Process for identification of compounds for modulating the activity of a sodium/calcium exchange transporter

The invention refers to a cell free assay for determining the activity or the modulation of the activity of a Na^+/Ca^{2+} exchanger (NCX) protein by means of a cell free electrophysiological sensor chip, a kit of parts comprising the sensor chip with a NCX protein as well as the manufacturing and use of the kit of parts.

General

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.

Most membrane transporters shift electrical charges while going through their transport cycle. This shift may originate either from the movement of charged substrates or from the movement of protein moieties carrying (partial) charges.

Monitoring of Transporter-Related Current

In some cases the transporter-related currents can either be directly monitored in a rather physiological environment by patch-clamp experiments or at artificial "black lipid membranes". In the latter case, a lipid bilayer is generated in a small hole between two buffer reservoirs, each of them containing an Ag/AgCl electrode. After incorporation of the protein into the bilayer, the biological activity (e.g. enzymatic activity) can be triggered e.g. by photoactivation of ATP derivatives. Yet, due to its lack of stability, no

rapid buffer exchange experiments can be conducted with this system, limiting the system to photoactivateable substrates. The lack of stability can be overcome by immobilizing protein-containing particles on a sensor surface. This fact is the rational behind a cell free electrophysiological technology called SurfE^{2}R ® (Surface Electrogenic Event Reader) of IonGate Biosciences GmbH, Frankfurt/Main that detects the resulting transporter-related currents.

In analogy also according to the present invention a sensor chip consists of a substrate carrying the transducer and a cover plate with a hole, forming a well similar to those of titer plates. Either glass or polymer plates serve as suitable substrates. In the case of a glass plate, the transducer consists of a thin, lithographically structured gold film which has been chemically modified (e.g. by means of mercaptane) on its surface, whereas with polymer substrates modified thick film gold electrodes can also be used. Due to the range of suitable substrates, single sensor chips can be manufactured as well as sensor strips or even sensor array plates with 96 or 384 sensors. Particularly the polymer-based sensors bear the potential for low cost mass production. Specific examples are disclosed in WO02/074983 and DE10244129.

It holds true for all sensor types that the gold surface is turned into a capacitor after the surface modification has taken place and the well has been filled with an aqueous solution. The properties of this capacitor can be determined by the aid of a current-carrying reference electrode such as Pt/Pt or Ag/AgCl or indium tin oxide (ITO) or others brought in contact with the solution. Furthermore, the sensor surface is very hydrophilic, i.e. sticky for membrane fragments and vesicles. Electrogenic proteins kept within their native or native-like environment, i.e. biological membrane sheets, vesicles or proteoliposomes readily adsorb to the hydrophilic sensor surface, forming compartments whose inner space with its solution is electrically isolated from both, the gold surface as well as the surrounding solution within the well. If inserted into a cuvette, the well of the chip (Fig. 2A) defines the inner volume of a flow cell, enabling a rapid solution exchange above the sensor surface.

Switching from a solution which does not contain a substrate of the investigated protein to a solution that does, induces a measurable, transient charging current of the above mentioned capacitor which is typically within the range of 100 pA to 4 nA.

In the used workstation all components necessary for carrying out solution exchange experiments are accommodated in a PC- or otherwise controlled workstation. In the conventional system, the non-activating (i.e. substrate-free) solution as well as the activating solution are stored in glass bottles. Air pressure applied to the bottles drives the solution through a system of electromechanically operated valves and through the flow cell. Alternatively, an autosampler can be used to process several solutions in a automated fashion.

Na^+/Ca^{2+} Exchanger (NCX)

The term "NCX protein" or "NCX" in context of the present invention shall mean any one of the list of the following Na^+/Ca^{2+} exchanger proteins either alone or in combination with each other: NCX1, NCX2, NCX3, NCX4, NCX5, NCX6, NCX7. Especially preferred are NCX1, NCX2 and/or NCX3. Such NCX protein 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 NCX 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 NCX protein.

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+/Ca2+ 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+-Ca2+ 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+/Ca2+ exchanger 3 isoforms).

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. 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 Na^+/Ca^{2+} exchanger electrogenically transports three to four Na^+ for every Ca^{2+} that moves in the opposite direction as e.g. shown by electrophysiological means in Hinata, M. et al. (2002) J. Physiol. 545, 453-461. The NCX is able to maintain the cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]_{in}$) three to four orders of magnitude below the extracellular Ca^{2+} concentration ($[Ca^{2+}]_{out}$). Nevertheless, the direction of net Ca^{2+} transport depends on the electrochemical gradient of Na^+. Simultaneous and consecutive transport models have been suggested for Na^+ and Ca^{2+} translocations, and a bulk of evidence favors the latter.

It is known from the state of the art that the NCX protein's activity can be determined by means of living cells. For this purpose the protein has to be expressed in cells and the cells have to be propagated. Such a system has been disclosed e.g. by Axxam S.r.l. (Mailand, Italy) during the "Pharmaconference 2003" in Pontresina on Poster Nr.

