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Development and validation of HTS assay for screening the calcium-activated chloride channel modulators in TMEM16A stably expressed CHO cells

Jinlong Qi · Yuan Wang · Yani Liu · Fan Zhang ·
Bingcai Guan · Hailin Zhang

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Abstract Calcium-activated chloride channels (CaCCs), for example TMEM16A, are widely expressed in a variety of tissues and are involved in many important physiological functions. We developed and validated an atomic absorption spectroscopy (AAS)-based detection system for high-throughput screening (HTS) of CaCC modulators. With this assay, Cl^- flux from CHO cells stably transfected with TMEM16A is assayed indirectly, by measuring excess silver ions (Ag^+) in the supernatant of AgCl precipitates. The screening process involved four steps: (1) TMEM16A CHO cells were incubated in high- K^+ and high- Cl^- buffer with test compounds, and with ionomycin as Ca^{2+} ionophore, for 12 min; (2) cells were washed with a low- K^+ , Cl^- -free and Ca^{2+} -free buffer; (3) CaCC/TMEM16A were activated in high- K^+ , Cl^- -free buffer with ionomycin ($10 \mu\text{mol L}^{-1}$) for 12 min; and (4) excess Ag^+ concentration was measured using an ion channel reader (ICR, an AAS system). The assay can be used to screen CaCC activators and inhibitors at the same time. With this assay, positive control drugs, including NPPB, CaCCinh-A01, flufenamic acid (Flu) and E_{act} , all had good

concentration-dependent effects on CaCC/TMEM16A. NPPB and CaCCinh-A01 inhibited the CaCC/TMEM16A currents completely at $300 \mu\text{mol L}^{-1}$, with IC_{50} values of $39.35 \pm 4.72 \mu\text{mol L}^{-1}$ and $6.35 \pm 0.27 \mu\text{mol L}^{-1}$, respectively; and E_{act} activated CaCC/TMEM16A, with an EC_{50} value of $3.92 \pm 0.87 \mu\text{mol L}^{-1}$.

Keywords CaCCs · TMEM16A · Activator · Inhibitor · HTS

Introduction

Calcium-activated chloride channels (CaCCs), a subtype of chloride channel, are ubiquitously expressed in epithelial and nonepithelial cell types, where they are involved in epithelial fluid secretion, sensory signal transduction, smooth muscle contraction, oocyte fertilization, and other functions [1–4]. TMEM16A (alternative names anoctamin-1 and ANO1) was recently identified as a possible molecular component of CaCCs [5–7]. Among the ten members of TMEM16 family found so far, TMEM16A and TMEM16B have been clearly shown to generate CaCCs [8–10]. It is not yet known whether other members of the TMEM16 family are also CaCCs.

CaCC/TMEM16A are regarded as potential drug targets for treating cystic fibrosis, hypertension, asthma, secretory diarrhea, pain, and some tumors [4, 11]. A substantial limitation when studying CaCCs (TMEM16A) has been the lack of potent and selective modulators, especially inhibitors. Although many well-known chloride channel inhibitors have been shown to inhibit CaCC/TMEM16A [4], only T16Ainh-A01 and MONNA have recently been described as selective TMEM16A inhibitors [12, 13]. This is partly because determination of the molecular identity of CaCCs has been slower than for other targets, because of technical challenges in screening for CaCC modulators.

J. Qi · Y. Wang · Y. Liu · B. Guan · H. Zhang
The Key Laboratory of Neural and Vascular Biology, Ministry of Education, Hebei Medical University, Shijiazhuang 050017, China

J. Qi · Y. Wang · Y. Liu · B. Guan · H. Zhang
The Key Laboratory of New Drug Pharmacology and Toxicology, Hebei Province, Hebei Medical University, Shijiazhuang 050017, China

J. Qi · Y. Wang · Y. Liu · B. Guan · H. Zhang (✉)
Department of Pharmacology, Hebei Medical University,
Shijiazhuang 050017, China
e-mail: z.hailin@yahoo.com

F. Zhang
Department of Biochemistry, Hebei Medical University,
Shijiazhuang 050017, China

Methods of assaying chloride-channel activity include:

1. Electrophysiological methods: The best method for the study of ion-channel function is the patch-clamp technique. Both single-channel and whole-cell patch-clamp techniques have been used to characterize a variety of subtypes of Cl^- channels and currents. However, these techniques suffer from low throughput. Automated patch-clamp processes also have limitations, including price, complexity, and reproducibility of data generated for high-throughput screening (HTS) [14, 15].
2. Radiotracer flux-based methods: Radiotracer flux measurement of ^{125}I and ^{36}Cl through these channels has proved to be a powerful technique for screening potential modulators of Cl^- channels [16–18]. Although the radiotracer flux methods achieve a higher throughput than the patch-clamp technique and are less prone to artefacts than fluorescence-based assays, they carry a significant radiation hazard.
3. Fluorescence-based methods: Fluorescence methods are best suited to high-throughput identification of chloride-channel modulators. Chemical-type chloride-sensitive fluorescent indicators and iodide-sensitive fluorescent indicators can be loaded into cells to provide a direct readout of cytoplasmic halide concentration. The fluorescence of these indicators is quenched by halides through a collisional mechanism [19, 20]. All the most recently discovered CaCC modulators were identified by the HTS method, which used a cell-based plate reader assay involving measurement of calcium-agonist-induced iodide influx in FRT cells co-expressing human TMEM16A and the fluorescent iodide-sensing protein YFP-H148Q/I152L/F46L [12, 21–23]. In addition, membrane-potential-sensing fluorescent indicators provide an alternative method of assaying chloride channel function [24, 25]. Unfortunately, these fluorescence-based assays have several disadvantages. These include multi-step screening procedures, significant background noise, being prone to false-positives and negatives, unsuitability for screening autofluorescent compounds, and fluorescence quenching [4, 26].

