TITLE OF THE INVENTION

Fluorescence based assay to detect sodium/calcium exchanger (NCX) "reverse mode" modulating compounds

FIELD OF THE INVENTION

The present invention relates to sodium/calcium exchangers (NCX) and methods for determining their activity. More specifically, the invention relates to a fluorescence-based assay for detecting NCX "reverse mode" modulating compounds. It further refers to a kit of parts comprising cells expriming NCX and the use of the kit of parts.

BACKGROUND OF THE INVENTION

A basic requirement for life is compartmentalization - with biological membranes being nature's tool to realize this principle. However, a lipid bilayer - the structure underlying the cell membrane - is impermeable to most ions and compounds whose transport is essential to sustain vital functions in cells and organisms. The answer to this paradox lies in the semi-permeable nature of the cell membrane - solutes that have to cross the membrane are transported by specific membrane proteins. These transporters are responsible for the generation and maintenance of ion gradients, the uptake of nutrients, the transport of metabolites, the reuptake of signaling molecules and the disposal of toxic and waste compounds. Therefore, transporters are potential drug targets that allow direct influence on disease-related abnormalities in this context.

Transporters are an emerging target family with enormous potential, offering scientific and economic opportunities. On the other hand, transporters are a difficult target class in terms of drug-discovery technologies.

The sodium/calcium exchanger human gene family (also known as NCX or SLC8) encompasses three distinct proteins, NCX1, NCX2 and NCX3. SLC8 together with SLC24 constitute a superfamily of Na^+/Ca^{2+} countertransporters. SLC24 family members also transport K^+, they are also known as NCKXs.

NCX1 is the most highly characterized member of the sodium/calcium exchanger human gene family, its expression is up regulated in failing human heart and is involved in ischemia-reperfusion damage after myocardial infarction. Inhibition of NCX1 normalizes heart muscle contractility in failing hearts and acts cardio-protective during post-ischemic reperfusion (Flesch et al., Circulation 1996; Komuro and Ohtsuka, Journal of Pharmacological. Sciences. 2004). NCX2 is mainly expressed in the brain and NCX3 in the brain and skeletal muscle, their physiological roles remain elusive.

The sodium/calcium exchanger can transport Ca^{2+} and Na^{+} in two directions depending on membrane potential and ion gradients. At the first direction, named as "forward mode" or "calcium export mode", Ca^{2+} is transported out of the cell and Na^{+} into the cell. At the other direction, named "reverse mode" or "calcium import mode", the transport directions are vice versa.

The sodium/calcium exchanger is an important mechanism for removing Ca^{2+} from diverse cells. In heart, it extrudes Ca^{2+} that has entered through Ca^{2+} channels to initiate contraction, while Na^{+} enters the heart cell. Its relevance in cardiovascular diseases is e.g. illustrated in Hobai, JA & O'Rourke,B (2004) Expert Opin. Investig. Drugs, 13, 653-664. Therefore, pharmaceutical industry has developed compounds inhibiting the NCX as e.g. described in Iwamoto, T. et al. (2004) J. Biol. Chem., 279, 7544-7553.
The inhibition of the cardiac sodium/calcium exchanger is important for cardio-protection (e.g. Pogwizd, 2003). It exists a medical need to discrimate between compounds inhibiting the "forward mode" and compounds inhibiting the "reverse mode" of the NCXs. Thus, an assay analyzing the calcium import mode is needed. In addition, compounds inhibiting NCX1 should be selective as NCX2 and NCX3 belong to the same transporter family. Thus, an assay allowing to do selectivity profiling is needed.

It is of considerable interest to identify compounds that modulate the sodium/calcium exchanger activity, for example, by blocking the flow of calcium and/or inhibiting the activation of NCX. One standard method to do so is through the use of patch clamp experiments.
In these experiments, cells must be evaluated individually and in sequence by highly skilled operators, by measuring the calcium current across the cell membrane in response to changes of the membrane potential and/or application of test compounds. The effect of Sea0400, a new specific inhibitor of NCX, on the action potential in dog ventricular papillary muscle was investigated and disclosed by K. Acsai during the "ESC Congress 2004" in Munich on Poster

Nr. 2886 (Title: Effect of a specific sodium-calcium exchanger blocker Sea0400 on the ventricular action potential and triggered activity in dog ventricular muscle and Purkinje fiber) and by C. Lee et al. (The journal of pharmacology and experimental therapeutics; Vol. 311: 748-757, 2004; Title: Inhibitory profile of SEA0400 [2-[4-[(2,5-Difluorophenyl)methoxy]phenoxy]-5-ethoxyaniline] assessed on the cardiac Na^+/Ca^{2+} exchanger, NCX1.1).

It was shown, using an ion-selective electrode technique to quantify ion fluxes in giant patches, that the cardiac Na^+/Ca^{2+} exchanger has multiple transport modes (Tong Mook Kang & Donald W. Hilgemann; Nature; Vol. 427, 5 February 2004; Title: Multiple transport modes of the cardiac Na^+/Ca^{2+} exchanger).

These experiments, while valid and informative, are very time consuming and not adaptable to high-throughput assays for compounds that modulate calcium ion channel activity.

Various techniques have been developed as alternatives to standard methods of electrophysiology. For example, radioactive flux assays have been used in which cells are exposed with a radioactive tracer (e.g., ^{45}Ca) and the flux of the radio-labled Ca is monitored. Cells loaded with the tracer are exposed to compounds and those compounds that either enhance or diminish the efflux of the tracer are identified as possible activators or inhibitors of ion channels in the cells' membranes. A specific example is enclosed in T. Kuramochi et al.; Bioorganic & Medicinal Chemistry; 12 (2004) 5039-5056; Title: Synthesis and structure-activity relationships of phenoxypyridine derivates as novel inhibitors of the sodium-calcium exchanger. EP1031556 discloses a method wherein Na^+/Ca^{2+} exchanger activity is measured using sarcolemmal vesicles, the concentration of Ca^{2+} uptake in the sarcolemmal vesicles being determined by measuring ^{45}Ca radioactivity.

Many radioactive ion-transporter assays have limited sensitivity and therefore insufficient data quality. In addition, the cost and safety issues associated with the radioactive screening technology are hurdles that hinder a broadened application.

Among the above cited drug-discovery technologies, the use of radioactive flux assays to identify compounds that modulate the activity of ion channels and ion transporters is the closest prior art to our invention as it is a technique in which a test compound can be identified as possible activator or inhibitor by monitoring the flux of Ca^{2+} from the cells.

The main issue for the radioactive assays is based on the difficulty of detecting the limited turnover of ion transporters of about 1 to 1000 molecules per second – about 10^4 times less than most ion channels.

The problem arising from the state of the art was therefore to identify a robust assay for high throughput screening and profiling of sodium/calcium exchanger modulators with a very good sensitivity, allowing to discriminate the "forward mode" and "reverse mode" modulating activity of compounds and allowing to profile identified modulators regarding their selectivity towards NCX1, NCX2 and NCX3, respectively. The present invention solves this problem.

SUMMARY OF THE INVENTION

One subject-matter of the present invention refers to an assay for determining the calcium import activity of a sodium/calcium exchanger wherein:

a) cells expressing a sodium/calcium exchanger are provided;
b) a colored substance for determining intracellular calcium is provided;
c) cells are contacted with a sodium/calcium exchanger activator; and
d) the calcium mediated change in the luminescent signal from said colored substance is compared to a luminescent signal produced in a control experiment.

