

Development of a QPatch Automated Electrophysiology Assay for Identifying KCa3.1 Inhibitors and Activators

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ABSTRACT

The intermediate-conductance Ca^{2+} -activated K^+ channel KCa3.1 (also known as KCNN4, IK1, or the Gárdos channel) plays an important role in the activation of T and B cells, mast cells, macrophages, and microglia by regulating membrane potential, cellular volume, and calcium signaling. KCa3.1 is further involved in the proliferation of dedifferentiated vascular smooth muscle cells and fibroblast and endothelium-derived hyperpolarization responses in the vascular endothelium. Accordingly, KCa3.1 inhibitors are therapeutically interesting as immunosuppressants and for the treatment of a wide range of fibroproliferative disorders, whereas KCa3.1 activators constitute a potential new class of endothelial function preserving antihypertensives. Here, we report the development of QPatch assays for both KCa3.1 inhibitors and activators. During assay optimization, the Ca^{2+} sensitivity of KCa3.1 was studied using varying intracellular Ca^{2+} concentrations. A free Ca^{2+} concentration of $1\ \mu\text{M}$ was chosen to optimally test inhibitors. To identify activators, which generally act as positive gating modulators, a lower Ca^{2+} concentration ($\sim 200\ \text{nM}$) was used. The QPatch results were benchmarked against manual patch-clamp electrophysiology by determining the potency of several commonly used KCa3.1 inhibitors (TRAM-34, NS6180, ChTX) and activators (EBIO, riluzole, SKA-31). Collectively, our results demonstrate that the QPatch provides a comparable but much faster approach to study compound interactions with KCa3.1 channels in a robust and reliable assay.

INTRODUCTION

The intermediate-conductance Ca^{2+} -activated K^+ potassium channel KCa3.1 is widely expressed throughout the body and found in cells of the hematopoietic system (i.e., erythrocytes, platelets, T cells, B cells, mast cells, monocytes/macrophages, microglia), epithelial tissues in the lung and gastrointestinal tracts, as well as in vascular endothelial cells, fibroblasts, and proliferating neointimal vascular smooth muscle cells.^{1–6} Similar to the related small-conductance KCa2 channels (SK), KCa3.1 channels have fewer charges in their S4 segment than voltage-gated

potassium channels and do not respond to changes in membrane voltage.¹ The channels instead are activated by Ca^{2+} binding to calmodulin,^{7,8} which functions as their β -subunit and induces Ca^{2+} -dependent channel opening with reported Ca^{2+} EC_{50} values for KCa3.1 ranging from 95 to 350 nM.⁹ KCa3.1 channels are accordingly able to hyperpolarize the membrane toward the K^+ equilibrium potential in response to increases in intracellular Ca^{2+} and often modulate Ca^{2+} influx during cellular activation and proliferation by sustaining Ca^{2+} entry through Ca^{2+} -release activated Ca^{2+} (CRAC) or transient receptor potential channels (see reviews by Cahalan and Chandy¹⁰ and Wulff and Castle¹¹). For example, in T lymphocytes, the expression of KCa3.1 channels is known to be upregulated following activation. Genetic deletion as well as pharmacological channel inhibition reduce T-cell Ca^{2+} signaling and inhibit IL-2 production and T-cell proliferation.^{12,13} KCa3.1 is similarly involved in the activation and proliferation of B cells, fibroblasts, and dedifferentiated vascular smooth muscle cells, making KCa3.1 blockers attractive potential drugs for restenosis, asthma, and immunosuppression.^{5,14–18} Based on the role of KCa3.1 in erythrocyte volume regulation and intestinal fluid and electrolyte secretion,^{6,19} KCa3.1 blockers have also been suggested for the treatment of sickle cell anemia and diarrhea in humans and farm animals.^{20,21}

In vascular endothelium, KCa3.1 is expressed together with the small-conductance KCa2.3 channel, and both channels are involved in generating endothelium-derived hyperpolarization (EDH), which then spreads to the underlying vascular smooth muscle cell layer, closes voltage-gated calcium channels, and finally produces relaxation and vasodilation.^{22–25} While mice deficient in KCa3.1 and/or KCa2.3 exhibit impaired EDH responses and an increased mean arterial blood pressure,²⁶ KCa3.1 activators have been shown to lower blood pressure in normotensive and hypertensive mice as well as in dogs, suggesting that KCa3.1 activators could potentially constitute novel antihypertensives.^{27–29}

Taken together, KCa3.1 is an attractive and, based on the fact that groups in both the pharmaceutical industry and academia have identified potent and selective KCa3.1 blockers, highly druggable target (see reviews by Wulff and colleagues^{11,30}). However, the existing compounds have multiple issues, such as short remaining patent-lives, low oral availability, and lack of brain penetration, which would be desirable to target microglial KCa3.1 channels in stroke and Alzheimer's disease.^{31–33} There is, therefore, a need for assays to identify KCa3.1 modulators with different pharmacophores.

Scientists at NeuroSearch A/S in Denmark recently described a 384-well high-throughput Ti^+ -influx assay capable of screening a 200,000 compound library in approximately 6 weeks.³⁴ Here, we report the development of a QPatch-based electrophysiology assay, which is suitable for secondary screening and produces concentration–response curves for both KCa3.1 blockers and activators comparable to manual electrophysiology.

MATERIALS AND METHODS

KCa3.1 Cell Line

HEK293 cells stably expressing hKCa3.1 were obtained from Khaled Houamed (University of Chicago, IL) in 2002 and have been maintained in the Wulff laboratory at the University of California since then. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM, 25 mM glucose) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 1 mM Na^+ pyruvate, 100 units/mL penicillin, 100 $\mu\text{g}/\text{mL}$ streptomycin, and 1 $\mu\text{g}/\text{mL}$ puromycin to maintain selection pressure. Cells were maintained at 37°C in a 5% CO_2 atmosphere and typically passaged when they were 70%–80% confluent.