P25 (Title: Application of FLIPR platform to study K^+ dependent Na^+/Ca^{2+} exchangers). These K^+ dependent Na^+/Ca^{2+} exchangers are different from NCX proteins since they have been isolated from eye's tissue, exhibit a different transport mechanism and are not found expressed in heart tissue. There are further measuring methods known for electrochemical determination of Na^+/Ca^{2+} symport systems in cells or in cellular assays. The disadvantage of such systems is that the protein activity has to be determined before a complex biological background containing a mixture of all sorts of macromolecules with potency of interfering with an assay to be applied. The Na^+ and Ca^{2+} efflux and influx currents are driven by several and different proteins. The measurement concerning one single protein is, therefore spoiled by a large background. A method free of that disadvantage is a method for electrochemical determination of Na^+/Ca^{2+} symport systems in proteo-liposomes (Eisenrauch, A. et al.; J. Membrane Biology (1995) 145:151-164; Title: Electrical Currents generated by a partially purified Na/Ca exchanger from lobster muscle reconstituted into liposomes and adsorbed on black lipid membranes: activation by photolysis of Ca^{2+}). The clear disadvantage of that method would be that no rapid exchange of solutions could happen.

The system of the present invention avoids these disadvantages with respect to the NCX protein by fixing the protein onto a device outside of a cell's background allowing for rapid solution exchange. The activity of the protein is determined then by measurement of a current. Furthermore, a rapid solution exchange is possible.

According to a first aspect, the present invention provides an assay as defined above wherein the cell free electrophysiological sensor chip is comprising at least one of the NCX proteins of the following list: NCX1, NCX2, NCX3, NCX4, NCX5, NCX6, NCX7.

The term "sensor chip" means a cell free electrophysiological sensor chip as for example described in WO02/074983, in particular in the claims and/or figures 1 and/or

2 including the description of the figures of said PCT application, which is hereby incorporated by reference, if not otherwise described in the present invention.

In particular, this assay comprises a protocol which allows efficient preparation membranes containing NCX protein, a protocol for efficient preparation of sensor chips containing a NCX protein as well as a solution exchange protocol allowing for measuring NCX activity.

In general, the NCX protein used was of mammalian origin, as described above, and in particular of human origin. The NCX protein is elected from NCX1, NCX2, NCX3, NCX4, NCX5, NCX6 and/or NCX7, in particular NCX1, NCX2 and/or NCX3. In a preferred embodiment the NCX protein is a human NCX1 protein. Such NCX proteins could be manufactured by means of recombinant methods known to a person skilled in the art, hereinafter referred to as "recombinant NCX protein" or harvested from native tissue probes, hereinafter referred to as "native NCX protein".

In a preferred embodiment the sensor chip contains a basic body of borofloate-glass that carries gold structures. Further the sensor chip can be preferably covered by a mercaptane layer and having one or several insulating layers. Such a sensor chip (e.g. Borofloate glass chip with rounded gold structures (1-3 mm diameter) and a contact area) is commercially available from IonGate Biosciences GmbH, Industriepark Höchst, D 528, D-65926 Frankfurt am Main, Germany.

A particularly preferred protocol is as follows:
10-30 µl, for example, of NCX-containing membrane fragments were applied and circulated on the sensor chip and preferably incubated for at least 12 hours at 4°C. In preferred cases of experiments the NCX protein is a human NCX1 protein. The capacitance of the protein-loaded sensor chip was preferably around 100 nF cm^{-2} and the conductance G_{1S} preferably around 10 nS cm^{-2}. The principal of the assay is to test the electrical activity of NCX protein in the absence of the inhibitor and afterwards in the presence of the inhibitor. For this reason the NCX-sensor chip is rinsed with a sequence of solutions, which activate NCX. In general, there is the possibility to test

whether an inhibitor is reversible. In this case after the inhibitor application the solutions are changed back to inhibitor-free conditions. Before the experiment the solution reservoirs of the chip are filled with the following preferred solutions: washing buffer: 40 mM KCl, 100 mM NaCl, 4 mM $MgCl_2$, 30 mM HEPES/NMG pH 7.4; non-activating solution: 140 mM KCl, 4 mM $MgCl_2$, 30 mM HEPES/NMG pH 7.4; activating solution: 140 mM KCl, 4 mM $MgCl_2$, 30 mM HEPES/NMG pH 7.4, 0.5 mM $CaCl_2$. For measurements with inhibitor all three solutions contained the inhibitor in the same concentration. The change from the non-activating to the activating solution leads to the activation of NCX causing a negative current, which decays to the base line. If the average amplitude of NCX-signal in the three measurement cycles is constant, the solutions are changed to the inhibitor-containing solutions and the same protocol is repeated. Afterwards the solutions can be changed back to inhibitor-free conditions, to test whether the inhibitor is reversible.