Flux assays to detect functional ionic flux through ion channels are becoming increasingly popular as tools for screening compounds. The non-radiolabeled rubidium-based flux assay coupled with an atomic absorption spectroscopy (AAS) system has been very successful in screening for potassium channel modulators in our lab [27, 28]. Based on this HTS technique, we here report development and validation of a flame-AAS-centered, accurate, and robust HTS assay for CaCC modulators, using measurement of excess Ag^+ as the readout [29]. It is obvious that our developed flux assays will not have the disadvantages related to intrinsic

fluorescence problems mentioned above. With this assay, TMEM16A stably expressed in CHO cells was screened for modulators of its activity.

Materials and methods

Materials

Molecular-biology-grade chemicals, tissue culture media, ionomycin, NPPB, and other chemicals, unless otherwise indicated, were purchased from Sigma. CaCCinh-A01 and E_{act} (purity > 98 % by HPLC–DAD) were synthesized and identified in our lab.

Cell cultures

Mouse TMEM16A cDNA was kindly provided by Professor Uhtak Oh (Sensory Research Center, College of Pharmacy, Seoul National University, Korea), and was stably expressed in CHO cells. The cells were grown by standard procedures. The cultures were maintained in Ham's F-12 K Medium, supplemented with 2 mmol L^{-1} glutamine, 10 % fetal calf serum, 100 U ml^{-1} penicillin or streptomycin, and 800 $\mu\text{g ml}^{-1}$ (geneticin) G418, at 37 °C and 5 % CO_2 . Cells were plated in 96-well polystyrene microplates at a density of 100,000 cells well^{-1} , in 200 μL of growth medium for 24 h in 5 % CO_2 at 37 °C.

Screening buffers

The buffers for the screening were as follows (in mmol L^{-1}), and were all adjusted to pH 7.4 with NaOH:

1. Incubation buffer (high K^+ and high Cl^-): NaCl 4, KCl 150, MgCl_2 1, CaCl_2 4, $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ 0.8, and Hepes 10;
2. Wash buffer (Cl^- -free, Ca^{2+} -free, and low K^+): Na gluconate 150, K gluconate 4, Mg gluconate 1, $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ 0.8, and Hepes 10;
3. Open buffer (Cl^- -free and high K^+): Na gluconate 54, K gluconate 100, Mg gluconate 1, Ca gluconate 4, $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ 0.8, and Hepes 10

Screening procedures

Cell incubation

The F-12 K medium used to incubate TMEM16A-expressing CHO cells was removed quickly and carefully, and 200 μL incubation buffer supplemented with 10 $\mu\text{mol L}^{-1}$ ionomycin

and test compounds was added, and was then placed at 37 °C for 12 min.

Washing

The incubation buffer was removed, and the wells were washed rapidly and carefully with wash buffer (200 μL well⁻¹ × 3).

Activation of CaCCs (TMEM16A)

After washing, 200 μL open buffer per well, supplemented with 10 $\mu\text{mol L}^{-1}$ ionomycin, was added and then placed for 12 min at 37 °C.

Measurement of Ag^+

190 μL open buffer from each well, added in step 3, was carefully transferred, using an eight-channel pipette, into another 96-well microplate, and 30 μL AgNO_3 (50 ppm) was added to each well and mixed sufficiently. The mixture was placed for 24 h in dark and still conditions. The next day, 100 μL supernatant was drawn into ICR to measure the free Ag^+ concentration, from which the Cl^- efflux was calculated.

For the control experiments, all procedures described above were performed with no TMEM16A-expressing cells present.

Measurements

Quantification of free- Ag^+ concentration in the extracellular samples was performed using the flame-AAS-based ICR8000 (Aurora Biomed Inc., Vancouver, BC, Canada). Settings were: detection wavelength 328.16 nm; average current 5 mA; entrance slit 0.6 nm; integration: 10 s; delay 0 s; autozero every.

Data analysis and statistics

Considering the K_{sp} effect of the AgCl at very low concentrations, the amount (ppm) of Cl^- present in each sample, at 25 °C, was calculated by the following formula:

$$\text{Cl}^- (\text{ppm}) = [(X - Y) / 108 + 0.01916 / Y] \times 35.5$$

Where “ Y ” = Ag^+ concentration (ppm) in the samples from the TMEM16A cell group, and “ X ” = average Ag^+ concentration (ppm) in the samples from the no-cell control group.

The Z' factor, which measures the overall assay quality as a screening window coefficient, was also determined. This factor evaluates the assay dynamic range of variation among the

replicates of the basal and activated efflux, and was calculated as follows [30]:

$$Z' = 1 - 3 \times (SD_{sam} + SD_{con}) / |M_{sam} - M_{con}|$$

where SD_{sam} is the standard deviation of the positive control (in the presence of modulator, activator, or inhibitor) and SD_{con} is the standard deviation of the blank control (in the absence of modulator). M_{sam} and M_{con} are the means of the positive control and blank control, respectively.