Cell Preparation

On the day of the experiment, cells grown in T175 tissue culture flasks to ~70% confluency were rinsed once in 15 mL of sterile phosphate-buffered saline containing 0.02% EDTA, and lifted with 2 mL of TrypLE™ Express (Gibco [Life Technologies]) for ~2 min. When cells were rounded but not detached, cells were dislodged from culture flask surface by gentle tapping. Cells were washed and suspended in 10–12 mL DMEM and centrifuged at 125 *g* for 5 min at room temperature. The supernatant was removed and cells resuspended in 2 mL of external solution. A portion of the cells (1 mL) were then placed into the Qfuge tube and resuspended in 150–200 μL extracellular solution after one additional spin on the QPatch. The remaining cells were refrigerated and sometimes used for running a second QPlate.

Test Compounds and Assay Plates

Charybdotoxin was obtained from Bachem Biosciences and prepared as a 100 μM stock solution in full DMEM with 10% serum. TRAM-34 and SKA-31 were synthesized in the Wulff laboratory as previously described.^{27,35} NS6180 was synthesized at NeuroSearch A/S as described.³⁶ Riluzole and 1-ethylbenzimidazolin-2-one (EBIO) were purchased from Sigma. All small molecules were dissolved in dimethyl sulfoxide (DMSO) at concentrations of 1 or 10 mM. Compound solutions were prepared immediately before use on the QPatch by dilution of DMSO stock in external solution. DMSO concentrations ranged from 0.01% to 0.1%. The only exception was EBIO, for which concentrations of up to 1% were necessary to test 25, 75, and 100 μM . (We previously determined that KCa3.1 currents are not affected by 1% of DMSO.²⁷) Charybdotoxin was diluted with external solution containing 0.1% of serum. Glass vial inserts (Sophion Biosciences) were filled with 350–400 μL of compound solution and placed into the glass insert base plate for use in the QPatch assay.

Electrophysiology

Whole-cell patch-clamp experiments were carried out on a QPatch-16 automated electrophysiology platform (Sophion Biosciences) using disposable 16-channel planar patch chip plates (QPlates; patch hole diameter ~1 μm , resistance $2.00 \pm 0.02 \text{ M}\Omega$). Cell positioning and sealing parameters were set as follows: positioning pressure –70 mbar, resistance increase for success 750%, minimum seal resistance 0.1 $\text{G}\Omega$, holding potential –80 mV, holding pressure –20 mbar. To avoid rejection of cells with large KCa3.1 currents, the minimum seal resistance for whole-cell requirement was lowered to 0.001 $\text{G}\Omega$. Access was obtained with the following sequence: (1) suction pulses in 29 mbar increments from –250 mbar to –453 mbar; (2) a suction ramp of an amplitude of –450 mbar; (3) –400 mV voltage zaps of 1 ms duration (10 \times). Following establishment of the whole-cell configuration, cells were held at –80 mV and KCa3.1 currents elicited by a voltage protocol that held at –80 mV for 20 ms, stepped to –120 mV for 20 ms, ramped from –120 to +40 mV in 200 ms, and then stepped back to –120 mV for 20 ms. This pulse protocol was applied every 10 s. The external solution was Na^+ -Ringer's and contained (in mM): 160 NaCl, 10 HEPES, 4.5 KCl, 1 MgCl_2 , 2 CaCl_2 (pH 7.2, 310 mOsm). The internal solution contained (in mM): 120 KCl, 10 HEPES, 1.75 MgCl_2 , 1 Na_2ATP , 10 EGTA and different amounts of CaCl_2 (4.6 for 150 nM; 5.4 for 200 nM and 8.6 for 1 μM) to achieve different concentrations of free Ca^{2+} (pH 7.4, 300 mOsm). Free Ca^{2+} concentrations were calculated using the 7/3/2009 online version of MaxChelator (<http://maxchelator.stanford.edu/webmaxc/webmaxcS.htm>).³⁷

Data Analysis

Current slopes (in amperes per second) were measured using the Sophion QPatch software and exported to Microsoft Excel and Origin 7.0 (OriginLab Corp.) for analysis. Each current slope is the average of three measurements taken at the end of the respective liquid period (marked by double lines in the figures). Increases or decreases of slopes between –85 and –65 mV were used to calculate KCa3.1 inhibition or activation. Data fitting to the Hill equation to obtain EC_{50} and IC_{50} values was performed with Origin 7.0. Data are expressed as mean \pm SD. For activator testing, each cell served as its own maximal control and the increases in current slope were normalized to the effect obtained with a saturating activator concentration applied during the last two liquid periods before the final washout.

RESULTS

KCa3.1 Assay Establishment

We first established the assay conditions using HEK293 cells stably transfected with human KCa3.1. Since KCa3.1 is voltage-independent, our group and many others typically record KCa3.1 currents with linear voltage ramps from –120 mV to +40 mV and sufficient free Ca^{2+} in the intracellular solution to activate these Ca^{2+} /calmodulin-gated channels. With physiological K^+ concentrations in the extracellular solution, KCa3.1 reverses close to the K^+ equilibrium potential, typically between –90 and –80 mV depending on

the exact composition of the solutions and the presence or absence of contaminating chloride background currents in the respective cell line. When manually recording KCa_{3.1} currents with the EPC10 amplifier, we never leak subtract since KCa_{3.1} produces sizable inward currents, which can reach amplitudes of several nA at -120 mV when fully activated by Ca^{2+} or a positive gating modulator. Seal quality during the experiment is accordingly judged not based on the membrane resistance in $\text{M}\Omega$ (which becomes inaccurate with large inward currents at negative potentials) but based on the position of the reversal potential. Any movement of the reversal potential to more positive values than -80 mV is taken as an indication of the cell getting leaky. We kept these observations in mind for adaptation to automated electrophysiology and set the minimum seal resistance for sealing to 0.1 $\text{G}\Omega$ but lowered the minimum seal resistance requirement during the ongoing whole-cell experiment to 0.001 $\text{G}\Omega$ to prevent the software from automatically terminating cells with large KCa_{3.1} currents due to activator action or high Ca^{2+} concentrations. The success rate of QPatch recording for KCa_{3.1} varied significantly in our hands, from 90% seal and 70% completion rate to occasional near complete failure. Since we kept the more easily controllable factors, such as cell density, internal and external solution composition, pH, and osmolarity constant, we attribute these failures, which also routinely occur for manual KCa channel patching in our hands, primarily to the fact that sealing and recording with internal solutions containing elevated concentrations of free intracellular Ca^{2+} requires the cells to be in absolute “prime” conditions. In contrast, success rates for KF containing internals were much more consistent in our hands and hardly showed any daily variation (data not shown).