Another subject-matter of the present invention refers to an assay for determining the calcium import activity of a sodium/calcium exchanger in response to the addition of a compound wherein:

a) cells expressing a sodium/calcium exchanger are provided;
b) a colored substance for determining intracellular calcium is provided;
c) cells are contacted with a compound, wherein said cells have been treated, prior to treating with said compound, with a sodium/calcium exchanger activator; and
d) the calcium mediated change in the luminescent signal from said colored substance is compared to a luminescent signal produced in a control experiment.

In general, the sodium/calcium exchanger used is of mammalian origin, and in particular of human origin. The sodium/calcium exchanger is selected from one of the following sodium/calcium exchanger proteins: NCX1, NCX2, NCX3, NCX4, NCX5, NCX6 and/or NCX7, in particular NCX1, NCX2 and/or NCX3; and/or from one of the following sodium/calcium/potassium exchanger proteins: NCKX1, NCKX2, NCKX3, NCKX4 and/or NCKX5.

In general, the cells used in the assay of the present invention can be derived from any eukaryotic organism. In a preferred embodiment, the cells are mammalian cells. In a more preferred embodiment, the cells are CHO (CCL-61), HEK (CCL-1573), COS7 (CRL-1651) and/or JURKAT (CRL-1990) cells.

In a preferred embodiment, said colored substance is added to the cells as a dye precursor capable of entering the cells and being hydrolyzed to a dye, whereby the dye complexes with calcium in said cells and provides a luminescent signal. Further said dye precursor can be preferably an acetoxymethylester derivate and said dye can be preferably the calcium sensitive fluorescence dye fluo-4. In a more preferred embodiment, said luminescent signal is fluorescence and said monitoring step c) employs a FLIPR device.

The invention pertains further to the use of an assay as mentioned before to test a compound for activity as an agonist or antagonist of the calcium import activity of a sodium/calcium exchanger. In another preferred embodiment, the invention pertains to the use of an assay as mentioned before for the diagnosis of a disease associated with a sodium/calcium exchanger altered expression.

The invention pertains further to a kit of parts comprising:

a) lyophilized cells expriming a sodium/calcium exchanger;
b) a colored substance;
c) a compound buffer; and
d) a colored substance buffer.

In a preferred embodiment of the kit of parts of the present invention, said colored substance is the calcium sensitive fluorescence dye fluo-4. In another preferred embodiment, the sodium/calcium exchanger used is of mammalian origin, and in particular of human origin. The sodium/calcium exchanger is selected from one of the following sodium/calcium exchanger

proteins: NCX1, NCX2, NCX3, NCX4, NCX5, NCX6 and/or NCX7, in particular NCX1, NCX2 and/or NCX3; and/or from one of the following sodium/calcium/potassium exchanger proteins: NCKX1, NCKX2, NCKX3, NCKX4 and/or NCKX5.

The invention pertains further to the use of a kit of parts as mentioned before to test a compound for activity as an agonist or antagonist of the calcium import activity of a sodium/calcium exchanger. In another preferred embodiment, the invention pertains to the use of a kit of parts as mentioned before for the diagnosis of a disease associated with a sodium/calcium exchanger altered expression.

DETAILED DESCRIPTION OF THE INVENTION

The term “assay” refers to a procedure where a property of a system or object is measured. Assay is a short hand commonly used term for biological assay and is a type of in vitro experiment. Assays are typically conducted to measure the effects of a substance on a living organism. Assays may be qualitative or quantitative, they are essential in the development of new drugs.
The subject assay provides a broad dynamic range so that the activity of a NCX protein can be determined. In particular the present invention makes available a rapid, effective assay for screening and profiling pharmaceutically effective compounds that specifically interact with and modulate the activity of a sodium/calcium exchanger.

The term " sodium/calcium exchanger " or “NCX” in context of the present invention shall mean any one of the list of the following Na^+/Ca^{2+} exchanger proteins: NCX1, NCX2, NCX3, NCX4, NCX5, NCX6, NCX7; or any one of the list of the following $Na^+/Ca^{2+}/K^+$ exchanger proteins: NCKX1, NCKX2, NCKX3, NCKX4, NCKX5, either alone or in combination with each other.
Especially preferred are the SLC8 family members NCX1, NCX2 and/or NCX3 which amino acid sequences correspond, respectively, to SEQ ID NO: 1, SEQ ID NO: 2 and SEQ ID NO: 3.

Such a sodium/calcium exchanger could be derived from any vertebrate and in particular mammalian species (e.g. dog, horse, bovine, mouse, rat, canine, rabbit, chicken, anthropoid, human or others). The sodium/calcium exchanger could be isolated from tissue probes of such

vertebrate organisms or could be manufactured by means of recombinant biological material that is able to express the sodium/calcium exchanger.

The term "sodium/calcium exchanger protein" refers to polypeptides, polymorphic variants, mutants, and interspecies homologues that have an amino acid sequence that has greater than about 80% amino acid sequence identity, 85%, 90%, preferably 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% or greater amino acid sequence identity, preferably over a region of at least about 25, 50, 100, 200, or 500, or more amino acids, to an amino acid sequence contained in SEQ ID NO: 1, SEQ ID NO: 2 and SEQ ID NO: 3.

The term "biological material" means any material containing genetic information and capable of reproducing itself or being reproduced in a biological system. Recombinant biological material is any biological material that was produced, has been changed or modified by means of recombinant techniques well known to a person skilled in the art.

The following references are examples of the cloning of particular NCX proteins: The canine Na^+/Ca^{2+} exchanger NCX1 has been cloned by Nicoll, DA. et al. (Science. 250(4980): 562-5, 1990; Title: Molecular cloning and functional expression of the cardiac sarcolemmal Na(+)-Ca2+ exchanger.). The human Na^+/Ca^{2+} exchanger NCX1 has been cloned by Komuro, I., et al. (Proc. Natl. Acad. Sci. U.S.A. 89 (10), 4769- 4773, 1992; Title: Molecular cloning and characterization of the human cardiac Na^+/Ca^{2+} exchanger cDNA) and by Kofuji, P. et al. (Am. J. Physiol. 263 (Cell Physiol. 32): C1241-C1249, 1992; Title: Expression of the Na-Ca exchanger in diverse tissues: a study using the cloned human cardiac Na-Ca exchanger). The human Na^+/Ca^{2+} exchanger NCX2 has been cloned by Li, Z. et al. (J. Biol. Chem. 269(26): 17434-9, 1994; Title: Cloning of the NCX2 isoform of the plasma membrane Na(+)-Ca2+ exchanger). The rat Na^+/Ca^{2+} exchanger NCX3 has been cloned by Nicoll, DA. et. al. (J. Biol. Chem. 271(40): 24914-21. 1996; Title: Cloning of a third mammalian Na^+/Ca^{2+} exchanger, NCX3). The human Na^+/Ca^{2+} exchanger NCX3 has been cloned by Gabellini, N. et. al. (Gene. 298: 1-7, 2002; Title: The human SLC8A3 gene and the tissue-specific Na^+/Ca^{2+} exchanger 3 isoforms).

