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

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**Running Title:** KCa3.1 modulator assay

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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 (EDH) responses in the vascular endothelium. Accordingly, KCa3.1 inhibitors are therapeutically interesting as immunosuppressants and for the treatment of a wide range of fibroproliferative disorder, while 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 μM was chosen to optimally test inhibtitors. To identify activators, which generally act as positive gating modulators, a lower $Ca^{2+}$ concentration (~200 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.

**ABBREVIATIONS:** DMSO, dimethyl sulfoxide; EBIO, 1-ethylbenzimidazolin-2-one; HEK, human embryonic kidney; KCa3.1, intermediate-conductance $Ca^{2+}$-activated $K^+$ channel; NS6180, (4-{[3-(trifluoromethyl)phenyl]methyl}-2*H*-1,4-benzothiazin-3(4*H*)-one); PBS, phosphate buffered saline; SKA-31, (naphtho[1,2-*d*]thiazol-2-ylamine), TRAM, triarylmethane; TRAM-34, (5-[(2-chlorophenyl)(diphenyl)methyl]-1*H*-pyrazole).

## 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 chanels 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 towards 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 (TRP) channels (for reviews see:[10,11]). For example, in T lymphocytes, expression of KCa3.1 channels is known to be up-regulated 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 KCa3.1's role 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 diarhea 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 in academia have identified potent and selective KCa3.1 blockers, highly druggable target (for reviews see:[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 $Tl^+$-influx assay capable of screening a 200,000 compound library in approximately six 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, 1 mM $Na^+$ pyruvate, 100 units/ml penicillin, 100 μg/ml streptomycin and 1 μg/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 PBS containing 0.02% EDTA, and lifted with 2 mL of TrypLE™ Express (Gibco [Life Technologies], Grand Island, NY) 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 0.2 rcf for 5 min at room temperature. The supernantant 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 placed in the fridge and sometimes used for run a second QPlate.

### Test Compounds and Assay Plates

Charybdotoxin was obtained from Bachem Biosciences (King of Prussia, PA) 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 (Ballerup, Denmark) as described.[36] Riluzole and 1-EBIO were purchased from Sigma (St Louis, MO). All small molecules were dissolved in DMSO at concentrations of 1 or 10 mM. Compound solutions were prepared immediately prior to use on the QPatch by dilution of DMSO stock in external solution (DMSO concentration ranged from 0.01 to 1%). Charybdotoxin was diluted with external solution containing 0.1% of serum. Glass vial inserts (Sophion Biosciences, Denmark) 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, Denmark) using disposable 16-channel planar patch chip plates (QPlates; patch hole diameter approximately 1 μm, resistance 2.00 ± 0.02 MΩ). Cell positioning and sealing parameters were set as follows: positioning pressure -70 mbar, resistance increase for success 750%, minimum seal resistance 0.1 GΩ, holding potential -80 mV, holding pressure -20 mbar. In order to avoid rejection of cells with large KCa3.1 currents the minimum seal resistance for whole-cell requirement was lowered to 0.001 GΩ. 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 (10x). 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).

**Data Analysis**

Slope conductances were measured using the Sophion QPatch software and exported to Microsoft Excel and Origin 7.0 (OriginLab Corp. MA) for analysis. Increases or decreases from slopes between -85 and -65 mV were used to calculate KCa3.1 inhibition or activation. Data fitting of $EC_{50}$ and $IC_{50}$ values was performed with Origin 7.0. Data are expressed as mean ± SD.

## 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 KCa3.1 currents with the EPC10 amplifier we never leak subtract since KCa3.1 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 MΩ (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 GΩ but lowered the minimum seal resistance requirement during the ongoing whole-cell experiment to 0.001 GΩ in order to prevent the software from automatically terminating cells with large KCa3.1 currents due to activator action or high $Ca^{2+}$ concentrations. The success rate of QPatch recording for KCa3.1 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 KCa3.1 currents, reaching maximal amplitudes of 100-400 pA at +40 mV were detectable (*Fig. 1A*). With slightly higher free $Ca^{2+}$ concentrations of 200 or 250 nM more sizable KCa currents developed in keeping with the steep $Ca^{2+}$-concentration response curve of KCa3.1, which typically stabilized after 3-8 min and 1-3 saline additions (*Fig. 1C and 1D*). With a higher intracellular free $Ca^{2+}$ concentration of 1 μM, KCa3.1 currents developed rapidly, reached average amplitudes of 10-20 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 testing. Based on our more than 10 years of manual patch-clamp experience with high intracellular $Ca^{2+}$ concentrations when testing KCa3.1 blockers on transfected HEK or COS cells or characterizing KCa3.1 in primary cells,[5, 12, 14, 27, 33, 35] we decided to design a relatively short application protocol for KCa3.1 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 KCa3.1 blockers and then two saline additions for washout (*Table 1*).

For KCa3.1 activator testing we picked 250 nM of free intracellular $Ca^{2+}$ in order 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,[37] a sufficiently large "signal window". However, since KCa3.1 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 followed by two additions of a low concentration of activator, two saline washes, two or three consecutive additions of higher activator concentrations and three additions of saline for washout (*Table 2*).