As expected, current amplitudes varied depending on the amount of free Ca^{2+} in the internal solution (Fig. 1). With 150 nM of free Ca^{2+} , only small KCa_{3.1} currents were detectable, which exhibited maximal amplitudes of 300 ± 198 pA at $+40$ mV within 100 ± 106 s after break-in ($n = 20$). With slightly higher free Ca^{2+} concentrations of 200 nM or 250 nM more sizable KCa currents developed in keeping with the steep Ca^{2+} -concentration-response curve of KCa_{3.1} (4.456 ± 4.554 nA, time to $I_{\text{max}} = 98.5 \pm 65$ s, $n = 20$), which typically stabilized after 3–8 min and 1–3 saline additions (Fig. 1C, D). With a higher intracellular-free Ca^{2+} concentration of 1 μM , KCa_{3.1} currents developed rapidly (time to $I_{\text{max}} = 21 \pm 35$ s, $n = 20$), reached average amplitudes of 14.056 ± 7.723 nA at $+40$ mV, and stabilized after just a few pulses in most cells (Fig. 1B).

According to these different current kinetics and amplitudes, we developed different application protocols for blocker and activator

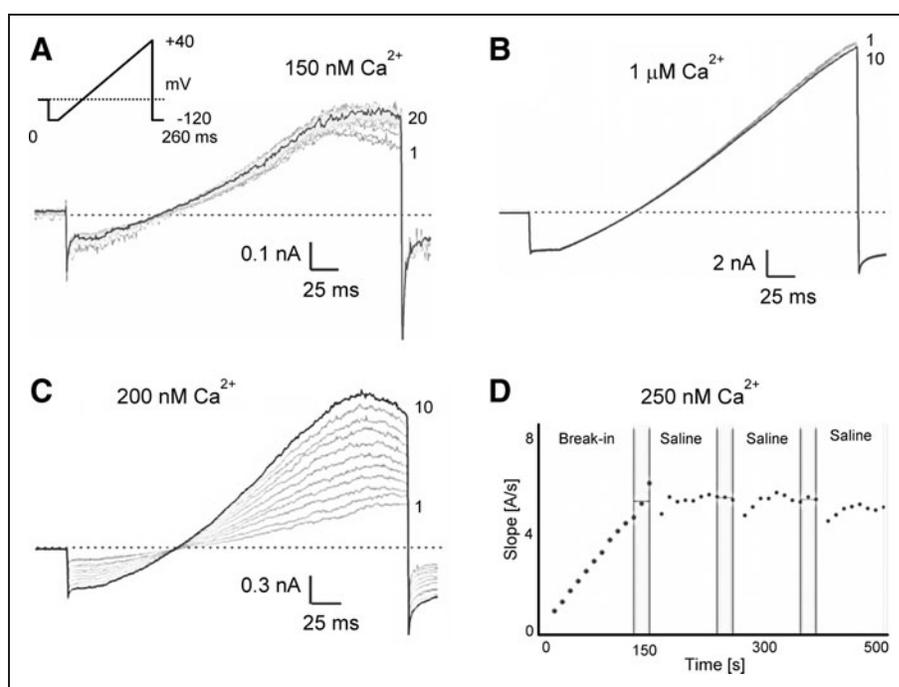


Fig. 1. KCa_{3.1} current amplitudes depend on the amount of free Ca^{2+} in the internal solution. **(A)** Current development following break-in with 150 nM free Ca^{2+} . **(B)** Much more rapid development of larger currents with 1 μM internal Ca^{2+} . **(C)** Current development following break-in with 200 nM free Ca^{2+} . **(D)** Plot of current slope (measured between -85 and -65 mV) versus time during the first 500 s after break-in with 250 nM of free Ca^{2+} . Vertical bars mark the window at the end of the liquid period from where three current slope values were averaged for subsequent calculations. KCa_{3.1}, intermediate-conductance Ca^{2+} -activated K^+ channel.

testing. Based on our more than 10 years of manual patch-clamp experience with high intracellular Ca^{2+} concentrations when testing KCa_{3.1} blockers on transfected human embryonic kidney (HEK) or COS cells or characterizing KCa_{3.1} in primary cells,^{5,12,14,27,33,35} we decided to design a relatively short application protocol for KCa_{3.1} blockers since cells rarely withstand 1 μM of free Ca^{2+} for more than 20–30 min. However, based on the attractively large assay window that control currents activated by 1 μM of Ca^{2+} produce even in the single hole mode in our assay, we decided against using lower Ca^{2+} concentrations for blocker testing. Initial break-in was followed by a short stabilization, one saline addition and then three cumulative additions of increasing concentrations of KCa_{3.1} blockers and then two saline additions for washout (Table 1).