The terms "polypeptide", "peptide" and "protein" are used interchangeably herein to refer to a polymer of amino acid residues. The terms apply to amino acid polymers in which one or more amino acid residue is an artificial chemical mimetic of a corresponding naturally occurring

amino acid, as well as to naturally occurring amino acid polymers and non-naturally occurring amino acid polymer.

The term "calcium import activity of a sodium/calcium exchanger" refers to the "reverse mode" of the sodium/calcium exchanger, i.e. the mechanism of transporting Ca^{2+} into the cell and Na^{+} out of the cell. This "reverse mode" transport occurs under certain plasma membrane-depolarizing conditions and high cytosolic Na^{+} concentration.
The activity of a sodium/calcium exchanger is determined by measuring the enhanced luminescence resulting from a suitable colored substance complexing with calcium.

The term "cells expressing a sodium/calcium exchanger" refers to cells expressing the exchanger of interest endogenously or recombinant cells.

The term "recombinant" when used with reference, e. g., to a cell, or nucleic acid, protein, or vector, indicates that the cell, nucleic acid, protein or vector, has been modified by the introduction of a heterologous nucleic acid or protein or the alteration of a native nucleic acid or protein, or that the cell is derived from a cell so modified. Thus, for example, recombinant cells express genes that are not found within the native (non-recombinant) form of the cell or express native genes that are otherwise abnormally expressed, under expressed or not expressed at all. In the present invention this typically refers to cells that have been transfected with nucleic acid sequences that encode a sodium/calcium exchanger.

The assay is performed simply by growing the cells in an appropriate container with a suitable culture medium. The cell may be a naturally occurring cell, a native cell, an established cell line, a commercially available cell, a genetically modified cell, etc. so long as the cell is able to be maintained during the assay and desirably growing in a culture medium.

Suitable cells for generating the subject assay include prokaryotes, yeast, or higher eukaryotic cells, especially mammalian cells. Prokaryotes include gram negative and gram positive organisms. The cells will usually be mammalian cells, such as human cells, mouse cells, rat cells, Chinese hamster cells, etc. Cells that are found to be convenient include CHO, COS7, JURKAT, HeLa, HEKs, MDCK and HEK293 cells.
Cells may be prepared with the well known methods (Current protocols in cell biology, John Wiley & Sons Inc, ISBN: 0471241059) or may be bought (Invitrogen Corp., Sigma-Aldrich Corp., Stratagene).

The term “colored substance” refers in particular to a calcium sensitive fluorescence dye. The dye precursor is characterized by not being luminescent under the conditions of the assay, being an ester capable of entering the cells and that is hydrolyzed intracellularly to the luminescent oxy compound, and providing enhanced luminescence upon complexing with calcium. The esters are chosen to be susceptible to hydrolysis by intracellular hydrolases.

The term “capable of entering the cells” means that the precursors are able to cross the cellular membrane and be hydrolyzed in the cells, the dye precursor enters the cells under specific conditions of pH, temperature, etc., enters the cells at different speeds or does not enter the cells under specific conditions.

The colored substance is added to the cells using the well known protocols (Current protocols in cell biology, John Wiley & Sons Inc, ISBN: 0471241059).
The use of a colored substance is conventional and commercially available reagents (Invitrogen Corp.) as well as reagents synthesized in laboratory can be used.
A number of commercially available dyes fulfilling the above requirements are known. Fluorescent dyes for monitoring Ca^{2+} are well known and described in detail in section 20.1-20.4 of the Molecular Probes catalog, 9th edition. They usually have two bis-carboxymethylamino groups attached to a fluorescent nucleus such as fluoresceins, rhodamines, coumarins, aminophenylindoles, and others. For the most part the compounds are 3,6-dioxy substituted xanthenes, where in the precursor the oxy groups are substituted and in the luminescent dye they are unsubstituted. Usually there are acetoxymethyl groups protecting the phenols and acids. See, for example, Fluo3/4, Fura2/3, calcein green, etc. Hydrolysis of the acetyl group results in the luminescent product. The precursors are able to cross the cellular membrane and be hydrolyzed in the cell.

The term “luminescence" refers to a "cold light", light from other sources of energy, which can take place at normal and lower temperatures. In luminescence, some energy source kicks an electron of an atom out of its "ground" (lowest-energy) state into an "excited" (higher-energy) state; then the electron gives back the energy in the form of light so it can fall back to its "ground" state. There are several varieties of luminescence, each named according to what the source of energy is, or what the trigger for the luminescence is.

The term “fluorescence” refers to a luminescence that is mostly found as an optical phenomenon in cold bodies, in which the molecular absorption of a photon triggers the

emission of another photon with a longer wavelength. The energy difference between the absorbed and emitted photons ends up as molecular vibrations or heat. Usually the absorbed photon is in the ultraviolet range, and the emitted light is in the visible range, but this depends on the absorbance curve and Stokes shift of the particular fluorophore. Fluorescence is named after the mineral fluorite, composed of calcium fluoride, which often exhibits this phenomenon.

Fluorescence from the indicator dyes can be measured with a luminometer or a fluorescence imager. One preferred detection instrument is the Fluorometric Imaging Plate Reader (FLIPR) (Molecular Devices, Sunnyvale, Calif.). The FLIPR is well suited to high throughput screening using the methods of the present invention as it incorporates integrated liquid handling capable of simultaneously pipetting to 96 or 384 wells of a microtiter plate and rapid kinetic detection using a argon laser coupled to a charge-coupled device imaging camera.

An alternative to the use of calcium indicator dyes is the use of the aequorin system. The aequorin system makes use of the protein apoaequorin, which binds to the lipophilic chromophore coelenterazine forming a combination of apoaequorin and coelenterazine that is known as aequorin. Apoaequorin has three calcium binding sites and, upon calcium binding, the apoaequorin portion of aequorin changes its conformation. This change in conformation causes coelenterazine to be oxidized into coelenteramide, CO2, and a photon of blue light (466 nm). This photon can be detected with suitable instrumentation.
For reviews on the use of aequorin, see Créton et al., 1999, Microscopy Research and Technique 46:390-397; Brini et al., 1995, J. Biol. Chem. 270:9896-9903; Knight & Knight, 1995, Meth. Cell. Biol. 49:201-216. Also of interest may be U.S. Pat. No. 5,714,666 which describes methods of measuring intracellular calcium in mammalian cells by the addition of coelenterazine co-factors to mammalian cells that express apoaequorin.

"Inhibitors" are compounds that, e. g., bind to, partially or totally block activity, decrease, prevent, delay activation, inactivate, desensitize, or down regulate the activity or expression of sodium/calcium exchanger proteins, e. g., antagonists.
"Activators" are compounds that increase, open, activate, facilitate, enhance activation, sensitize, agonize, or up regulate sodium/calcium exchanger activity. A preferred activator in the present invention is gramicidine, which triggers the elevation of the intracellular sodium concentration, thus leading to an increase of the sodium/calcium exchanger activity in the "reverse" transport mode.

Inhibitors, activators, or modulators also include genetically modified versions of sodium/calcium exchanger proteins, e. g., versions with altered activity, as well as naturally occurring and synthetic ligands, antagonists, agonists, peptides, cyclic peptides, nucleic acids, antibodies, antisense molecules, ribozymes, small organic molecules and the like.