The term "current" in context of this invention shall mean the peak current in response to the replacement of non-activating by activating solution, including but not limited to the maximal peak current. The current amplitude rises within 10 to 100 ms, followed by a slower decay within about 2 seconds. The polarity of the current may be positive or negative, depending on the polarity of the transported ions and/or the polarity of the shifted moieties of the protein and the vectorial orientation of their transport or shift across or within the membranes of the compartments. Currents resulting from the replacement of the activating solution by non-activating solution or from the replacement of the non-activating solution by the washing solution are not taken into consideration with respect to the determination of the NCX activity. Flow rates and intervals are chosen such that the current response to the replacement of the non-activating solution by activating solution remains unbiased by current responses provoked by the other replacement steps.

The replacing of the washing solution by the non-activating solution will preferably induce a Na^+-gradient across membranes harboring NCX protein. Thereafter, replacement of the non-activating solution by activating solution (i.e. Ca^{2+}-containing

solution) will selectively trigger the NCX activity. Replacing solutions subsequently in reverse order returns the sensor chip into its initial state.

Washing of the sensor chip means generally incubation of the sensor chip in a sodium-containing, calcium-free buffer solution (washing solution) causing preferably an accumulation of Na^+ in the vesicular compartments. Again, establishing a Na^+-gradient across the membrane is performed by replacing the washing solution by a non-activating solution thereby exposing the sensor chip to a rapid variation of the Na^+-concentration. The activation of the NCX is preferably performed by replacing the non-activating solution by an activating solution thereby exposing the sensor chip to a rapid variation of the Ca^{2+} - concentration.

In another preferred embodiment the invention pertains to an assay for determining the activity of NCX protein wherein a first solution replacement is performed by replacing the washing solution by non-activation solution and/or a second replacement is performed by replacing the non-activating solution by activating solution and/or a third solution replacement is performed by replacing the activating solution by non-activating solution and/or a fourth solution replacement is performed by replacing the non-activating solution by washing solution.

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According to a second aspect, the present invention provides an assay for identifying of a compound that modulates the activity of a NCX protein wherein

a] a cell free electrophysiological sensor chip is provided that comprises NCX protein;

b] a washing solution, a non-activating solution and an activating solution is provided;

c] a washing solution, a non-activating solution and an activating solution is provided which all of these three solutions contain additionally a chemical compound with the same concentration in all of the three solutions;

d] a cell free electrophysiological sensor chip from a] is treated stepwise consecutively by washing solution, non-activating solution, activating solution, non-activating solution and washing solution from b];

e] a current is determined when changing from non-activating to activating solution in d];

f] a cell free electrophysiological sensor chip from e] is treated stepwise consecutively by washing solution, non-activating solution, activating solution, non-activating solution and washing solution from c];

g] a current is determined when replacing the non-activating by activating solution in f];

thereby proving the modulation of the activity of the NCX protein in case the current from e] is of different strength as the current from g].

The consecutive stepwise treatment in steps d), e) and f) could be performed in a continuous flow or an almost continuous flow.

Modulation of the activity of the NCX protein could consist of stimulating or inhibiting of the activity of the NCX protein.

The stimulation of activity is demonstrated when the current from e] is larger than the current from g],

The inhibition of activity is demonstrated when the current from e] is smaller than the current from g].

Preferred embodiments of this protocol are already described above, in the following Examples and the claims.

According to a third aspect, the present invention provides a kit of parts comprising

a] a cell free electrophysiological sensor chip comprising NCX protein;

b] a washing solution;

c] a non-activating solution;

d] an activating solution.

Preferred embodiments of this kit are also already described above, in the following Examples and the claims.

According to a fourth aspect, the present invention provides the manufacturing of a kit of parts as defined above wherein a cell free electrophysiological sensor chip is manufactured, the NCX protein is manufactured, the manufactured NCX protein is fixed onto the surface of the cell free electrophysiological sensor chip, a washing solution is manufactured, a non-activating solution is manufactured, an activating solution is manufactured and the cell free electrophysiological sensor chip comprising NCX protein, the washing solution, the non-activating solution and the activating solution are combined to a kit unit.

The kit unit could consist of one or several parts comprising e.g. notifications for the user.

The invention pertains further to the use of a kit of parts as mentioned before for identifying of a compound that modifies (= inhibits or activates) the activity of a NCX protein or of determining the activity of NCX1 protein.

According to a fifth aspect, the present invention provides the use of a kit of parts as defined above for identifying of a compound that modifies the activity of a NCX protein.

According to a sixth aspect, the present invention provides the use of a kit of parts as defined above for determining the activity of NCX protein.

According to a seventh aspect, the present invention provides an assay for determining the activity of NCX protein wherein a cell free electrophysiological sensor chip comprising adsorbed NCX protein containing membranes is exposed to a rapid change in the concentration of at least one NCX substrate.

The NCX substrate is Ca^{2+} or Na^{+}.

The following Figures and Examples shall describe the invention in further details without limiting the scope of protection.