For KCa_{3.1} activator testing, we picked 250 nM of free intracellular Ca^{2+} to allow these positive gating modulators, which increase the apparent Ca^{2+} sensitivity of KCa channels by stabilizing the interaction between calmodulin and the channel,³⁸ a sufficiently large signal window. However, since KCa_{3.1} takes some time to turn on with lower calcium concentrations and we often observe when patching manually that mechanically disturbing the cell with repeated perfusions helps with obtaining full access and presumably with Ca^{2+} diffusion through the cell, we designed a longer application protocol than for the blockers. Three saline perfusions were

Table 1. Application Protocol for KCa3.1 Blocker Testing on the QPatch

Liquid Period	Liquid	Volume (μL) ^a	VP runs
1	Saline	15	10
2	Concentration 1	15	10
3	Concentration 2	15	10
4	Concentration 3	15	10
5	Saline	15	10
6	Saline	15	10

The VP protocol consisted of 260-ms voltage ramps applied every 10 s.
^aThe volume of the recording chamber on the QPatch is $\sim 1 \mu\text{L}$.
 KCa3.1, intermediate-conductance Ca^{2+} -activated K^+ channel; VP, voltage protocol.

followed by two additions of a low concentration of activator, two saline washes, two or three consecutive additions of higher activator concentrations (including a concentration producing maximal activation), and three additions of saline for washout (Table 2). This long protocol was chosen to achieve the following three objectives. (1) The control current is really fully developed and has stabilized. Calcium in the intracellular solution promotes resealing and lower concentrations in the range of 200–500 nM free Ca^{2+} typically require sometime to really diffuse through the cell. When manually patching KCa3.1, we have therefore often observed highly variable control currents that increased as access to the cell improved. (2) The effect of the activator is “real” and not just an increase in access or a reduction in Ca^{2+} perfusion barriers as a consequence of the mechanical disturbance from the perfusion. We therefore included two saline washes after the first activator concentration and three saline washes at the end of the experiment. (3) The absolute current increases measured as fold-increases for KCa3.1 activators are highly dependent on the exact free intracellular Ca^{2+} concentration because of KCa3.1’s steep Ca^{2+} dependence. When patching manually, we therefore previously found that it is necessary to average data from many cells for fitting EC_{50} s. As an alternative, we here developed a protocol in which each cell has its own maximum control at the highest activator concentration. This is of course only possible with previously characterized compounds known to be “full agonists.” For compounds from a chemically new series working with fold-increases or bench-marking against a saturating concentration of EBIO or SKA-31 would be more appropriate.

Evaluation of Assay Performance for KCa3.1 Blockers and Activators

To compare the QPatch assay to manual electrophysiology, we decided to obtain concentration–response curves for several widely used KCa3.1 modulators and selected three blockers and three activators for which we had previously determined IC_{50} and EC_{50} values manually. As exemplary blockers, we chose the scorpion toxin peptide

Table 2. Application Protocol for KCa3.1 Activator Testing on the QPatch

Liquid Period	Liquid	Volume (μL) ^a	VP runs
1	Saline	15	10
2	Saline	15	10
3	Saline	15	10
4	Concentration 1	15	10
5	Concentration 1	15	10
6	Saline	15	10
7	Saline	15	10
8	Concentration 2	15	10
9	Concentration 2	15	10
10	Concentration 3	15	10
11	Concentration 3 ^b	15	10
12	Saline	15	10
13	Saline	15	5
14	Saline	15	5

The VP protocol consisted of 260-ms voltage ramps applied every 10 s.
^aThe volume of the recording chamber on the QPatch is $\sim 1 \mu\text{L}$.
^bSometimes a fourth concentration of activator was used and the number of VP runs per liquid period reduced to 5 or 7 in the later parts of the protocol.

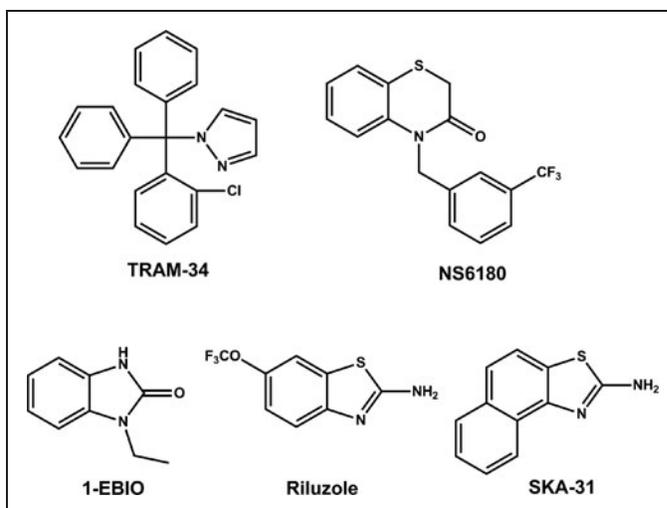


Fig. 2. Chemical structures of small molecule KCa3.1 blockers and activators. TRAM-34, 5-[(2-chlorophenyl)(diphenyl)methyl]-1H-pyrazole; NS6180, 4-[[3-(trifluoromethyl)phenyl]methyl]-2H-1,4-benzothiazin-3(4H)-one; SKA-31, naphtho[1,2-d]thiazol-2-ylamine; EBIO, 1-ethylbenzimidazolin-2-one.

charybdotoxin (ChTX) and the small molecules TRAM-34³⁵ and NS6180.³⁹ Similar to clotrimazole and senicapoc (ICA-17043),^{40,41} TRAM-34 is a “classic” triarylmethane-type KCa_{3.1} blocker, whereas NS6180 is a recently described benzothiazinone (Fig. 2). Both TRAM-34 and NS6180 are potent, with reported IC₅₀ values in the range of 11–25 nM,^{35,39} lipophilic and highly plasma protein-bound. The two compounds accordingly seemed good choices for exploring if our assay exhibits any of the right-shifting effect on the concentration–response curves often observed for “sticky” lipophilic compounds in

automated assays. As activators (Fig. 2), we selected the “classic” KCa-activator 1-EBIO, the neuroprotectant riluzole and the more potent riluzole derivative SKA-31, which was developed in our laboratory as a more selective KCa_{3.1}/KCa₂ channel activator.²⁷

As shown in Figure 3, KCa_{3.1} currents typically stabilized soon after break-in or after saline application when using 1 μM of free internal calcium (see Fig. 3C for an example of a cell where saline perfusion establishes full access). Subsequent applications of increasing concentrations of charybdotoxin (Fig. 3A), TRAM-34 (Fig.