The term “compound” or “test compound” or “test candidate” or grammatical equivalents thereof describes any molecule, either naturally occurring or synthetic, e. g., protein, oligopeptide, small organic molecule, polysaccharide, lipid, fatty acid, polynucleotide, oligonucleotide, etc., to be tested for the capacity to modulate sodium/calcium exchanger activity (Current protocols in molecular biology, John Wiley & Sons Inc, ISBN: 0471250961). The test compound can be in the form of a library of test compounds, such as a combinatorial or randomized library that provides a sufficient range of diversity (Current protocols in molecular biology, John Wiley & Sons Inc, ISBN: 0471250937). Test compounds are optionally linked to a fusion partner, e. g., targeting compounds, rescue compounds, dimerization compounds, stabilizing compounds, addressable compounds, and other functional moieties. Conventionally, new chemical entities with useful properties are generated by identifying a test compound (called a "lead compound") with some desirable property or activity, e. g., enhancing activity, creating variants of the lead compound, and evaluating the property and activity of those variant compounds. Preferably, high throughput screening (HTS) methods are employed for such an analysis.

Said inhibitor, activator and test compound may be added to the cells by injection into the culture medium after the cells have grown or they may be present in the culture medium prior to the cell growth (Current protocols in cell biology, John Wiley & Sons Inc, ISBN: 0471241059).
The cells may be grown to the appropriate number on the inhibitor, activator and/or test compound or they may be placed on it and used without further growth. The cells may be attached to the inhibitor, activator and/or test compound or, in those embodiments where the cells are placed or grown in wells, the cells may be suspension cells that are suspended in the fluid in the wells.

The term “control experiment” refers to different kinds of experiments that should be run together. The skilled person will recognize that it is generally beneficial to run controls together with the methods described herein.

For example, it will usually be helpful to have a control for the assay for determining the activity of a sodium/calcium exchanger in which the cells are preferably essentially identical to the cells that are used in the assay except that these cells would not express the sodium/calcium exchanger of interest.

Furthermore, it will usually be helpful to have a control for the assay for determining the activity of a sodium/calcium exchanger in response to the addition of a compound in which the compounds are tested in the assay of the invention against cells that preferably are essentially identical to the cells that are used in the assay except that these cells would not express the sodium/calcium exchanger of interest. In this way it can be determined that compounds which are identified by the assay are really exerting their effects through the sodium/calcium exchanger of interest rather than through some unexpected non-specific mechanism. One possibility for such control cells would be to use non-recombinant parent cells where the cells of the actual experiment express the sodium/calcium exchanger of interest.

Other controls for the assay for determining the activity of sodium/calcium exchanger in response to the addition of a compound would be to run the assay without adding a test compound (low control) and to run the assay with a high concentration of test compound (high control).

Other types of controls would involve taking compounds that are identified by the assay of the present invention as agonists or antagonists of sodium/calcium exchangers of interest and testing those compounds in the methods of the prior art in order to confirm that those compounds are also agonists or antagonists when tested in those prior art methods. Furthermore, one skilled in the art would know that it also desirable to run statistical analysis by comparing the assay values to standard values.

The terms “agonist” and “antagonist” refer to receptor effector molecules that modulate signal transduction via a receptor. Receptor effector molecules are capable of binding to the receptor, though not necessarily at the binding site of the natural ligand. Receptor effectors can modulate signal transduction when used alone, i.e. can be surrogate ligands, or can alter signal transduction in the presence of the natural ligand, either to enhance or inhibit signaling by the natural ligand. For example, "antagonists" are molecules that block or decrease the signal transduction activity of receptor, e.g., they can competitively, noncompetitively, and/or allosterically inhibit signal transduction from the receptor, whereas "agonists" potentiate, induce or otherwise enhance the signal transduction activity of a receptor.

The term "disease associated with a sodium/calcium exchanger altered expression" refers to dilated cardiomyopathy, coronary heart disease, arrhythmia, heart failure, etc.

For convenience, the colored substance and other components of the assay may be provided in kits, where the colored substance may be present as a reconstitutable powder or as a cooled solution on ice, in a buffer. The kit may also include buffer, activator, inhibitor, test compound, cells expriming a sodium/calcium exchanger protein, etc.. Cells may be present as lyoplilized cells.
Said kit of parts can be used as a diagnostic kit for diagnosing dilated cardiomyopathy, coronary heart disease, arrhythmia, heart failure, etc.

The following figures and examples shall describe the invention in further details, describing the typical results of the fluorescence based cellular sodium/calcium exchanger assay, without limiting the scope of protection.

EXEMPLIFICATION

1. Material and methods

1.1. Chemicals and equipment

Equipment	*Manufacturer*	*Function*
Biomek FX	Beckman Coulter	liquid handling
Biomek 2000	Beckman Coulter	liquid handling
16°C-CO_2-Incubator Cytomat	Heraeus	Cell handling
CO_2-Incubator Hera Cell	Heraeus	Cell handling
$FLIPR_{384}$	Molecular Devices	fluorescent imaging plate reader

Materials	*Supplier*	*Cat.No.*	*Function*

CHO-hNCX1 CHO-hNCX2 CHO-hNCX3	Steinbeis Transfer Zentrum	-	Cell line transfected with human Na^+/Ca^{2+} exchanger (NCX1, NCX2, NCX3)
A000135933	ACR	-	NCX1 blocker
Fluo4-AM	Molecular Probes	F14201	Monitoring of intracellular Ca^{2+}
Pluronic F-127	Sigma	P2443	Detergent
Geneticin	Gibco	10131-027	Cell selection
F-12 (Ham) + L-Glutamin	Gibco	21765-029	Cell culture
PBS w/o calcium and magnesium	Gibco	14200-067	Buffer
FCS	PAA	A15-649	Cell culture
Gramicidine	Sigma	G-5002	Na/K ionophor
96-well PP-microplate, U-Form	Greiner	650201	Compound plate
96-well black clear flat bottom plate (TC treated)	Costar	3904	Assay plate
Trypsin	Biochrom	L2143	Cell culture

1.2. Cell lines

The recombinant CHO-K1 cell lines stably expressing, respectively, the human NCX1, NCX2 and NCX3 were delivered by *Steinbeis-Transferzentrum für Angewandte Biologische Chemie*, Mannheim.

The cells were kept in continuous culture under standard conditions (37°C, air supplemented with 5% CO_2). The CHO-K1 NCX1, CHO-K1 NCX2 and CHO-K1 NCX3 cells were kept in HAM'S-F12 medium plus glutamine supplemented with 10% fetal calf serum (FCS) and 450 µg/ml G418. Cells were passed every 3-4 days after detachment using a Trypsin solution and reseeded with a concentration of 150.000 cells/ml.

1.3. Electrophysiological cell line characterization

The electrophysiological characterization was accomplished at Iongate Biosciences GmbH, Frankfurt.

The cultured cells were detached by application of iced PBS (phosphate buffered saline) or Trypsin at least 18 hours prior to electrophysiological experiments and replated on cover slips.

