
    (c) measuring or detecting a change of the TRPC channel activity.

16. The method of claim 15 further comprising the step of:
    (d) selecting a test compound with an activity against a cardiovascular disease by comparing the changes of the TRPC channel activity in the absence of the test compound.

17. The method according to any of the claims 15-16, wherein the expression of the TRPC channel or an inactivating mutant thereof in the cell is controlled by an inducible promoter.

18. The method according to claim 17, wherein the inducible promoter is selected from a tetracycline-inducible promoter.

19. The method according to any of the claims 15-18, wherein the cell is a fluorescent cell.

20. The method according to any of the claims 15-19, wherein the cell is selected from a MDCK, HEK 293, HEK 293 T, BHK, COS, NIH3T3, Swiss3T3 or CHO cell.

21. The method according to any of the claims 15-20, wherein the TRPC channel activity is measured or detected by measuring or detecting a change in ion fluxes, in particular Ca^{2+} fluxes, by preferably patch clamp techniques, whole cell currents, radiolabeled ion fluxes, or in particular fluorescence preferably using voltage-sensitive dyes or ion-sensitive dyes.

22. The method according to any of the claims 15-21, wherein the channel activator is a compound selected from diacylglycerols, in particular 1-Oleyl-2acetyl-sn-glycerol (OAG), G_q-coupled receptor agonists, especially phenylephrine and in particular trypsin, an agonist that stimulates receptor tyrosine kinases, expecially epidermal growth factor (EGF), or diacylglycerol generating enzymes, especially phospholipases or activators thereof.

23. The method according to any of claims 15-22, wherein the TRPC is selected from TRPC3, TRPC6, TRPC7, or an inactivating mutant thereof.

24. The method according to claim 23, wherein the inactivating mutant is $TRPC3^{DN}$ with the amino acid sequence of SEQ ID NO: 3, $TRPC6^{DN}$ with the amino acid sequence of SEQ ID NO: 7, or $TRPC7^{DN}$ with the amino acid sequence of SEQ ID NO: 11.

25. The method according to any of the claims 15-24, wherein the test compound is provided in the form of a chemical compound library.

26. The method according to any of the claims 15-25, wherein the method is carried out on an array.

27. The method according to any of the claims 15-26, wherein the method is carried out in a robotics system.

28. The method according to any of the claims 15-27, wherein the method is a method of high-through put screening of the test compound.

29. The method according to any of the claims 15-28, wherein the test compound detected is an inhibitor.

30. The method according to any of the claims 15-29, wherein the cells are seeded onto a well of a multi-well test plate.

31. A method for producing a medicament for the treatment of a cardiovascular disease, wherein the method comprises the steps of:

(a) carrying out the method according to any of the claims 15-30,

(b) isolating a detected test compound suitable for the treatment of a cardiovascular disease, and

(c) formulating the detected test compound with one or more pharmaceutically acceptable carriers or auxiliary substances.

32. The method according to any of the claims 15-31, wherein the cardiovascular disease is atherosclerosis.