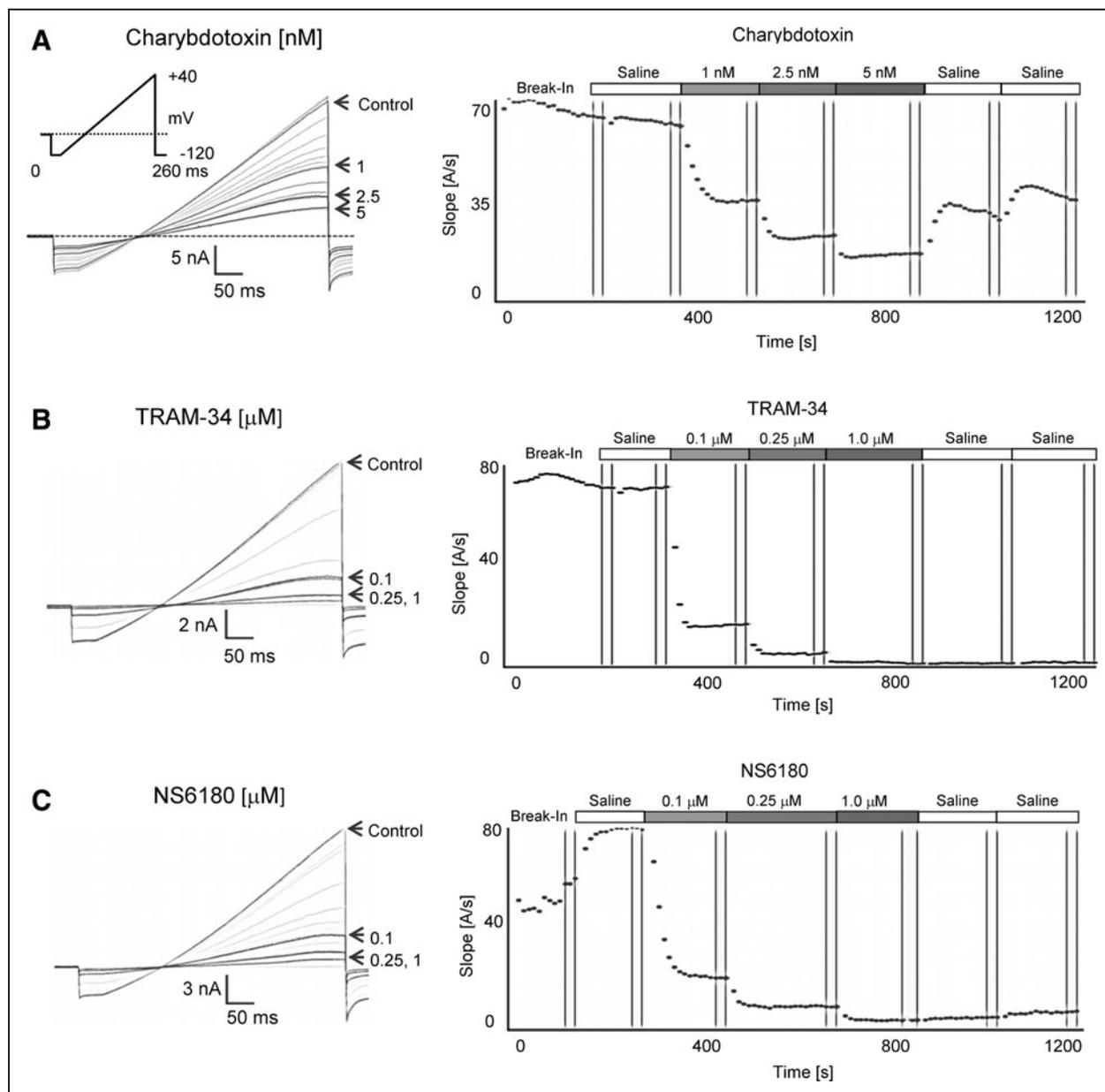


Fig. 3. KCa_{3.1} blocker testing with 1 μM of free Ca²⁺ in the internal solution. **(A)** Raw current traces and plot of current slope (measured between –85 and –65 mV) versus time for KCa_{3.1} block by charybdotoxin (1, 2.5, and 5 nM). **(B)** Effect of TRAM-34 (0.1, 0.25, and 1 μM). **(C)** Effect of NS6180 (0.1, 0.25, and 1 μM).

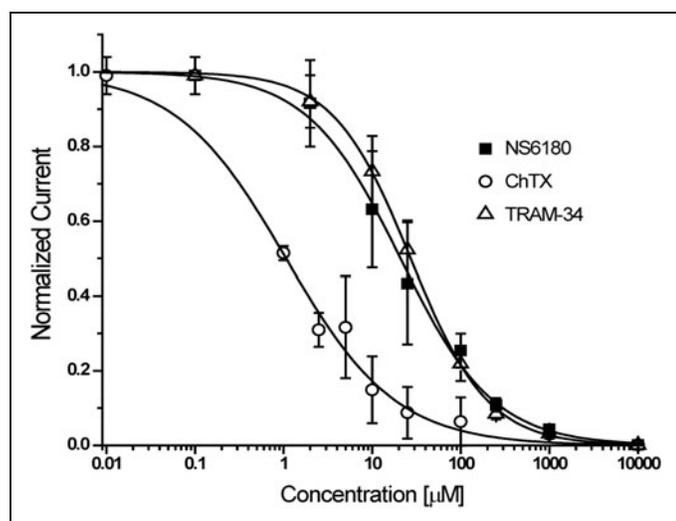


Fig. 4. Concentration–response curves for KCa_{3.1} blockers. Plots are normalized current slopes measured between -85 and -65 mV versus compound concentration. ChTX: $IC_{50} = 1.1 \pm 0.2$ nM, $n_H = 0.7$ ($n = 3$ per concentration point); TRAM-34: $IC_{50} = 27.2 \pm 0.6$ nM, $n_H = 1.0$ ($n = 5$ per concentration point); NS6180: $IC_{50} = 20.9 \pm 1.7$ nM, $n_H = 0.83$ ($n = 5$ per concentration point). Data are given as mean \pm SD.