Two bath solutions and pipette solutions were prepared for patch clamp experiments with the following composition:

Bath (external) solution "low calcium":

135 mM NMG, 5 mM MgCl2, 2 mM CaCl2, 4 mM EGTA, 30 mM HEPES, pH 7.4 (HCl → pH 7.0, then CsOH → pH 7.4)

Bath (external) solution "high calcium":

139 mM NMG, 5 mM MgCl2, 2 mM CaCl2, 30 mM HEPES, pH 7.4 (HCl → pH 7.0, then CsOH → pH 7.4)

Pipette (internal) solution:

100 mM NaCl, 5 mM KCl, 2 mM CaCl2, 2 mM MgCl2, 2.1 mM EGTA, 35 mM TEA-Cl, 4 mM Mg-ATP, 0.5 mM Na-GTP, 5.63 mM Phosphocreatine, 30 mM HEPES, 3.5 U/ml Creatine Phosphokinase (70 ml pipette solution + 3500 U filtered Creatine Phosphokinase, ad 100 ml pipette solution), pH 7.4 (NaOH)

To inhibit NCX currents, 5 mM nickel chloride was dissolved in bath solution "high calcium". NCX activity was measured using the patch clamp technique in the whole cell configuration. The cells were clamped at room temperature (20-25°C) to a holding potential of 0 mV. NCX currents were evoked by application of 2 mM external calcium (bathsolution "high calcium") for 4 seconds (=control). Calcium application was repeated every 20 s. After 5 stimulations, inhibition by nickel was tested using the same protocol but activation with calcium in the presence of 5 mM nickel (5 replicates). Finally, 5 calcium pulses were applied again for washout studies. To characterize NCX currents, peak currents, transported charge and the time constant of signal decay were investigated and analyzed according to the report. All data are presented as mean ± SEM.

1.4. Assay reagents

Reagent	*Chemicals*	*Remarks*

Assay buffer	133.8 mM NaCl 4.7 mM KCl 1.25 mM MgCl 3.5 mM CaCl2 5 mM Glucose 10 mM Hepes/ NaOH, pH 7.5 0,01% Pluronic F-127 2.5 mM Probenecid	Probenecid has to be prepared freshly every day
Dye loading buffer	Fluo4-AM, 0,02 % Pluronic F-127, 0.1%BSA	12 µl Pluronic F-127 (20%) added to 12 µM Fluo4-AM (2 mM) and then dissolved in assay buffer
Gramicidine stock solution	1 mM gramicidine in Ethanol (4°C)	prepared freshly every day and stored at 4°C until measurement
Gramicidine assay solution	60 µM gramicidine in assay buffer	Plated on PP-plates and stored at 16°C (stable for 3h); 3x concentrated, 20 µM final concentration; 2% ethanol concentration on cells;

1.5. Final assay procedure

1.5.1. Preparation of cell plates

One day prior to the experiment, the cells were detached with Trypsin and plated with a density of 35.000 cells/100 µl medium/well in 96-well microplates and were incubated for ~22 h at 37°C, 5% CO_2 and 90% humidity.

1.5.2. Preparation of compound plates

The compounds were prepared in assay buffer with a concentration 1.5x of the final concentration. The compound solution was preincubated and stored at 16°C. For IC_{50} determinations a dilution series with a starting concentration of 45 µM (final: 30 µM) was prepared with the Biomek2000.

1.5.3. Preparation of gramicidine plates

A 1 mM gramicidine stock solution was prepared every day using cold ethanol (4°C) as solvent. Starting from the stock solution a 60 µM gramicidine assay solution was prepared using assay buffer. A 96 well polypropylene plate was filled with 150 µl per well of the gramicidine assay solution and stored at 4°C. The addition plate could only be used two times before refilling was required because the gramicidine solution loses activity very fast at RT.

1.5.4. Assay procedure

1. Medium is removed from the cell plates
2. 100 µl/well of dye loading buffer is added
3. Cells are incubated for 90 min at RT
4. Wash 3x with 100µl assay buffer per well, discard buffer completely afterwards
5. 80 µl of test compounds are added at different concentrations (1.5x concentrated) using the Biomek FX
6. Cells are incubated for 45 min at 16°C and 5% CO_2 in a CO_2 incubator
7. Cell plates are transferred to the FLIPR and recorded by adding 40 µl of gramicidine assay solution (final concentration: 20 µM gramicidine) during the measurement

10 µM of the NCX inhibitor A000135933 is used as high control and buffer as low control.

1.5.5. FLIPR Setup Parameters

FLIPR Experimental Setup Parameters	
Exposure	1.2 sec (at 0.6 W)
Aperture	2.8
Filter	515-575 ("Calcium")
Defaults (Graphs Setup)	

Subtract bias	From sample 1
Spatial uniformity correction	off
Negative control correction	off
First Sequence	
Initial Period	2 sec
Initial Count	240 (480 sec)
Add After Frame	5
Add Height	70 µl
Add Speed	40 µl/sec
Add Volume	40 µl
Mix	2x 40 µl

1.6. Data analysis

The following calculations are based on the data obtained using the statistic export function of the FLIPR software: Statistic 1= max-min (sample1-240). The calculations are corrected for edge effects if necessary.

The inhibition of NCX as primary result refers to the inhibition of the fluorescence increase after addition of gramicidine and is derived from statistic 1. The high control refers to the inhibition of NCX activity with 10 µM A000135933 and the low control to no NCX inhibition after addition just of buffer. Thus, % inhibition is calculated in reference to the controls (0% inhibition = low control, 100% inhibition = high control). Results are calculated from the corrected raw data and intraplate controls with the following formula:

$$\text{Inhibition}(\%) = 100 \circ \left(1 - \frac{(\text{sample} - \text{low control})}{(\text{high control} - \text{low control})}\right)$$

2. Results

2.1. Electrophysiological cell line characterisation

2.1.1. Parental cell line

The electrophysiological data were obtained from Iongate GmbH. CHO-K1 cells were used to establish the stable CHO-NCX1, CHO-NCX2 and CHO-NCX3 cell lines. The parental cells exhibited no significant current response (0.12 ± 0.07 pA/pF, figure 2) when extracellular Ca^{2+} was applied under high intracellular Na^{+} concentrations. The use of nickel did not produce artifacts under the described conditions. Typical currents are shown in figure 3.

2.1.2. CHO-NCX cell lines

The CHO-NCX1 cells revealed outward steady-state currents after Ca^{2+} application with almost no deactivation after four seconds. The relative peak current was 1.40 ± 0.18 pA/pF (n=3, figure 4a). Currents could be completely and reversible blocked by application of 5 mM nickel (91.3 ± 3.3 %).

The CHO-NCX2 cells revealed outward currents under reverse mode conditions. The relative peak current was 2.95 ± 0.70 pA/pF (n=6, figure 4b). Currents decayed with a time constant of 1.26 ± 0.11 s and could be completely and reversible blocked by application of 5 mM nickel (washout: 100.2 ± 1.9 %).

The CHO-NCX3 cells revealed outward currents under reverse mode conditions decaying with a time constant of 0.80 ± 0.08 s. The relative peak current was 3.46 ± 1.11 pA/pF (n=4, figure 4c). Currents could be completely and reversible blocked by application of 5 mM nickel (washout: 111.5 ± 2.4 %). Typical currents are shown in figure 5.