The inhibition ratio (%) of Cl^- influx for the inhibitors in each sample was calculated as follows:

$$\text{Inhibition ratio}(\%) = (A - B) / (A - C) \times 100\%$$

Where “ A ” = average Cl^- efflux of the blank-control group (TMEM16A-expressing CHO, with no test compounds added in step 1 of the screening procedures); “ B ” = Cl^- efflux of each test compound sample; “ C ” = average Cl^- efflux of the blank-control-without-incubation group (blank-control group, without step 1 of the screening procedures). To explain further: “ $A - B$ ” gives the Cl^- influx inhibited by the test compound, and “ $A - C$ ” gives the total Cl^- influx from CaCCs when the cells were incubated.

The activation ratio (%) Cl^- influx for the activators in each sample was calculated as follows:

$$\text{Activation ratio}(\%) = B / A \times 100\%$$

Where “ A ” and “ B ” have the meanings given above.

The concentration-response curve was fitted by logistic equation, using Originpro 7.0 (Origin Lab Corporation, US). The EC_{50} and IC_{50} results are expressed as means \pm SEM. Statistical analysis of differences between groups was performed using student's t -test or paired t -test. P -values ≤ 0.05 were regarded as significant.

Electrophysiology

TMEM16A currents in CHO cells were recorded using whole-cell recording, with an EPC 10 amplifier (HEKA Electronic) and PULSE software (HEKA). The acquisition rate was 10 kHz, and signals were filtered at 2.5 kHz. Patch electrodes were pulled with a Flaming/Brown micropipette puller (Sutter Instruments) and fire polished. The recording electrodes had a resistance of 2–4 $\text{M}\Omega$ when filled with an internal solution of composition (mmol L^{-1}): 130 CsCl, 10 EGTA, 1 MgCl_2 , 10 HEPES, and 8 CaCl_2 (447 nmol L^{-1} free- Ca^{2+} concentration). Before experiments, the pH of this solution was adjusted with CsOH to 7.3 before addition of 2 mmol L^{-1} ATP. The bath solution contained (mmol L^{-1}):