Description of the Figures

Figure 1:

Figure 1 shows the polynucleotide sequence of vector pVL1393 harboring the cDNA sequence of human NCX1. The according open reading frame is marked by exhibiting the related amino acid sequences. The depicted sequence corresponds to SEQ ID NO.: 1. DNA of pVL1393 harboring cDNA for the human NCX1 has been deposited with DSMZ (Deutsche Sammlung für Mikroorganismen und Zellkulturen GmbH; Mascheroder Weg 1b; D-38124 Braunschweig) under DSM 16588.

SEQ ID NO.: 1: Polynucleotide sequence of pVL1393

Deposited biological material:
DSM 16588: DNA of pVL1393 harboring cDNA for the human NCX1
DSM ACC2670: Flp-In-T-Rex-293-NCX!

Deposits were carried out by transferring the biological material to DSMZ (Deutsche Sammlung für Mikroorganismen und Zellkulturen GmbH; Mascheroder Weg 1b; D-38124 Braunschweig) according to the rules of the Budapest Treaty.

Figure 2:
A) A 3 mm biosensor chip with gold structure and fluid compartment B – D) Scheme of the biosensors sandwich structure assembly B) gold suface C) coating with chemical linker and lipid D) adsorption of the membranes

Figure 3:
A) Electrical charging current induced by ion transport activity of the recombinantly expressed Na^+/Ca^{2+}-exchanger. B) IC_{50} of a Na^+/Ca^{2+}-exchanger inhibitor as acquired with the cell free electrophysiological sensor chip in accordance with the present invention. All errors are quoted as standard error of mean (SEM).

EXAMPLES

1. Mammalian Cell Line Generation

Rapid generation of stably transfected cell lines was achieved utilizing the Flp-In™ T-Rex™ expression system (Invitrogen Corporation, 1600 Farraday Avenue, Post Box 6482 Carlsbad, California 92008, USA). For this purpose cDNA encoding for the (human) cardiac sodium calcium exchanger (NCX-1, i.e., SLC8A1 PDB-entry NM_021097) was cloned into the Flp-In™ T-Rex™ expression vector (Invitrogen Corporation, 1600 Farraday Avenue, Post Box 6482 Carlsbad, California 92008, USA) and subsequently transfected into HEK-293 cells. The cells were kept in culture under standard conditions (37°C, air supplemented with 8% CO2) in D-MEM (Invitrogen Corporation, 1600 Farraday Avenue, Post Box 6482 Carlsbad, California 92008, USA) supplemented with 10% fetal calf serum (Biochrom AG, Post Box 46 03 09, 12213 Berlin, Germany), 50 mg/ml Hygromycin (Invitrogen Corporation, 1600 Farraday Avenue, Post Box 6482 Carlsbad, California 92008, USA) and 10 mg/ml Blasticidin (Invitrogen Corporation, 1600 Farraday Avenue, Post Box 6482 Carlsbad, California 92008, USA).

Cells were passed every 3 to 4 days using a Trypsin/EDTA solution Biochrom AG, Post Box 46 03 09, 12213 Berlin, Germany) to detach the cells. At least 12 to 24 hours prior to a membrane preparation cells were treated with 1 µg/ml Doxicycline (Beckton Diccinson Bioscience Clontech, 1290 Terra Bella Avenue
Mountain View, CA 94043, USA) to boost NCX expression.

The Flp-In-T-Rex-293-NCX1 cell line expressing NCX-1 is deposited at DSMZ (Deutsche Gesellschaft von Mikroorganismen und Zellkulturen GmbH; Mascheroder Weg 1b, D-38124 Braunschweig) under DSM ACC2670.

2. Insect Cell Line Generation

Rapid generation of transiently transfected cell lines was achieved utilizing the Bac3000 expression system (EMD Biosciences, Inc., Novagen Brand, 441 Charmany Drive, Madison, WI 53719, USA). For this purpose cDNA encoding for the (human) cardiac sodium calcium exchanger (NCX-1, i.e., SLC8A1 PDB-entry NM_021097) was cloned into the Bac3000 expression vector (EMD Biosciences, Inc., Novagen Brand, 441 Charmany Drive, Madison, WI 53719, USA). After generating virus according to the manufactures procedures viral stock in an threefold excess was used to transfect Sf9 insect cell. The cells were kept in culture under standard conditions (27°C) in appropriate medium. Membranes were harvested after 3 days posttransfection. The stock cell culture was maintained according to manufactures procedures.

Specifically, the method can be carried out as follows:

2.1 Co-Transfection to prepare recombinant virus

For co-transfection approximately 10µg of highly purified and sterile plasmid DNA should be prepared. To provide cells for the co-transfection a six-well plate is set up for each transfection as well as for the positive and negative control. 1x10e6 cells Sf9 cells in serum-free medium are seeded on each cavity. Cells are allowed to attach, usually for one hour. While cells are attaching the transfection mix can be prepared, which consists of 15µl BacVector 3000 from Novagen [0,02µg/µl], 30µl Cellfectin (InVitrogen), 0,4µg recombinant donor plasmid DNA pVL_NCX1 and distilled water ad 50µl. The incubation time takes 20 min. at room temperature. After this time 0,5 ml medium is added to each mix. If the cells are attached the old medium is removed and the transfection mix is added drop-by drop to the cavity. After 4h, 27°C incubation time 1ml fresh medium (containing 10% serum) is added and another incubation follows for .4-5 days at 27°C.