2.2. Assay parameter

2.2.1. NCX1 assay

HTS-based NCX1 assays analysing the "reverse" mode were not known in the literature. The application of gramicidine is known out of combined patch clamp and fluorescence measurements. All parameters had to be optimized. By reason of flexibility between the "forward" and the "reverse" mode assay, running at the same time, the buffer conditions (e.g. Ca^{2+} concentration, pH) and cell number per well were adapted from the "forward" mode assay and showed very good results.

In the first experiments the influence of the gramicidine concentration and storage on the Ca^{2+}-import activity of NCX1 were investigated (Fig. 6a,b).

A gramicidine concentration of 20 µM showed the best results on the Ca^{2+}-import activity regarding S/B and z´-factor. Lower concentrations were not sufficient due to slow kinetics and higher concentrations caused cellular side effects (Fig. 6a,b). The IC_{50} of the tool compound A000135933 (also Figure 11a,b) was unaffected by different gramicidine concentrations (Fig. 7a). Gramicidine was solved in ethyl alcohol and the influence of the solvent had to be characterized. Up to 2 % ethyl alcohol showed no effect on the background fluorescence of the assay while increasing concentrations led to a slight increase in fluorescence (Fig. 7b).

The preparation of the gramicidine solution and the storage was a critical step, because gramicidine is degraded at higher temperatures. In the following experiments gramicidine was solved and stored at different temperatures. The gramicidine solution had to be prepared and stored at 4°C otherwise gramicidine was degraded very fast (Figure 8).

Different FLIPR settings concerning pipette height, presoak and mixing were checked to improve the S/B and z´-factor (Figure 9a,b). The best z´factor was achieved with a pipette height of 70 µl with two mixing cycles.
Using the parameters described above two temperatures (16 and 22°C) were checked for compound incubation (Figure 10a). The best results were obtained with 16°C and the best incubation time regarding S/B, z´ and IC_{50} (A000135933) results was 45 min (Figure 10b).

One of the key requirements of the "reverse" mode assay was the sensitivity of compounds. The tool compound for the NCX1 project is A000135933, an iminothiazole derived out of the screen with the "forward" mode assay. The compound has an IC_{50} of 5.9 µM in the "forward" assay and 1.44 µM in patch clamp experiments. The compound is in use as standard control in the "forward" assay. To examine the effect, A000135933 was tested under different gramicidine concentrations in the "reverse" assay. Typical inhibitory effects on the NCX1 activity after activation with 20 µM gramicidine and the calculated dose response relationship is shown in Figure 11a and b.

The NCX1 cell line was stable concerning S/B and IC_{50} of the standard (A000135933) for two month. The effect of the tool compound A000135933 on the Ca^{2+}-import activity of NCX1 is shown in Figure 11a and the IC_{50} was around 1 µM (Figure 11b). The z´ values were between 0.7 and 0.8. To compare this assay ("reverse") with the fluorescence based cellular Ca^{2+}-export mode assay ("forward") and with an electrophysiological technique called SURFE^{2}R

(Iongate Biosciences, Frankfurt) the IC_{50} of 18 or the Inhibition at 10 µM of 11 compounds were tested respectively (Fig. 12a,b).

The "reverse" assay was in general 3-5 fold more sensitive than the "forward" assay (Fig. 12a, black dots) and the data correlates with a r^2 of 0.86. Some compounds do not show a significant increase in inhibition (Fig. 8a, red dots). Other compounds inhibit the NCX1 with a much higher potency (Fig. 8a, blue dots) perhaps indicating a transport-mode specific effect. The percent Inhibition of the compounds at 10 µM derived from the "reverse" assay was higher (up to 20%) than the SURFE^{2}R values (Fig. 12b), but the correlation with the SURFE^{2}R-technique was good with a r^2 of 0.83.

2.2.2. NCX2 assay

No NCX2 assays analysing the "forward" or "reverse" mode were known in the literature. The establishment of the "forward mode" assay was not successful due to limitations in the sensitivity and robustness of the assay. For clarity – no data is shown in this report. The "reverse mode" assay is based on the application of gramicidin. Gramicidin forms alkali-permeable pores. Gramicidin-induced Ca^{2+} influx is indirect and NCX dependent. The activation of NCX1 after gramicidin addition is known from radioactive ion flux or fluorescence measurements on primary neurons (Kiedrowski et al., Journal of Neurochemistryl, 2004). By reason of comparability between the NCX assays of the different subtypes, running at the same time, the buffer conditions (e.g. Ca^{2+} concentration, pH), cell number, incubation times and gramicidin concentrations were adapted for the NCX2 assay from the "reverse" mode NCX1 assay.

In the first experiments the buffer conditions, incubation times, gramicidin concentration and storage were checked on the Ca^{2+}-import activity of NCX2 (Fig. 13a,b).

The kinetic of the fluorescence increase is a little bit slower for NCX2 compared to NCX1. The measurement time had to be extended to 10 min. The buffer system, cell number, incubation times, gramicidin concentration (20 µM) and storage showed very good results. These parameters must not be further optimized. Gramicidin was solved in ethyl alcohol and the influence of the solvent had to be characterized. Up to 3 % ethyl alcohol showed no effect on the background fluorescence of the assay (Fig. 14).

One of the key requirements of the "reverse" mode assay was the sensitivity to compounds but there were no electrophysiological data available for the tool compounds on NCX2. The main

tool compound for the NCX1 project is A000135933, an iminothiazole derived out of the NCX1-screen. The compound has an IC_{50} on NCX1 of 1.8 µM in the "reverse" assay and 1.44 µM in patch clamp experiments. The effect of A000135933 after activation with 20 µM gramicidin on the NCX2 activity was examined (Fig 16a). The calculated dose response relationship is shown in Figure 15b.

The NCX2 cell line was stable concerning S/B and IC_{50} of the standard (A000135933) for two month. The effect of the tool compound A000135933 on the Ca^{2+}-import activity of NCX2 is shown in Figure 15a and the IC_{50} (1.25 ± 0.19 µM, n=26, example Fig. 15b) was a little bit lower on NCX2 than on NCX1 (1.78 ± 1.03 µM, n=55) indicating that the compound is more potent for NCX2. The z´ values were between 0.75 and 0.85. 125 compounds from the NCX1 project were tested with the NCX2 "reverse mode" assay and compared with the NCX1 data. The results are summarized in Figure 16.

Nearly all compounds with an effect on the NCX1 activity also inhibited NCX2 (Figure 16). A lot of compounds inhibited NCX2 with a more than two-fold higher potency than NCX1. Only 4 compounds showed a more than two-fold selectivity for NCX1. These data indicates that there is a possibility to obtain NCX1 selective compounds.

2.2.3. NCX3 assay

NCX3 assays were not known in the literature. The establishment of the "forward mode" assay was not successful due to limitations in the sensitivity and robustness of the assay. For clarity – no data is shown in this report. The "reverse mode" assay is based on the application of gramicidin. Gramicidin forms alkali-permeable pores. Gramicidin-induced Ca^{2+} influx is indirect and NCX dependent. The activation of NCX1 after gramicidin addition is known from radioactive ion flux or fluorescence measurements on primary neurons (Kiedrowski et al., Journal of Neurochemistry, 2004). By reason of comparability between the NCX assays of the different subtypes, running at the same time, the buffer conditions (e.g. Ca^{2+} concentration, pH), cell number, incubation times and gramicidin concentrations were adapted for the NCX3 assay from the "reverse" mode NCX1 assay.