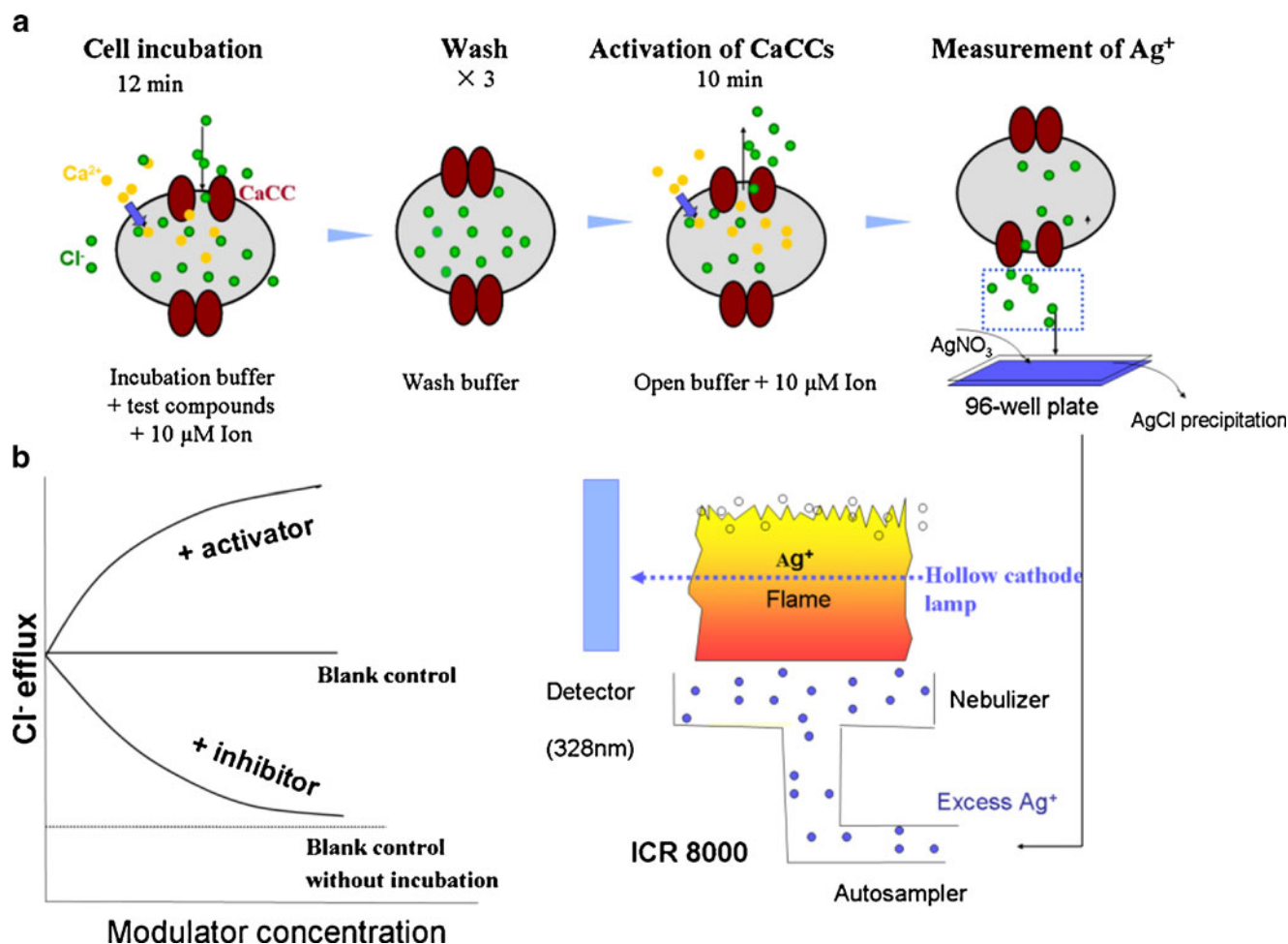


Fig. 1 Atomic-absorption-based Cl⁻ flux assay (ICR 8000 system) for screening CaCC/TMEM16A modulators. **(a)** Mechanism and process for CHO cells stably expressing TMEM16A Cl⁻ channels; the procedures were divided into cell incubation, wash, activation of CaCC/TMEM16A, and measurement of Ag⁺. Here Ion means

ionomycin. **(b)** Schematic illustration showing expected results for designed experiments measuring final Cl⁻ efflux, calculated from excess concentration of Ag⁺ after addition of an activator (*upper curve*) and an inhibitor (*lower curve*) of CaCC/TMEM16A, and a blank control with or without an incubation step

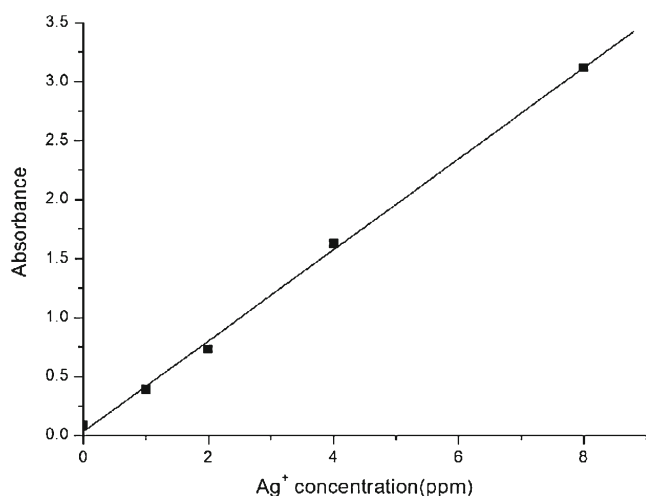


Fig. 2 Calibration curve for the concentration-dependent absorbance of free Ag⁺, measured by atomic absorption (ICR8000; wavelength 328.16 nm), $r^2=0.998$

140 NMDG, 1 CaCl₂, 1 MgCl₂, 10 glucose, and 10 HEPES, and was adjusted to pH 7.4 with HCl (12 mol L⁻¹). All recordings were performed at room temperature.

Results and discussion

Screening procedure

The non-radiolabel-based ion flux assay has been successfully used for ion channel activity analysis [16, 29]. At first, we attempted to develop a similar assay via indirect measurement of Cl⁻ efflux, which could be affected by the added test compounds acting on the Cl⁻ channels (CaCC/TMEM16A in this study). The modulatory effects of the test compounds on the Cl⁻ channels tested were evident from the change in Cl⁻

Table 1 Accuracy and precision data for the Cl^- -efflux assay using the ICR 8000 system

Amount added (ppm)	Intra-day ($n=6$)			Inter-day ($n=6$)		
	Measured concentration ^a (ppm)	Accuracy (%)	Precision (%)	Measured concentration ^a (ppm)	Accuracy (%)	Precision (%)
0.25	0.298±0.0135	19.2	4.55	0.288±0.0122	15.3	4.23
0.75	0.742±0.0219	−10.5	2.96	0.815±0.0313	8.6	3.84
1.5	1.389±0.0789	−7.4	5.68	1.353±0.0832	−9.8	6.15

^a Means±SD

efflux after these compounds were applied to the tested channels. The concentration of Cl^- was measured indirectly via the concentration of Ag^+ , which, having a known concentration and known reaction with effluxed Cl^- , could be easily measured by an automated atomic absorption system. However, we soon found that the designed assay had two serious disadvantages. First, the assay had poor sensitivity because of a relatively low concentration of Cl^- in cells, meaning the Cl^- efflux from the cells was too low to be measured reliably, especially when the concentration-dependent effects of the test compounds were studied. Second, it was prone to false positive or negative results, because Ag^+ can react with many tested compounds which are not uncommon in compound libraries, including: polyhydroxy compounds, for example tannic acid; halogen acid salts; and even Cl^- residue from synthesis of the test compounds.

Then, as shown Fig. 1a, we improved our screening procedure. We first introduced an incubation step, in which the

test compounds were added to an incubation buffer with an ionic composition (high Cl^- and K^+ concentration, ionomycin, and Ca^{2+}), promoting the opening of CaCC/TMEM16A (expressed in CHO cells) and Cl^- influx. Under these conditions the concentration of intracellular Cl^- increased, in accordance with the blocking or activating property and the concentrations of the test compounds (Fig. 1b). After this initial incubation, the Cl^- in the extracellular space was washed away using wash buffer, and an open buffer and 10 $\mu\text{mol L}^{-1}$ ionomycin were then added to the cells. The open buffer has a similar composition to the incubation buffer, but with the Cl^- replaced by gluconate. The initial influx of Cl^- observed during the incubation step was thus reversed to an efflux, which again was dependent on the blocking or activating property and on the concentrations of the test compounds present in the incubation buffer. Finally, the extracellular solutions from these open buffers were sampled to determine their concentration of Cl^- efflux, measured indirectly via their reaction with Ag^+ .