2.2 Plaque-Purification to isolate a monoclonal recombinant virus clone

After these 5 days the supernatant of the co-transfection plates is collected (so called virus stock VS0). For the identification of recombinant virus by plaque screening another six-well plate is set up with 1x10E-6 Sf9 cells for each cavity. Cells should settle for at least 30 min. Meanwhile virus is diluted to 1 ml aliquots at dilutions of 10E-3, 10E-4, 10E-5, 10E-6 and 10E-7. After cells have attached well to the plates the media is aspirated off. Quickly 1 ml of diluted virus is added to each well of the 6-well plate. The plates are transferred to a rocking platform and slowly rocked for at least a couple hours. Now the overlay agarose with low melting agarose is prepared (just before use). The following mix is used: 1 part 2X Grace's Medium supplemented with 20% Fetal Calf Serum and 1 part 3% SeaPlaque Agarose in ddH2O. The agarose is completely melted in a microwave. The 1:1 mixture is placed in a 38°C water bath. Now all the medium is aspirated off from the cavities and 1ml overlay mix is added to each well by allowing it to slide down the far wall of the well and onto the plate. After overlaying the cells, the plates stay for 30 minutes in the hood. After this time 1 ml medium is added to each cavity to avoid drying up. The plates are placed in a 27°C, 98% humidity controlled incubator for at least 3 days.

After this the plaques are stained with a solution of 1ml 1% Neutral red. to each well of a 6-well dish. Plates are returned to the incubator for at least 4 hours. During this time the plaques will begin to appear as clear spots among stained cells. With a sterile pasteur pipette one plaque (ore more) is picked and transferred into a 25cm^2 T-flask with 2e6 Sf9 cells in total and a culture volume of 4ml with 5%FCS. Incubation follows for 5-6 days at 27°C. The culture supernatant is collected (so called virus stock VS1) and used for the next step: the determination of the virus titer.

2.3. Plaque Assay for virus titer determination

Same procedure as described under 2.2.

The plaques are counted and the titer is calculated by using the following equation (for example 20 plaques are counted)

$$\text{Titer (pfu/ml)} = 20 \text{ plaques at 10E-6} \times \left(\frac{1}{1\text{ml inoculum/well}}\right)$$

Titer (pfu/ml) = 2x10e7 pfu/ml (plaque forming units /ml)

2.4. Amplification of the recombinant virus in a larger scale (i.e. 1L)

For the virus amplification usually a M.O.I. (multiplicity of infection) of 0,1 is used, that means a ratio of 1 virus for 10 Sf9 cells. Since the virus has the possibility for secondary infection cycles the amplification time should be approximately 7 days. During this time the virus will be amplified exponentially and the vitality of the cell culture can decrease to 20-30%. For generating high virus titer a 5% FCS supplementation is recommended and the use of a super-spinner culture vessel with bubble-free aeration. After one week the culture supernatant is collected (so called virus stock VS2). This is a larger volume of virus stock which is sufficient for several expression studies and which can be stored for several month (up to 1 year) at 4°C and light protected.

2.5. Expression of the recombinant protein NCX1

For all expression experiments a M.O.I of 3 is recommended (ratio: 3 virus particles for 1 Sf9 cell) and an incubation time of 72h, at 27°C.

3. Membrane/Protein Preparation

Cells from native tissue or recombinant expression systems were harvested by mechanical separation of the surrounding environments (bottle or body) surface.

Membrane fragments were prepared by cell rapture and subsequent centrifugation steps and/or sucrose gradient centrifugation.

Specifically, the method can be carried out as follows:

3.1 Insect cell Membrane/Protein Preparation

After harvesting the cells via centrifugation and aliquots of approx. 2g wet weight cells from Sf9 suspension culture are quick-frozen in liquid Nitrogen and stored at –80°C for further preparation.

The cell pellet is thawed on ice and transferred to ice-cold buffer (0.25M sucrose, 5mM Tris pH 7.5, 2mM DTT, one complete protease inhibitor cocktail tablet per 50ml (Roche Diagnostics GmbH, Mannheim, Germany).

The membrane fragments were prepared by cell rapture. Cells are homogenized by the nitrogen cell disruption method utilizing a Parr Cell Disruption Bomb (Parr Instrument Company, Illinois, USA) or the Dounce homogenisation method utilizing a Dounce Homogenisator (7ml from Novodirect GmbH, Kehl/Rhein, Germany) and the suspension centrifuged 10min at 4°C and 680g. The supernatants are collected and again centrifuged for 1h at 4°C and 100000g in SW41 swing-out rotor.