In the first experiments the buffer conditions, cell number, incubation times, gramicidin concentration and storage were checked on the Ca^{2+}-import activity of NCX3 (Fig. 18a,b).

The kinetic of the fluorescence increase is a faster in comparison to NCX1 and NCX2. The buffer system, incubation times, gramicidin concentration (20 µM) and storage showed very

good results. These parameters must not be further optimized. Gramicidin was solved in ethyl alcohol and the influence of the solvent had to be characterized. Up to 2 % ethyl alcohol showed no effect on the background fluorescence of the assay (Fig. 19) more than 2% leads to slight increase in background fluorescence.

One of the key requirements of the "reverse" mode assay was the sensitivity to compounds but there were no electrophysiological data available for the tool compounds on NCX3. The main tool compound for the NCX1 project is A000135933, an iminothiazole derived out of the NCX1-screen. The compound has an IC_{50} on NCX1 of 1.8 µM in the "reverse" assay and 1.44 µM in patch clamp experiments. The IC50 on NCX2 is 1.25 µM in the "reverse" assay. The effect of A000135933 after activation with 20 µM gramicidin on the NCX3 activity was examined (Fig 21a) and the calculated dose response relationship is shown in Figure 19b.

The NCX3 cell line was stable concerning S/B and IC_{50} of the standard (A000135933) for two month. The effect of the tool compound A000135933 on the Ca^{2+}-import activity of NCX3 is shown in Figure 19a and the IC_{50} was 1.63 ± 0.30 µM (n=23, example Fig. 19b). These data indicates that the tool compound is not selective for a specific subtype (IC_{50} NCX1: 1.78 ± 1.03; IC_{50} NCX2: 1.25 ± 0.19). The z´ values were between 0.70 and 0.85. 125 compounds from the NCX1 project were tested with the NCX3 "reverse mode" assay and compared with the NCX1 and NCX2 data. The results are summarized in Figure 20a and 20b.

Nearly all compounds with an effect on the NCX1 activity also inhibited NCX3 (Figure 20a). A lot of compounds inhibited NCX3 with a more than two-fold higher potency than NCX1. Six compounds showed a more than two-fold selectivity for NCX1. In comparison to NCX2 - nine compounds were more potent on NCX2 and two substances on NCX3 (Figure 20b). These data indicates that there is a possibility to obtain NCX subtype selective compounds.

DESCRIPTION OF THE FIGURES

Figure 1:

(a) Amino acid sequence of NCX1 represented by SEQ ID NO: 1;

(b) Amino acid sequence of NCX2 represented by SEQ ID NO: 2;

(c) Amino acid sequence of NCX3 represented by SEQ ID NO: 3.

Figure 2:
Peak currents of CHO-K1 cells after addition of 2 mM Ca^{2+} (n = 3).

Figure 3:
Typical current of CHO-K1 cells after addition of 2 mM Ca^{2+} and application of 5 mM nickel.

Figure 4:
(a) Peak currents of CHO-NCX1 cells after addition of 2 mM Ca^{2+} (n = 3);
(b) Peak currents of CHO-NCX2 cells after addition of 2 mM Ca^{2+} (n = 6);
(c) Peak currents of CHO-NCX3 cells after addition of 2 mM Ca^{2+} (n = 6).

Figure 5:
(a) Typical current of CHO-NCX1 cells after addition of 2 mM Ca^{2+}, application of 5 mM nickel and washout;
(b) Typical current of CHO-NCX2 cells after addition of 2 mM Ca^{2+}, application of 5 mM nickel and washout;
(c) Typical current of CHO-NCX3 cells after addition of 2 mM Ca^{2+}, application of 5 mM nickel and washout.

Figure 6:
(a) Effect of different concentrations of gramicidine (—— 0 µM, ·····10 µM, - - -15 µM, ·-· 20 µM and — —25 µM) on the Ca^{2+}-import activity of NCX1. The effect of the addition of the NCX1 inhibitor A000135933 is shown at 20 µM gramicidine (·- ·-);
(b) Effect of different concentrations of gramicidine (10 µM, 15 µM, 20 µM and 25 µM) on the Ca^{2+}-import activity of NCX1 without (■, high control) and with 10 µM (□, low control) of the NCX1 inhibitor A000135933. The z´-factor is indicated as ▽.

Figure 7:
(a) Effect of different concentrations of gramicidine (10 µM, 15 µM, 20 µM and 25 µM) on the IC_{50} (■) of the NCX1 inhibitor A000135933;
(b) Effect of different concentrations of ethyl alcohol on the Fluo4-fluorescence of the CHO-NCX1 cells (■) and with the NCX inhibitor A000135933 (□) without gramicidine stimulation.

Figure 8:

Effect of 20 µM gramicidine solved and stored at different temperatures on the Ca^{2+}-import activity of NCX1 without (■, high control) and with 10 µM (□, low control) of the NCX1 inhibitor A000135933.

Figure 9:
(a) High controls (■, buffer), low controls (□, 10 µM A000135933) and z´-factor (▽) at different pipette heights;
(b) High controls (■, buffer), low controls (□, 10 µM A000135933) and z´-factor (▽) with different numbers of mixing cycles.

Figure 10:
(a) High controls (■, buffer), low controls (□, 10 µM A000135933) and z´-factor (▽) with different temperatures during compound incuba-tion (30 min);
(b) High controls (■, buffer), low controls (□, 10 µM A000135933) and z´-factor (▽) with different compound incubation times at 16°C.

Figure 11:
(a) Effect of different concentrations of A000135933 (·····30 µM, - - -15 µM, ·-·-·-7.5 µM, — — 3.75 µM, ·-·-·-1.88 µM, - - -0.94 µM, ——0.5 µM) on the Ca^{2+}-import activity of NCX1 after addition of 20 µM gramicidine (Incubation: 22°C, 30 min);
(b) Dose response relationship of A000135933. The IC_{50} value is 0.97 µM.

Figure 12:
(a) Correlation of the IC_{50} of 18 compounds tested with the fluorescence based cellular Ca^{2+}-export ("forward") and Ca^{2+}-import ("reverse") assay;
(b) Correlation of the %-Inhibition at 10 µM of 11 compounds tested with the fluorescence based cellular Ca^{2+}-import assay ("reverse") and the electrophysiological SURFE^{2}R technique.

Figure 13:
(a) Ca^{2+}-import activity of NCX2 without (——) and with 20 µM gramicidin (– – –) in comparison to NCX1 (·-·-·-). The effect of the addition of the NCX inhibitor A000135933 on NCX2 activity is shown at 20 µM gramicidin (·····);
(b) Effect of 20 µM gramicidin on the Ca^{2+}-import activity of NCX2 at 5 days without (■, high control) and with 10 µM (□, low control) of the NCX1 inhibitor A000135933. The z´-factor is indicated as ▽.

Figure 14:
Effect of different concentrations of ethyl alcohol on the Fluo4-fluorescence of the CHO-NCX2 cells (■) without gramicidin stimulation.