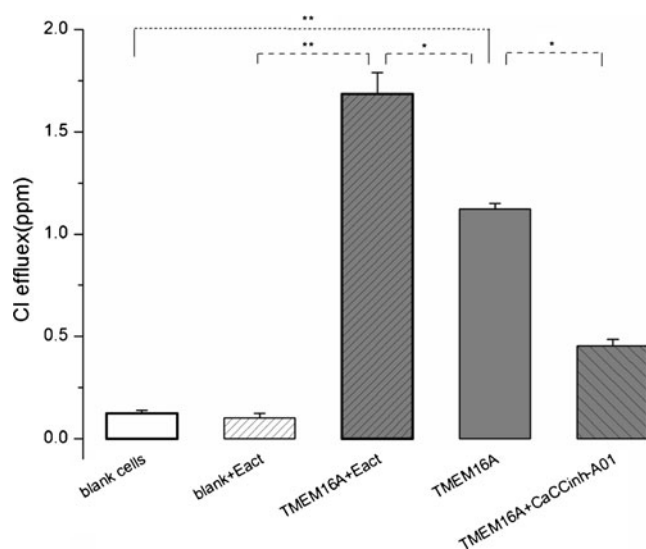


Fig. 3 Specificity of the Cl^- -efflux assay. Blank cells were CHO cells, without TMEM16A overexpression. Concentrations of inhibitor CaCCinh-A01 and activator E_{act} of CaCC/TMEM16A were both 25 $\mu\text{mol L}^{-1}$. Values are expressed as means±SD ($n=6$); significance was calculated by use of a t -test (* $p<0.05$, ** $p<0.01$)

Validation of analytical methods for indirect measurement of Cl^-

The Ag^+ concentration in the samples was measured with an automated flame atomic absorption system ICR 8000, using a 96-well Microplate. A calibration curve covering the range 0–8 mg L^{-1} (ppm) Ag^+ (Fig. 2) was generated, with standard concentrations of Ag^+ solution prepared from AgNO_3 each time the 96-well microplate was subjected to measurement of the Ag^+ concentration. All the correlation coefficients (r^2) of these calibration curves were between 0.995 and 0.999. The limit of detection (LOD) for Ag^+ was less than 0.25 ppm compared with the blank. Accuracy and precision for the indirect detection of Cl^- were assessed by the experiments performed in standard solutions of NaCl and AgNO_3 . The concentrations of Cl^- standard solution used were a high concentration of 1.5 ppm, a medium concentration of 0.75 ppm, and a low concentration of 0.25 ppm, reflecting the possible concentrations of Cl^- effluxed from the cells. The Cl^- concentration was calculated by the formula described in

the method, from the Ag^+ concentration measured and compared with the known standard Cl^- concentrations. In each run, the samples were evaluated in sets of six replicates, which were conducted on six consecutive days. The relative standard deviation (RSD) and relative error (RE) were taken as measures of precision and accuracy, respectively. As presented in Table 1, the intra-day and inter-day variability (RSD) ranged from 2.96 % to 5.68 % and 3.84 % to 4.23 %, respectively, and RE were all less than 20 %.

Cl^- efflux

In this cell-based assay, the relationship between extracellular and intracellular Cl^- may be interpreted in terms of free Ag^+ or

Cl^- concentration, or as percentage efflux of Cl^- . For example, in response to an inhibitory effect of a blocker, a rise in free Ag^+ concentration would occur, corresponding to a blocking of Cl^- efflux. In this case, efficient amounts of final Cl^- efflux were crucial for resolution and sensitivity of the assay.

We first tested Cl^- efflux under different cell conditions. As shown in Fig. 3, the blank CHO cells without TMEM16A overexpression had little Cl^- efflux, even in the presence of a CaCC activator, E_{act} , indicating that the CHO cells have few endogenous CaCCs. This result also indicated that the ionic species, for example Na^+ , K^+ , Ca^{2+} , and Mg^{2+} , and other components, including gluconate and HEPES, present in the medium did not react with free Ag^+ , because no observed precipitation was indicated. Moderate Cl^- efflux, which was