3B), or NS6180 (Fig. 3C) resulted in a rapid and concentration-dependent block of current. Although charybdotoxin could be partially washed out with two saline applications (Fig. 3A), current inhibition by high concentrations of TRAM-34 and NS6180 could not be reversed despite the fact that each saline application of $15 \mu\text{L}$ theoretically exchanges the bath volume of $\sim 1 \mu\text{L}$ about 15 times. Sigmoidal concentration–response curve fitting rendered IC_{50} values of 1.1 ± 0.2 nM for charybdotoxin, 27.2 ± 0.6 nM for TRAM-34, and 20.9 ± 1.7 nM for NS6180 (Fig. 4).

For KCa_{3.1} activator testing, the longer application protocol shown in Table 2 was used. Following stabilization of the current during three saline applications, the same activator concentration was typically applied twice and a washout was included after the first compound application (Fig. 5). Activator effects became visible shortly after application and reversed completely on washout even after two or three cumulative additions of increasing concentrations. As described above, a saturating concentration of activator was used last to obtain a “full effect” value for normalization purposes. The fastest washout was observed for EBIO, the most soluble and least potent of the tested activators (see Fig. 5C washout out $100 \mu\text{M}$ EBIO). Sigmoidal concentration–response curve fitting rendered Hill coefficients close to 2 and EC_{50} values of 226 ± 31 nM for SKA-31, $1.2 \pm 0.4 \mu\text{M}$ for riluzole, and $9.6 \pm 2.0 \mu\text{M}$ for EBIO (Fig. 6).

DISCUSSION

Using the QPatch-16 system, we developed a medium-throughput automated electrophysiology assay for secondary testing of KCa_{3.1} blockers and activators. In contrast to the many publications describing high-content automated electrophysiology assays for sec-

ondary screening and mechanism of action studies of voltage-gated potassium, sodium, and calcium channels, there are currently very few reports of such assays for voltage-independent calcium-activated potassium channels. In fact, the only comparable assays we are aware of are a study from Korsgaard *et al.* at NeuroSearch A/S describing the use of the QPatch for high-content screening on Nav, Kv11.1 (hERG) and small-conductance KCa_{2.3} channels⁴² and an advertisement for a KCa_{3.1} assay using CHO cells and $10 \mu\text{M}$ of free internal Ca^{2+} on the Chantest website. Other previously reported assays for KCa_{3.1} or the related small-conductance KCa₂ channels have used either Rb^+ efflux analyzed by atomic absorption spectroscopy⁴³ or Tl^+ influx triggered by a Ca^{2+} ionophore.^{34,44,45} There further is one report of using the 384-well IonWorks platform in the multi-hole population patch-clamp mode for screening of KCa_{3.1} and KCa_{2.3} activators.⁴⁶ Although very useful for high throughput screening, these assays only allow for a limited number of solution exchanges and tend to show right-shifts in the potency of lipophilic compounds, like TRAM-34, compared to manual electrophysiology.

The automated assay described here produced high-quality KCa_{3.1} electrophysiology data comparable to manual whole-cell patch-clamp. For the KCa_{3.1} blockers, charybdotoxin (1.1 ± 0.2 nM), TRAM-34 (27.2 ± 0.6 nM) and NS6180 (20.9 ± 1.7 nM), our assay rendered IC_{50} values similar to the ones previously reported for these compounds in manual patch-clamp (Table 3). The peptide toxin charybdotoxin had been found to block native KCa_{3.1} currents in T cells or transiently expressed human or mouse KCa_{3.1} in COS or HEK cells with IC_{50} values ranging from 2.5 to 5 nM in manual recordings,^{12,47,48} while previously reported IC_{50} values for the small molecules TRAM-34 and NS6180 ranged from 20 to 25 nM or 8 to 20 nM, respectively.^{35,36} While the two lipophilic small molecules were slightly less potent than in manual electrophysiology, charybdotoxin was slightly more potent. The latter observation can likely be explained by the fact that we used a new batch of lyophilized charybdotoxin that was only reconstituted 1 h before the assay and then used immediately for preparing the assay plate. We also would like to point out here, that all our assays were performed in glass inserts with freshly prepared compound dilutions and that assay plates were typically used within 1 h of preparation. This practice probably contributed to the tight correlation between manual and automated electrophysiology we observed on the QPatch. For the notoriously sticky TRAM-34 population, patch-clamp on the IonWorks Quattro⁴⁶ or Tl^+ flux³⁴ had reported 15–35 times higher IC_{50} values in the range of 300–700 nM. NS6180 had also been found to have a lower IC_{50} in Tl^+ flux³⁴ (80 nM vs. 8–20 nM in electrophysiology), but the difference was not as striking as with the triarylmethane TRAM-34.

Similar to the KCa_{3.1} blocker testing, the QPatch assay also rendered potencies for KCa_{3.1} activators that were in good agreement with previously reported EC_{50} values from manual electrophysiology. With the exception of the KCa_{2.1} selective GW542573x, which seems to have some direct channel opening properties independent of Ca^{2+} ,⁴⁹ most known KCa activators act as positive gating modulators by increasing the apparent Ca^{2+} sensitivity of channel activation^{44,50–52} and require intracellular Ca^{2+} levels to be at least slightly