Figure 15:
(a) Effect of different concentrations of A000135933 (— —30 µM, – – – 15 µM, - - -7.5 µM, ·····3.75 µM, ·-·-·-1.88 µM, ··-··-0.94 µM, ——0.5 µM and ——0 µM) on the Ca^{2+}-import activity of NCX2 after addition of 20 µM gramicidin (Incubation: 22°C, 45 min);
(b) Dose response relationship of A000135933. The IC_{50} value of this example is 1.4 µM.

Figure 16:
Comparison of the IC_{50} of 125 compounds on NCX2 and NCX1. Compounds were tested with the fluorescence based cellular Ca^{2+}-import assay ("reverse"). The dark grey line (——) marks the line of equal potency and the light grey dashed line (– – –) indicates a two-fold split in potency on a particular subtype.

Figure 17:
(a) Ca^{2+}-import activity of NCX3 without (··-··-) and with 20 µM gramicidin (- - -) in comparison to NCX1 (——) and NCX2 (·····). The effect of the addition of the NCX inhibitor A000135933 on NCX3 activity is shown at 20 µM gramicidin (— —);
(b) Effect of 20 µM gramicidin on the Ca^{2+}-import activity of NCX3 at 5 days without (■, high control) and with 10 µM (□, low control) of the NCX inhibitor A000135933. The z´-factor is indicated as ▽.

Figure 18:
Effect of different concentrations of ethyl alcohol on the Fluo4-fluorescence of the CHO-NCX3 cells (■) without gramicidin stimulation.

Figure 19:
(a) Effect of different concentrations of A000135933 (·····30 µM, ·-·-·-15 µM, - - -7.5 µM, ··-··-3.75 µM, - - -1.88 µM, ·····0.94 µM, ——0.5 µM and ——0 µM) on the Ca^{2+}-import activity of NCX3 after addition of 20 µM gramicidin (Incubation: 22°C, 45 min);
(b) Dose response relationship of A000135933. The IC_{50} value for this example is 1.76 µM.

Figure 20:

(a) Comparison of the IC_{50} of 125 compounds on NCX3 and NCX1. Compounds were tested with the fluorescence based cellular Ca^{2+}-import assay ("reverse"). The dark grey line (——) marks the line of equal potency and the light grey dashed line (– – –) indicates a two-fold split in potency on a particular subtype;

(b) Comparison of the IC_{50} of 125 compounds on NCX3 and NCX2. Compounds were tested with the fluorescence based cellular Ca^{2+}-import assay ("reverse"). The dark grey line (——) marks the line of equal potency and the light grey dashed line (– – –) indicates a two-fold split in potency on a particular subtype.

CLAIMS

1. An assay for determining the calcium import activity of a sodium/calcium exchanger comprising:

a) providing cells expressing a sodium/calcium exchanger;

b) providing a colored substance for determining intracellular calcium;

c) contacting cells with a sodium/calcium exchanger activator; and

d) comparing the calcium mediated change in the luminescent signal from said colored substance to a luminescent signal produced in a control experiment.

2. The assay according to claim 1, wherein the sodium/calcium exchanger is a NCX protein of the following list: NCX1, NCX2, NCX3; or a NCKX protein of the following list: NCKX1, NCKX2, NCKX3, NCKX4, NCKX5.

3. The assay according to claim 1, wherein the sodium/calcium exchanger is a NCX protein of the following list: NCX1, NCX2, NCX3.

4. The assay according to claims 1 to 3, wherein the sodium/calcium exchanger is of mammalian origin, preferably from rat, mouse, dog, bovine, pig, ape or human.

5. The assay according to claims 1 to 4, wherein the cells are selected from the group consisting of: CHO, HEK, COS7 and JURKAT cells.

6. The assay according to claims 1 to 5, wherein said colored substance is added to the cells as a dye precursor capable of entering the cells and being hydrolyzed to a dye, whereby the dye complexes with calcium in said cells and provides a luminescent signal.

7. The assay according to claims 1 to 6, wherein said luminescent signal is fluorescence and said monitoring step c) employs a FLIPR device.

8. The assay according to claim 6, wherein said dye precursor is an acetoxymethylester derivate.

9. The assay according to claim 6, wherein said dye is the calcium sensitive fluorescence dye fluo-4.

10. Use of the assay according to claims 1 to 9 to test a compound for activity as an agonist or antagonist of the calcium import activity of a sodium/calcium exchanger.

11. Use of the assay according to claims 1 to 9 for the diagnosis of a disease associated with a sodium/calcium exchanger altered expression.

12. An assay for determining the calcium import activity of a sodium/calcium exchanger in response to the addition of a compound comprising:

a) providing cells expressing a sodium/calcium exchanger;
b) providing a colored substance for determining intracellular calcium;
c) contacting cells with a compound, wherein said cells have been treated, prior to treating with said compound, with a sodium/calcium exchanger activator; and
d) comparing the calcium mediated change in the luminescent signal from said colored substance to a luminescent signal produced in a control experiment.

13. The assay according to claim 12, wherein the sodium/calcium exchanger is a NCX protein of the following list: NCX1, NCX2, NCX3; or a NCKX protein of the following list: NCKX1, NCKX2, NCKX3, NCKX4, NCKX5.

14. The assay according to claim 12, wherein the sodium/calcium exchanger is a NCX protein of the following list: NCX1, NCX2, NCX3.

15. The assay according to claims 12 to 14, wherein the sodium/calcium exchanger is of mammalian origin, preferably from rat, mouse, dog, bovine, pig, ape or human.

16. The assay according to claims 12 to 15, wherein the cells are selected from the group consisting of: CHO, HEK, COS7 and JURKAT cells.

17. The assay according to claims 12 to 16, wherein said colored substance is added to the cells as a dye precursor capable of entering the cells and being hydrolyzed to a dye, whereby the dye complexes with calcium in said cells and provides a luminescent signal.

18. The assay according to claims 12 to 17, wherein said luminescent signal is fluorescence and said monitoring step c) employs a FLIPR device.

19. The assay according to claim 17, wherein said dye precursor is an acetoxymethylester derivate.

20. The assay according to claim 17, wherein said dye is the calcium sensitive fluorescence dye fluo-4.

21. The assay according to claims 12 to 20, wherein said compound is a sodium/calcium exchanger antagonist.

22. A kit of parts comprising:

a) lyophilized cells expriming a sodium/calcium exchanger;
b) a colored substance;
c) a compound buffer; and
d) a colored substance buffer.

23. The kit of parts according to claim 22, wherein said colored substance is the calcium sensitive fluorescence dye fluo-4.

24. The kit of parts according to claims 22 and 23, wherein the sodium/calcium exchanger is a NCX protein of the following list: NCX1, NCX2, NCX3; or a NCKX protein of the following list: NCKX1, NCKX2, NCKX3, NCKX4, NCKX5.

25. The kit of parts according to claims 22 and 23, wherein the sodium/calcium exchanger is a NCX protein of the following list: NCX1, NCX2, NCX3.

26. The kit of parts according to claims 22 to 25, wherein the sodium/calcium exchanger is of mammalian origin, preferably from rat, mouse, dog, bovine, pig, ape or human.

27. Use of a kit of parts according to claims 22 to 26 to test a compound for activity as an agonist or antagonist of the calcium import activity of a sodium/calcium exchanger.

28. Use of a kit of parts according to claims 22 to 26 for the diagnosis of a disease associated with a sodium/calcium exchanger altered expression.