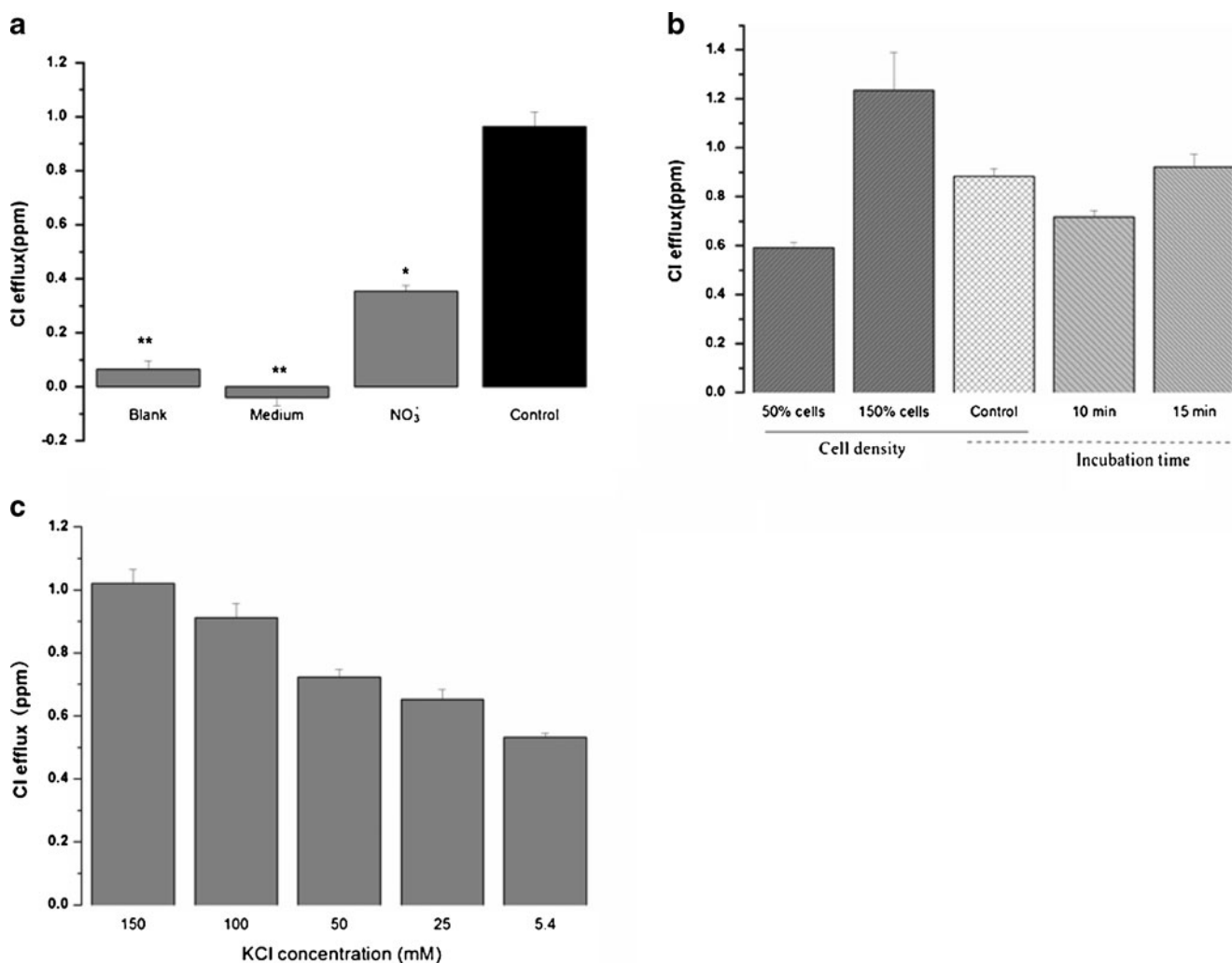
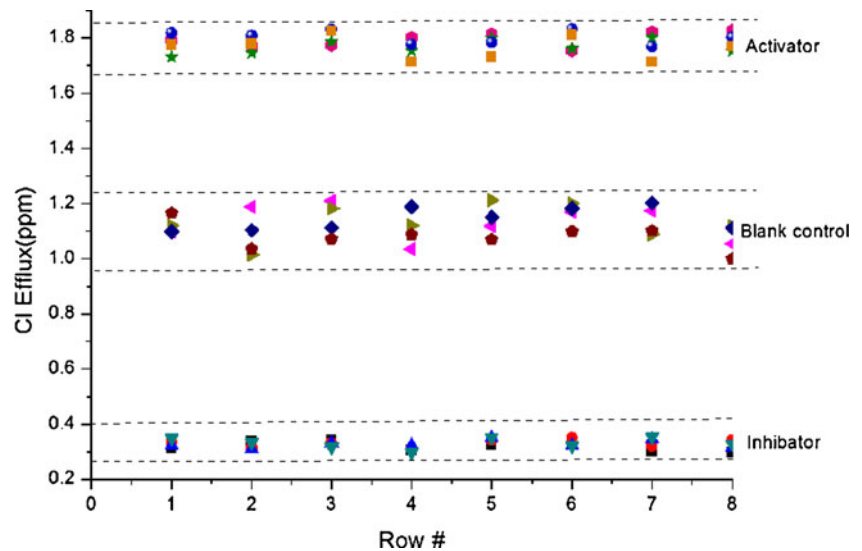


Fig. 4 Optimization of the Cl^- efflux assay. CHO cells stably expressing TMEM16A were used. **(a)** Blank (no cell control) and medium (no cell control, additional 200 μL F-12 K culture medium) did not interfere with the final Cl^- efflux. NO_3^- results indicated that use of nitrate instead of gluconate (control) in Cl^- -free buffer reduced Cl^- efflux significantly. **(b)** Effect of cell density and incubation time on

Cl^- efflux. Control indicated a cell density of 100,000 cells per well (100 % cells), and incubation time of 12 min. **(c)** KCl in the incubation buffer stimulated Cl^- efflux, in a concentration-dependent manner. Values are expressed as means \pm SD ($n=6$), and significance compared with the control was calculated by use of the t -test (* $p<0.05$, ** $p<0.01$)

Fig. 5 Windows of detection in a 96-well plate (12×8 rows). Signals of screening for activator (upper rows, $n=32$), inhibitor (lower rows, $n=32$), and blank control (middle rows, $n=32$)



increased by E_{act} and decreased by a CaCC inhibitor (CaCCinh-A01), was detected from CHO cells stably overexpressing TMEM16A. These results suggest this screening system could be used for studying CaCC/TMEM16A modulators.