Pellets are suspended in approximately 2ml of 5mM Tris pH 7.5. With 87% sucrose (in 5mM Tris) the suspension is adjusted to 56%. The sucrose gradient is now built up beginning with 2ml of the 56% fraction at the bottom, following 3ml 45% sucrose, 3ml 35% and 2ml 16% sucrose.

Again centrifugation for 2.5 h (or even more) at 4°C and 100000g the gradient-bands are aspirated carefully with a pasteur pipette and collected in fresh tubes together with 5 ml of 100mM NaCl, 1.5mM EDTA, 40mM Hepes pH 7.5.

Another centrifugation step follows: 30min 150000g, 4°C.

The resulting pellet is resuspended in 100mM NaCl, 1.5mM EDTA, 2mM DTT, 40mM Hepes pH 7.5, 10% glycerol.

4. Sensor chip preparation

The chip comprises the NCX protein in all instances the protein sticks or is attached to the chip. This may occur e.g. by hydrophobic, hydrophilic, ionic or covalent forces.

The sensor chip of such an assay consists e.g. of a basic body of borofloate glass that carries gold structures. This device would be further covered by a mercaptane layer and having one or several insulating layers.

In a preferred embodiment the borofloatee glass with gold structures was coated with a mercaptane layer and a lipid film consisting of 60 weight units of ,2-Diphytanoyl-sn-Glycero-3-Phosphocholin (AVANTI 850 356) and 1 weight unit of octadecylamine (FLUKA) dissolved in 800 weight units n-decane (in detail: 150 µl PC (Stock 20 mg/ml in hexane or $CHCl_3$ as distributed by AVANTI + 10 µl octadecylamine (5 mg/ml in hexane) are evaporate with nitrogen, and taken up in 400 µl n-decane.). To achieve this the sensor chip was incubated with 30µL mercaptane for 15 min and then washed with isopropanol (3x70µL) and vacuum dried. After several hours 2µL lipide + 30µL DTT-buffer (2mM, 1,542mg DTT/50mL Puffer C) was pipetted on top of the sensor chip and incubated for 20min. To create a functional sensor chip 10µL of the membrane of interest + 120µL DTT-Puffer was mixed and the ultrasonicated 2x10 units (0,5/30) with a 30s break on ice. The buffer is then removed from the sensor chip and replaced by 30µL membrane containing buffer, which is pipetted up and down and stored at least for 12 h at 4°C.

5. Solution Exchange Protocol

For the determination of its activity, the NCX protein was treated consecutively with a washing, non-activating and activating solution and the electrical current was measured when changing from charging to activating treatment. The replacement of

the pre-incubation solution (sodium-containing, calcium-free buffer solution) with the charging solution (reduced sodium, calcium-free buffer solution) induces a Na^+-gradient across membranes harboring NCX protein. Thereafter, replacement of the solution by activating solution (Ca^{2+}-containing solution) triggers the NCX activity. Subsequently replacing solutions in reverse order returns the sensor chip into its initial state.

After buffer containers A, B, and C of the biosensor system had been filled with "activating" buffer (30 mM HEPES/NMG pH 7.4, 140 mM KCl, 4 mM $MgCl_2$, 0,5 mM $CaCl_2$.), "non-activating" buffer (30 mM HEPES/NMG pH 7.4, 140 mM KCl, 4 mM $MgCl_2$), and "washing" buffer (30 mM HEPES/NMG pH 7.4, 40 mM KCl, 100 mM NaCl, 4 mM $MgCl_2$) respectively, a dummy was mounted to the sensor holder and the system was flushed with all buffers to remove air bubbles from the entire fluidic system. An empty or blind sensor was then replaced by a standard glass-based sensor preloaded with NCX1-containing HEK membrane fragments (chemically modified gold surface of 3 mm diameter; IonGate Biosciences GmbH, Frankfurt/M., Germany). Liquid transport through the fluidic system, including the sensor flow cell, was achieved by applying air pressure to the buffer containers.

Measurements were usually carried out at 200 mbar overpressure, resulting in a flow rate of about 300 µl s^{-1}. For the determination of its activity, the membranes harboring NCX protein were treated consecutively by an "washing," "non-activating" and "activating" solution. The replacement of the "washing" solution by the "non-activating" solution induces a Na^+-gradient across membranes harboring NCX protein. Thereafter, replacement of the "non-activating" solution by "activating" solution triggers the NCX activity and thus the induced electrical current was measured when changing from "non-activating" to "activating" treatment. Subsequently replacing solutions in reverse order returns the sensor chip into its initial state. By means of the control software, a sequence was defined in which "washing" buffer flowed over the sensor surface for 0.5 s, followed by "non-activating" buffer (2.0 s), "activating" buffer (2.0 s), "non-activating" buffer (2.0 s), and "washing" buffer (2.0 s). During the whole sequence, the current response was digitized (2000 samples s^{-1}) and saved to data

files. For dose-response experiments inhibitors were dissolved in "activating," "non-activating" and "activating" buffer, respectively. All chemicals were of analytical grade or better.