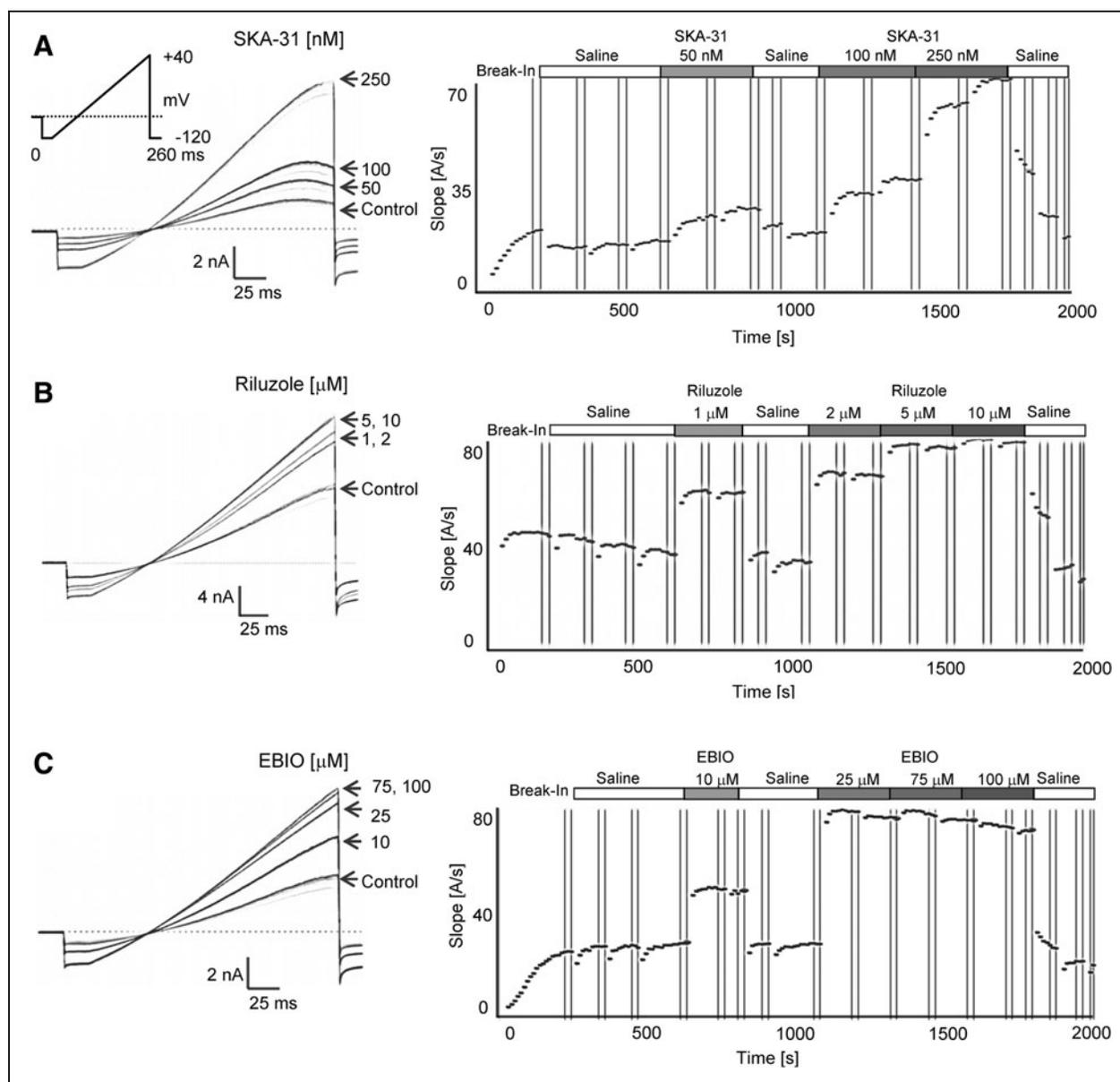


Fig. 5. KCa_{3.1} activator testing with 250 nM of free Ca²⁺ and 1 mM ATP in the internal solution. **(A)** Raw current traces and plot of current slope (measured between -85 and -65 mV) versus time for KCa_{3.1} activation by SKA-31 (50, 100, and 250 nM). **(B)** Effect of riluzole (1, 2, 5, and 10 μ M). **(C)** Effect of EBIO (10, 25, 75, and 100 μ M).

above resting Ca²⁺ levels to exert their effects. In keeping with this requirement, we found that internal Ca²⁺ concentrations in the range of 50–150 nM were too low and resulted in very high assay variability (data not shown). Cells either did not exhibit any perceivable control currents making it impossible to judge whether the activators were ineffective or the cells simply had no current. Or, if they exhibited perceivable control currents, activators elicited very varied responses and the same compound concentration could sometimes increase currents between 5- and 80-fold. This observation is in line with the high variability reported for KCa_{3.1} activators on the IonWorks in the single-hole mode.⁴⁶ Since the IonWorks uses the amphotericin-

perforation method, intracellular free Ca²⁺ concentrations in the assay reported by John *et al.* could not be controlled and were estimated to be in the range of 100 nM or lower despite 2 mM of unbuffered Ca²⁺ in the internal solution.⁴⁶ This low intracellular Ca²⁺ resulted in a variability that was too high for dose-response curve fitting in the single-hole mode and could only be overcome by switching to the multi-hole mode and averaging the signal from up to 64 cells. In our case, we decided to use 250 nM of intracellular free Ca²⁺ and 1 mM of ATP to obtain sufficiently large and reliable control currents. Using these conditions, the QPatch assay reproduced the Hill coefficient of 2 generally reported for KCa_{2/3}