Next, to further optimize the conditions for measurement of Cl^- efflux, the effects of cell density, type of anion used for replacement of Cl^- in the Cl^- -free buffer, and concentration of K^+ in the incubation buffer were studied. Although gluconate salts were not ideal for the ICR8000 system, because of their larger viscosity and the difficulty of storing them for long times at room temperature in solution, replacing Cl^- with gluconate salts produced a larger Cl^- efflux than replacement with NO_3^- (Fig. 4a). This is probably because CaCCs have a higher permeability to NO_3^- than to Cl^- , and the resulting

NO_3^- influx reduces Cl^- efflux [31]. As expected, the Cl^- efflux increased with increased cell density and with increased incubation time. After comparison study, we determined the optimum cell density and incubation time to be 100,000 cells $well^{-1}$, cultured for 24 h and incubated for 12 min in the high Cl^- buffer. Although higher cell density and longer incubation time generated stronger signals, it led to poor data reproducibility (see Fig. 4b). The incubation step, which promoted the influx of extracellular Cl^- , was vital to the success of the assay. For this, CHO cells expressing TMEM16A were incubated with 150 $mmol\ L^{-1}$ KCl in the presence of 4 $mmol\ L^{-1}$ Ca^{2+} and 10 $\mu mol\ L^{-1}$ ionomycin. A depolarizing high concentration of KCl increased Ca^{2+} influx. The influx was further increased by the presence of ionomycin [32–34], which

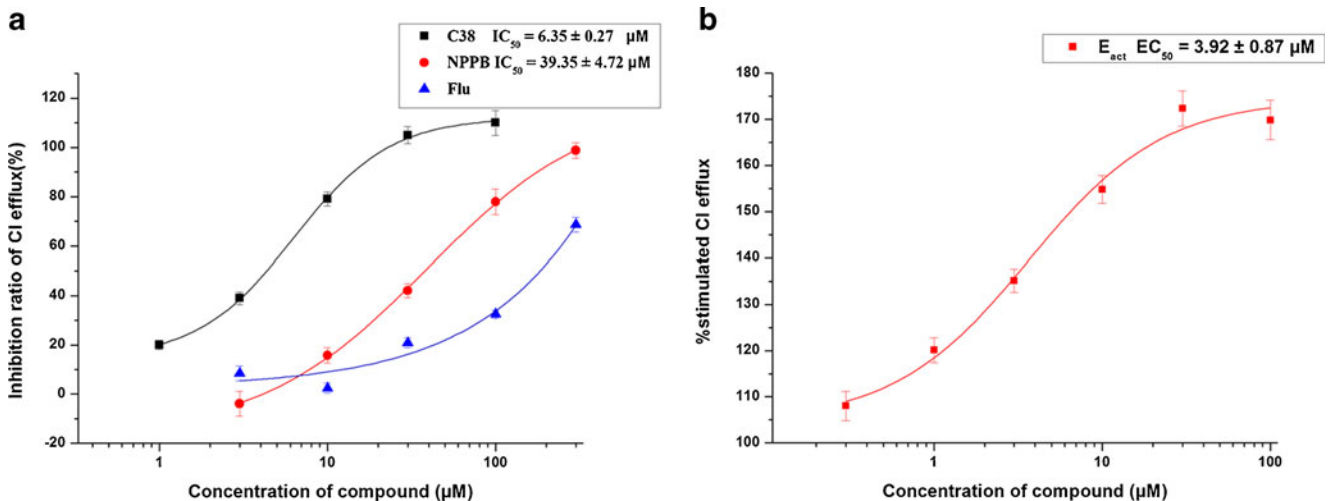


Fig. 6 Effect of known modulators of CaCC/TMEM16A on Cl^- efflux from CHO cells expressing TMEM16A. (a) Effect of CaCC/TMEM16A inhibitors. The maximum inhibitory effect of CaCCinh-A01 (squares)

was used to normalize all the inhibitory effects, including NPPB (circles) and Flu (triangles). (b) Effect of CaCC/TMEM16A activator E_{act} . Values are expressed as means \pm SEM ($n=3$)

increased Cl^- influx by activating CaCC/TMEM16A; also, high extracellular KCl increased the driving force for the Cl^- influx. As clearly shown in Fig. 4c, the final Cl^- efflux was significantly increased when the TMEM16A CHO cells were first incubated with a high (150 mmol L^{-1}) than with a low (5.4 mmol L^{-1}) concentration of KCl. Therefore, an incubation buffer with 150 mmol L^{-1} KCl was used for the rest of the experiments.