The following settings are used for the measurements of NCX1:

Cycle 1:

washing buffer	non-activating solution	activating solution	non-activating solution	washing buffer	break
0.5s	2s	2s	2s	4s	42
0.5s	2s	2s	2s	4s	42
0.5s	2s	2s	2s	4s	42
0.5s	2s	2s	2s	4s	1

5 minutes break

Cycle 2:

washing buffer	non-activating solution	activating solution	non-activating solution	washing buffer	break
0.5s	2s	2s	2s	4s	42
0.5s	2s	2s	2s	4s	42
0.5s	2s	2s	2s	4s	42
0.5s	2s	2s	2s	4s	1

5 minutes break and addition of a compound to be analyzed

Cycle 3:

Washing buffer	non-activating solution	activating solution	non-activating solution	washing buffer	break
0.5s	2s	2s	2s	4s	42
0.5s	2s	2s	2s	4s	42
0.5s	2s	2s	2s	4s	42
0.5s	2s	2s	2s	4s	1

5 minutes break

Cycle 4:

Washing buffer	non-activating solution	activating solution	non-activating solution	washing buffer	break
0.5s	2s	2s	2s	4s	42
0.5s	2s	2s	2s	4s	42
0.5s	2s	2s	2s	4s	42
0.5s	2s	2s	2s	4s	1

5 minutes break and addition of the same compound in another concentration or of another compound, etc.

6. Experiment

Due to the protocol the NCX-containing membrane fragments bound to the biosensors surface are exposed to a rapid Ca^{2+} concentration jump leading to a transient charging current (Fig. 3 a). It should be noted again, that a particular preference for this experimental outcome is an extra-vesicular Na^{+} reduction applied directly before the experiment to the biosensor to generate a sodium gradient. The reason for the fast decay of the current is due to inactivation and/or capacitive coupling of the transporter to the surface of the membrane fragments. In effect, a stationary current charges the

sandwich structure of the biosensor and therefore generates an electrical field that allows charging only to a limited state - similar to an DC current source charging a capacitor.

Figure 3 A shows a typical cell-free electrophysiological NCX biosensor recording before (black trays) and after (grey trays) inhibition with a NCX-specific inhibitor. A biosensor subjected to rapid Ca concentration jumps, in the absence or presence of 10 µM inhibitor A in all solutions generated a NCX peak current of 300 pA or 20 pA respectively, showing the specificity of the signal. The application of 10 µM of the inhibitor resulted in a decrease of the NCX-specific signal of 90.5 % ± 5.1 % (n = 5) The resulting dose-response curve is shown in Figure 3 B. IC_{50} values (n = 5) were recorded with application of the inhibitors' concentrations of 0.01, 0,1, 1, 5, and 10 µM. The value for 50 % inhibition can be calculated to 0.86 µM ± 0.22 µM. The Hill coefficient could be calculated to 0.7 ± 0.03. Subsequent experiments in the absence of the inhibitor resulted in NCX peak currents with up to 100 % of the amplitude of the initial signal, proving the inhibitors' reversibility.

In control experiments the current could be diminished by reduction of the applied Ca^{2+} jump and by application of the unspecific NCX1 blocker of Ni^{2+} (5 mM). Furthermore, membranes of cells not expressing NCX1 did not yield currents under similar experimental conditions.

Claims

1. An in vitro assay for determining the activity of NCX protein wherein a cell free electrophysiological sensor chip comprising a NCX protein is treated stepwise consecutively by washing, non-activating and activating and then the current is measured when changing from non-activating to activating treatment.

2. Assay as claimed in claim 1 wherein the cell free electrophysiological sensor chip is comprising at least one of the NCX proteins of the following list: NCX1, NCX2, NCX3, NCX4, NCX5, NCX6, NCX7.

3. Assay as claimed in claim 2 wherein the cell free electrophysiological sensor chip comprises NCX protein of mammalian origin, preferably from rat, mouse or human.

4. Assay as claimed in claim 3 wherein the NCX protein is human NCX1 protein.

5. Assay as claimed in claims 1 to 4 wherein the NCX protein is a recombinant NCX protein or a native NCX protein.

6. Assay as claimed in claims 1 to 5 wherein the cell free electrophysiological sensor chip contains a basic body of borofloate-glass that carries gold structures.

7. Assay as claimed in claims 1 to 5 wherein the cell free electrophysiological sensor chip is covered by a mercaptane layer and having one or several insulating layers.

8. Assay as claimed in claims 1 to 7 wherein the washing of the cell free electrophysiological sensor chip is performed by a washing solution.

9. Assay as claimed in claims 1 to 8 wherein a first solution replacement is performed by replacing the washing solution by non-activation solution.

10. Assay as claimed in claims 1 to 9 wherein a second replacement is performed by replacing the non-activating solution by activating solution.

11. Assay as claimed in claims 1 to 10 wherein a third solution replacement is performed by replacing the activating solution by non-activating solution.

