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.

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

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 ▽.

CHO-hNCX1 CHO-hNCX2 CHO-hNCX3	Steinbeis Transfer Zentrum	-	Cell line transfected with human Na^+/Ca^{2+} exchanger (NCX1, NCX2, NCX3)
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Geneticin	Gibco	10131-027	Cell selection
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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.

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
Gramicidin stock solution	1 mM gramicidin in Ethanol (4°C)	prepared freshly every day and stored at 4°C until measurement
Gramicidin assay solution	60 µM gramicidin 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 gramicidin assay solution was prepared using assay buffer. A 96 well polypropylene plate was filled with 150 µl per well of the gramicidin assay solution and stored at 4°C. The addition plate could only be used two times before refilling was required because the gramicidin 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 gramicidin 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.

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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 %).

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

A gramicidin 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 gramicidin concentrations (Fig. 7a). 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 while increasing concentrations led to a slight increase in fluorescence (Fig. 7b).

The preparation of the gramicidin solution and the storage was a critical step, because gramicidin is degraded at higher temperatures. In the following experiments gramicidin was solved and stored at different temperatures. The gramicidin solution had to be prepared and stored at 4°C otherwise gramicidin 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 gramicidin concentrations in the "reverse" assay. Typical inhibitory effects on the NCX1 activity after activation with 20 µM gramicidin 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

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;
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Figure 6:
(a) Effect of different concentrations of gramicidin (—— 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 gramicidin (·- ·-);
(b) Effect of different concentrations of gramicidin (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 gramicidin (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 gramicidin stimulation.

Figure 8:

Effect of 20 µM gramicidin 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.

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(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 gramicidin (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 ▽.

CLAIMS

1. An assay for determining the calcium import reverse mode 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, wherein as the sodium/calcium exchanger activator gramicidin is used at a concentration of 15 to 25 µM.

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 any one of 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 any one of 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 any one of 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 any one of claims 1 to 6, wherein said luminescent signal is fluorescence and determination of fluorescence 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. The assay according to any one of claims 1 to 9, wherein the cells are CHO cells, the assay buffer comprises about 133.8 mM NaCl and about 3.5 mM $CaCl_2$, and the sodium/calcium exchanger is selected from NCX1, NCX2 and NCX3.

1110. Use of the assay according to any one of claims 1 to 109 to test a compound for activity as an agonist or antagonist of the calcium import activity of a sodium/calcium exchanger, wherein the assay furthermore comprises contacting the cells with a compound, wherein the cells have been treated, prior to treating with said compound, with a sodium/calcium exchanger activator.

1211. Use of the assay according to any one of claims 1 to 109 for determining an altered calcium import activity of the sodium/calcium exchanger for the diagnosis of a disease associated with a sodium/calcium exchanger altered expression.

1312. An assay for determining the calcium import reverse mode 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,

wherein as the sodium/calcium exchanger activator gramicidin is used at a concentration of 15 to 25 µM.

1413. The assay according to claim 1312, 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.

1514. The assay according to claim 1312, wherein the sodium/calcium exchanger is a NCX protein of the following list: NCX1, NCX2, NCX3.

1615. The assay according to any one of claims 1312 to 1514, wherein the sodium/calcium exchanger is of mammalian origin, preferably from rat, mouse, dog, bovine, pig, ape or human.

1716. The assay according to any one of claims 1312 to 1615, wherein the cells are selected from the group consisting of: CHO, HEK, COS7 and JURKAT cells.

1817. The assay according to any one of claims 1312 to 1716, 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.

1918. The assay according to any one of claims 1342 to 1847, wherein said luminescent signal is fluorescence and determination of fluorescence employs a FLIPR device.

2019. The assay according to claim 1847, wherein said dye precursor is an acetoxymethylester derivate.

2120. The assay according to claim 1847, wherein said dye is the calcium sensitive fluorescence dye fluo-4.

2221. The assay according to any one of claims 1342 to 2120, wherein said compound is a sodium/calcium exchanger antagonist.

23. The assay according to any one of claims 13 to 22, wherein the cells are CHO cells, the assay buffer comprises about 133.8 mM NaCl and about 3.5 mM $CaCl_2$, and the sodium/calcium exchanger is selected from NCX1, NCX2 and NCX3.

2422. A kit of parts comprising:

a) lyophilized cells expressing a sodium/calcium exchanger;

b) a colored substance for determining intracellular calcium;

c) a compound buffer; and

d) a colored substance buffer,

e) gramicidin, whereby gramicidin is indicated for use at a concentration of 15 to 25 µM.

2523. The kit of parts according to claim 2422, wherein said colored substance is the calcium sensitive fluorescence dye fluo-4.

2624. The kit of parts according to claim 2422 or 2523, 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.

2725. The kit of parts according to claim 2422 or 2523, wherein the sodium/calcium exchanger is a NCX protein of the following list: NCX1, NCX2, NCX3.

2826. The kit of parts according to any one of claims 2422 to 2725, wherein the sodium/calcium exchanger is of mammalian origin, preferably from rat, mouse, dog, bovine, pig, ape or human.

29. The kit of parts according to any one of claims 24 to 28 comprising:

f) an assay buffer

wherein the cells are CHO cells, the assay buffer comprises about 133.8 mM NaCl and about 3.5 mM $CaCl_2$, and the sodium/calcium exchanger is selected from NCX1, NCX2 and NCX3.

3027. Use of a kit of parts according to any one of claims 2422 to 2826 to test a compound for activity as an agonist or antagonist of the calcium import activity of a sodium/calcium exchanger.

3128. Use of a kit of parts according to any one of claims 2422 to 2826 for determining an altered calcium import activity of the sodium/calcium exchanger for the diagnosis of a disease associated with a sodium/calcium exchanger altered expression.