Z' factor

Suitability or quality of the screening assay determines confidence in the ability of the HTS assay to screen compounds. This can be assessed by determining the screening window coefficient known as the Z' factor. An assay with a Z' value of 0.5 or higher is designated as an excellent assay. To calculate the Z' factor for our assay, the window of detection was determined after running replicate ($n=32$) experiments for the Cl^- efflux, under the effects of an activator (E_{act}) or an inhibitor (CaCCinh-A01) and under the blank control (basal efflux), in the same 96-well plate (Fig. 5). Z' factor values were calculated to be 0.71 for the inhibitor and 0.62 for the activator, which are both above 0.5, indicating that this assay is a robust assay and can be regarded as suitable for the HTS format.

Validation by comparative study of known modulators of CaCC/TMEM16A

Finally, to further validate confidence in the ability of HTS assay to screen compounds, a few known CaCC/TMEM16A modulators, in five-gradient concentrations (inhibitor) or six-gradient concentrations (activator), and mixed with some

inactive compounds, were studied using this newly developed assay. The results reveal that the known CaCC/TMEM16A modulators reduced or increased the Cl^- efflux as expected, and that their effects were concentration-dependent. In contrast, the inactive compounds had no modulatory activity (data not shown). Of the inhibitors, NPPB and CaCCinh-A01 were found to have IC_{50} of $39.35 \pm 4.72 \text{ } \mu\text{mol L}^{-1}$ and $6.35 \pm 0.27 \text{ } \mu\text{mol L}^{-1}$, respectively; flufenamic acid (Flu) did not inhibit CaCCs completely even at a concentration of $300 \text{ } \mu\text{mol L}^{-1}$ (Fig. 6a). A newly reported CaCC activator, E_{act} , increased the Cl^- efflux, with an EC_{50} value of $3.92 \pm 0.87 \text{ } \mu\text{mol L}^{-1}$ (Fig. 6b). All above values are basically consistent with reported IC_{50} or EC_{50} values from studies using other methods, for example fluorescence plate reader assay [21, 35].

Conclusion

The HTS screening system we establish here, using measurement of free Ag^+ with an automated atomic absorption system (ICR8000 system), can be reliably used to screen and study modulators of CaCC/TMEM16A. This assay proved to be an accurate and robust HTS system for screening and studying CaCC/TMEM16A modulators. The assay can be easily extended to study modulators of other Cl^- channel subtypes, establishing a common technique for studying pharmacological modulation of Cl^- channels using HTS, and thus promoting the discovery of Cl^- channel modulators. Using this system we have found novel CaCC/TMEM16A modulators, and the reliability of these screening results was verified by the best-practice patch-clamp method (Fig. 7).

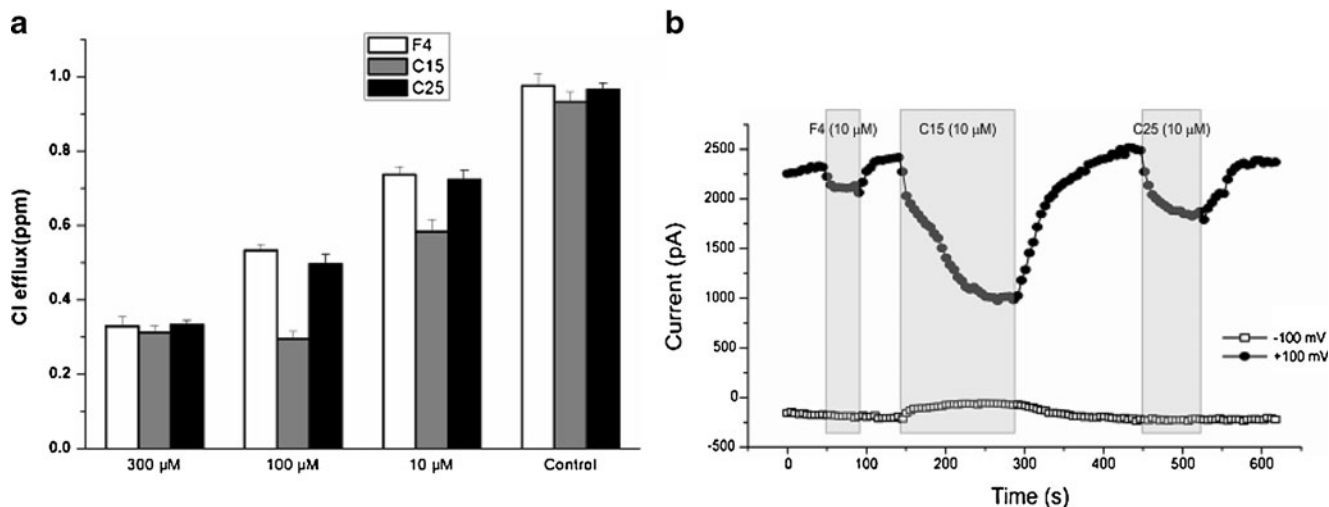


Fig. 7 Discovery of novel modulators of CaCC/TMEM16A. **(a)** Preliminary results from screening of novel modulators by use of the developed efflux assay. The concentration-dependent effects of three tested compounds (F4, C15, C25) on Cl^- efflux are shown, and are compared with the control (no drug). Values are expressed as means \pm SD ($n=3$). **(b)** The

effects of three tested compounds, shown in panel a, on TMEM16A currents assessed by whole-cell patch clamp. The currents of TMEM16A expressed in CHO cells were induced by a ramp voltage from -100 mV to $+100 \text{ mV}$, and the time courses of the TMEM16A currents measured at $+100 \text{ mV}$ (upper line) and -100 mV (lower line) are shown

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