12. Assay as claimed in claims 1 to 11 wherein a fourth solution replacement is performed by replacing the non-activating solution by washing solution.

13. Assay for identifying of a compound that modulates the activity of a NCX protein wherein

a] a cell free electrophysiological sensor chip is provided that comprises NCX protein;

b] a washing solution, a non-activating solution and an activating solution is provided;

c] a washing solution, a non-activating solution and an activating solution is provided which all of these three solutions contain additionally a chemical compound with the same concentration in all of the three solutions;

d] a cell free electrophysiological sensor chip from a] is treated stepwise consecutively by washing solution, non-activating solution, activating solution, non-activating solution and washing solution from b];

e] a current is determined when changing from non-activating to activating solution in d];

f] a cell free electrophysiological sensor chip from e] is treated stepwise consecutively by washing solution, non-activating solution, activating solution, non-activating solution and washing solution from c];

g] a current is determined when replacing the non-activating by activating solution in f];

thereby proving the modulation of the activity of the NCX protein in case the current from e] is of different strength as the current from g].

14. Assay as claimed in claim 13 wherein the cell free electrophysiological sensor chip is comprising at least one of the NCX proteins of the following list: NCX1, NCX2, NCX3, NCX4, NCX5, NCX6, NCX7.

15. Assay as claimed in claim 14 wherein the cell free electrophysiological sensor chip is comprising NCX protein of mammalian origin as e.g. from rat, mouse or human.

16. Assay as claimed in claim 15 wherein the NCX protein is human NCX1 protein.

17. Assay as claimed in claims 13 to 16 wherein the cell free electrophysiological sensor chip comprises NCX protein that was produced by means of recombinant biological material.

18. Assay as claimed in claims 13 to 17 wherein the cell free electrophysiological sensor chip consists of a basic body of borofloate-glass that carries gold structures.

19. Assay as claimed in claims 13 to 18 wherein the cell free electrophysiological sensor chip is covered by a mercaptane layer and having one or several insulating layers.

20. Kit of parts comprising

a] a cell free electrophysiological sensor chip comprising NCX protein;

b] a washing solution;

c] a non-activating solution;

d] an activating solution.

21. Kit of parts as claimed in claim 20 wherein the cell free electrophysiological sensor chip is comprising at least one of the NCX proteins of the following list: NCX1, NCX2, NCX3, NCX4, NCX5, NCX6, NCX7.

22. Kit of parts as claimed in claim 21 wherein the NCX protein is of mammalian origin as e.g. from rat, mouse or human.

23. Kit of parts as claimed in claim 22 wherein the NCX protein is human NCX1 protein.

24. Kit of parts as claimed in claims 20 to 23 wherein the cell free electrophysiological sensor chip comprises human NCX protein that was produced by means of recombinant biological material.

25. Kit of parts as claimed in claims 20 to 24 wherein the cell free electrophysiological sensor chip consists of a basic body of borofloate-glass that carries gold structures.

26. Kit of parts as claimed in claims 20 to 25 wherein the cell free electrophysiological sensor chip is covered by a mercaptane layer and harboring one or several insulating layers.

27. Kit of parts as claimed in claims 18 to 24 wherein the washing solution comprises 30 ± 15 mM HEPES/NMG pH 7.4 ± 1.0; 40 ± 20 mM KCl; 100 ± 50 mM NaCl; 4 ± 2 mM $MgCl_2$.

28. Kit of parts as claimed in claims 20 to 26 wherein the non-activating solution comprises 30 ± 15 mM HEPES/NMG pH 7.4 ± 1.0; 140 ± 70 mM KCl, 4 ± 2 mM $MgCl_2$.

29. Kit of parts as claimed in claims 18 to 26 wherein the activating solution comprises 30 ± 15 mM HEPES/NMG pH 7.4 ± 1.0, 140 ± 70 mM KCl, 4 ± 2 mM $MgCl_2$, 0.5 ± 0.25 mM $CaCl_2$.

30. Manufacturing of a kit of parts as claimed in claims 20 to 29 wherein a cell free electrophysiological sensor chip is manufactured, the NCX protein is manufactured, the manufactured NCX protein is fixed onto the surface of the cell free electrophysiological sensor chip, a washing solution is manufactured, a non-activating solution is manufactured, an activating solution is manufactured and the cell free electrophysiological sensor chip comprising NCX protein, the washing

solution, the non-activating solution and the activating solution are combined to a kit unit.

31. Use of a kit of parts as claimed in claims 20 to 30 for identifying of a compound that modifies the activity of a NCX protein.

32. Use of a kit of parts as claimed in claims 20 to 30 for determining the activity of NCX protein.

33. Assay for determining the activity of NCX protein wherein a cell free electrophysiological sensor chip comprising adsorbed NCX protein containing membranes is exposed to a rapid change in the concentration of at least one NCX substrate.

34. Assay as claimed in claim 33 wherein the NCX substrate is Ca^{2+} or Na^{+}.

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