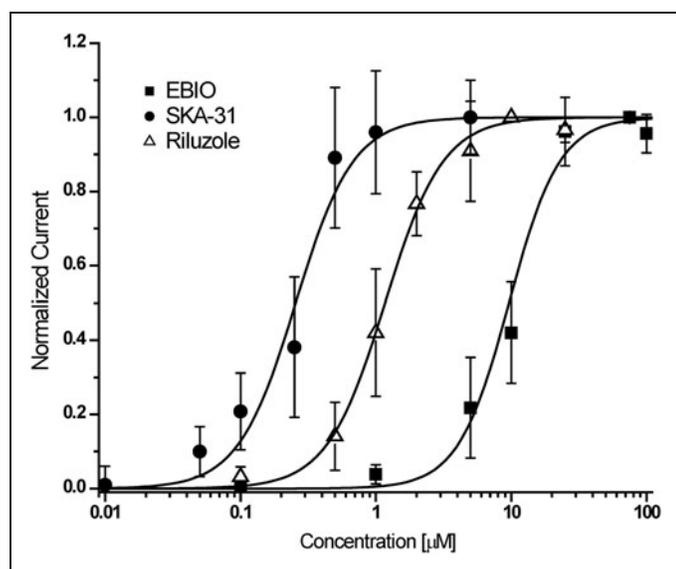


Fig. 6. Concentration–response curves for KCa_{3.1} activators. Plots are normalized current slopes measured between -85 and -65 mV versus compound concentration. SKA-31: $EC_{50} = 226 \pm 31$ nM, $n_H = 2.1$, ($n = 6$ per concentration point); riluzole: $EC_{50} = 1.2 \pm 0.4$ μ M, $n_H = 2.0$ ($n = 5$ per concentration point); EBIO: $EC_{50} = 9.6 \pm 2.0$ μ M, $n_H = 1.9$ ($n = 6$ per concentration point). Data are given as mean \pm SD.

channel activators^{27,42,44,51,52} and rendered EC_{50} values for SKA-31 (226 nM), riluzole (1.2 μ M), and EBIO (10 μ M), which are close to the values previously reported for KCa_{3.1} by manual whole-cell patch-clamp with 250 nM of free Ca^{2+} in the pipette solution (SKA-31: 260 ± 40 nM; riluzole: 1.9 ± 0.3 μ M; EBIO: ~ 20 μ M).^{27,30} ATP was added to prevent KCa_{3.1} run-down. However, we found that an ATP concentration of 4 mM, the amount usually used on the QPatch when

recording hERG (Kv11.1), is too high and elicited KCa_{3.1} currents with amplitudes of 10–20 nA at +40 mV even when used in combination with just 250 nM of free internal Ca^{2+} . This effect, which does not occur with KCa_{2.3} channels (data not shown) and therefore is not due to an alteration of the free Ca^{2+} concentration by the ATP addition, is most likely caused by an increase in KCa_{3.1} channel open probability following phosphorylation.^{53,54} KCa_{3.1} activity is known to be increased by protein kinase A⁵⁵ and nucleoside diphosphate kinase B (NDPK-B)⁵⁶ and inhibited by the histidine phosphatase PHPT1.⁵⁷ NDPK-B and PHPT1 directly phosphorylate/dephosphorylate KCa_{3.1} on histidine 358 in the C-terminus making KCa_{3.1} one of the rare examples of a histidine kinase/phosphatase regulated protein in mammals. Similar to the calmodulin-mediated Ca^{2+} activation of KCa_{3.1}, ATP-mediated KCa_{3.1} activation seems to have a very high degree of cooperativity with a steep concentration–response curve in the whole-cell mode where kinases and phosphatases are likely to be present, and we therefore settled on 1 mM of ATP as a compromise between having control currents that are too large to leave activators a sufficient assay window and having some ATP present to prevent current rundown.

In summary, we present a QPatch-based electrophysiology assay, which produces concentration–response curves for both KCa_{3.1} blockers and activators comparable to manual electrophysiology. The assay is therefore suitable for secondary screening and if used with varying intracellular Ca^{2+} concentrations should also be useful for mechanism of action studies. The ability of the QPatch to obtain whole-cell access and to effectively dialyze the cell with internal solution allows KCa currents to develop with time courses comparable to manual whole-cell experiments. We here used the assay for KCa_{3.1} modulator testing but would like to suggest that the assay can also be applied to the related small-conductance KCa₂ channels (KCa_{2.1}, KCa_{2.3}, and KCa_{2.3}) as well as to sodium-activated K^+ channels (KCa_{4.1} and KCa_{4.2}) after altering the composition of the internal solution. The assay should also be transferable to the higher throughput QPatch48 without any major adjustments.

Table 3. Comparison of the Half-Maximal Inhibitory and Effective Concentrations Obtained on the QPatch and by Manual Electrophysiology

KCa _{3.1} blocker	IC_{50} QPatch	IC_{50} manual
Charybdotoxin	1.1 ± 0.2 nM	2.5–5 nM ^{12,46,47}
TRAM-34	27.2 ± 0.6 nM	20–25 nM ^{35,36}
NS6180	20.9 ± 1.7 nM	8–20 nM ³⁶
KCa _{3.1} activator	EC_{50} QPatch	EC_{50} manual
SKA-31	226 ± 31 nM	260 ± 40 nM ²⁷
Riluzole	1.2 ± 0.4 μ M	1.9 ± 0.3 μ M ²⁷
EBIO	9.6 ± 2.0 μ M	~ 20 μ M ³⁰

TRAM-34, 5-[(2-chlorophenyl)(diphenyl)methyl]-1H-pyrazole; NS6180, 4-[[3-(trifluoromethyl)phenyl]methyl]-2H-1,4-benzothiazin-3(4H)-one; SKA-31, naphtho[1,2-d]thiazol-2-ylamine; EBIO, 1-ethylbenzimidazol-2-one.

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DISCLOSURE STATEMENT

W.Y. and L.D.L. are full-time employees of Sophion Bioscience.

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Abbreviations Used

DMEM	= Dulbecco's modified Eagle's medium
DMSO	= dimethyl sulfoxide
EBIO	= 1-ethylbenzimidazolin-2-one
EDH	= endothelium-derived hyperpolarization
HEK	= human embryonic kidney
KCa3.1	= intermediate-conductance Ca ²⁺ -activated K ⁺ channel
NDPK-B	= nucleoside diphosphate kinase B
NS6180	= 4-{{3-(trifluoromethyl)phenyl}methyl}-2H-1,4-benzothiazin-3(4H)-one
SKA-31	= naphtho[1,2-d]thiazol-2-ylamine
TRAM-34	= 5-[[2-chlorophenyl](diphenyl)methyl]-1H-pyrazole