**Evaluation of Assay Performance for KCa3.1 Blockers and Activators**

In order 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 charybdotoxin (ChTX) and the small molecules TRAM-34[35] and NS6180[38]. Similar to clotrimazole and senicapoc (ICA-17043),[39,40] TRAM-34 is a "classic" triarylmethane-type KCa3.1 blocker, while NS6180 is a recently described benzothiazinone (*Fig. 2*). Both TRAM-34 and NS6180 are potent, with reported $IC_{50}$-values in the range of 11-25 nM,[35,38] 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 KCa3.1/KCa2 channel activator.[27]

As shown in *Fig. 3* KCa3.1 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. 3B*) or NS6180 (*Fig. 3C*) resulted in rapid and concentration-dependent block of current. While 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 μL theoretically exchanges the bath volume of ~1 μl 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 KCa3.1 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. The fastest washout was observed for EBIO, the most soluble and least potent of the tested activators (see *Fig. 5C* washout out 100 μM 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 ± 0.4 μM for riluzole, and 9.6 ± 2.0 μM for EBIO (*Fig. 6*).

## DISCUSSION

Using the QPatch-16 system we developed a medium-throughput automated electrophysiology assay for secondary testing of KCa3.1 blockers and activators. In contrast to the many publications describing high content automated electrophysiology assays for secondary 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 report we are aware of is 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 KCa2.3 channels.[41] Other previously reported assays for KCa3.1 or the related small-conductance KCa2 channels have used either $Rb^+$ efflux analyzed by atomic absorption spectroscopy[42] or $Tl^+$ influx triggered by a $Ca^{2+}$ ionophore.[34, 43,44] There further is one report of using the 384-well IonWorks platform in the multi hole population patch-clamp mode for screening of KCa3.1 and KCa2.3 activators.[45] 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 KCa3.1 electrophysiology data comparable to manual whole-cell patch-clamp. For the KCa3.1 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. The peptide toxin charybdotoxin had been found to block native KCa3.1 currents in T cells or transciently expressed human or mouse KCa3.1 in COS or HEK cells with $IC_{50}$ values ranging from 2.5 to 5 nM in manual recordings,[12,46,47] 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 one hour 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 one hour 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 Quattro[45] or $Tl^+$ flux[34] 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 $Tl^+$ flux[34] (80 nM versus 8-20 nM in electrophysiology) but the difference was not as striking as with the triarylmethane TRAM-34.

Similar to the KCa3.1 blocker testing, the QPatch assay also rendered potencies for KCa3.1 activators that were in good agreement with previously reported $EC_{50}$ values from manual electrophysiology. With the exception of the KCa2.1 selective GW542573X, which seems to have some direct channel opening properties independent of $Ca^{2+}$,[48] most known KCa activators act as positive gating modulators by increasing the apparent $Ca^{2+}$-sensitivity of channel activation[43,49-51] and require intracellular $Ca^{2+}$ levels to be at least slightly above resting $Ca^{2+}$ levels to exert their effects. In keeping with this requirement, we found that internal $Ca^{2+}$ 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 KCa3.1 activators on the IonWorks in the single-hole mode.[45] Since the IonWorks uses the amphotericin-perforation method, intracellular free $Ca^{2+}$ 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 $Ca^{2+}$ in the internal solution.[45] This low intracellular $Ca^{2+}$ 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 $Ca^{2+}$ and 1 mM of ATP in order to obtain sufficiently large and reliable control currents. Using these conditions, the QPatch assay reproduced the Hill coefficient of 2 generally reported for KCa2/3 channel activators[27,41,43,50,51] and rendered $EC_{50}$ values for SKA-31 (226 nM), riluzole (1.2 μM) and EBIO (10 μM), which are close to the values previously reported for KCa3.1 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 μM; EBIO: ~20 μM).[27,30] ATP was added to prevent KCa3.1 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 KCa3.1 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 KCa2.3 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 KCa3.1 channel open probability following phosphorylation.[52,53] KCa3.1 activity is known to be increased by protein kinase A (PKA)[54] and nucleoside diphoshate kinase B (NDPK-B)[55] and inhibited by the histidine phosphatase PHPT1.[56] NDPK-B and PHPT1 directly phosphorylate/dephosphorylate KCa3.1 on histidine 358

in the C-terminus making KCa3.1 one of the rare examples of a histidine kinase/phosphatase regulated protein in mammals. Similar to the calmodulin mediated $Ca^{2+}$ activation of KCa3.1, ATP mediated KCa3.1 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 KCa3.1 blockers and activators comparable to manual electrophysiology. The assay is therefore suitable for secondary screening and 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 KCa3.1 modulator testing, but would like to suggest that the assay can also be applied to the related small-conductance KCa2 channels (KCa2.1, KCa2.3 and KCa2.3) as well as to sodium-activated $K^+$ channels (KCa4.1 and KCa4.2) after altering the composition of the internal solution. The assay should also be transferable to the higher throughput QPatch48 without any major adjustments.

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**DISCLOSURE STATEMENT**

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

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**Table 1.** Application protocol showing the different liquid periods, application volumes and number of voltage protocol (VP) runs for KCa3.1 blocker testing on the QPatch. The VP protocol consisted of 260-ms voltage ramps applied every 10 s.

| **KCa3.1 Blocker Protocol** | | |
|---|---|---|
| **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 |

[a]The volume of the recording chamber on the QPatch is approximately 1 µl.

**Table 2.** Application protocol showing the different liquid periods, application volumes and number of voltage protocol (VP) runs for KCa3.1 activator testing on the QPatch. The VP protocol consisted of 260-ms voltage ramps applied every 10 s.

| **KCa3.1 Activator Protocol** | | |
|---|---|---|
| **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 |

[a]The volume of the recording chamber on the QPatch is approximately 1 µl.

[b]Sometimes a 4$^{th}$ 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.

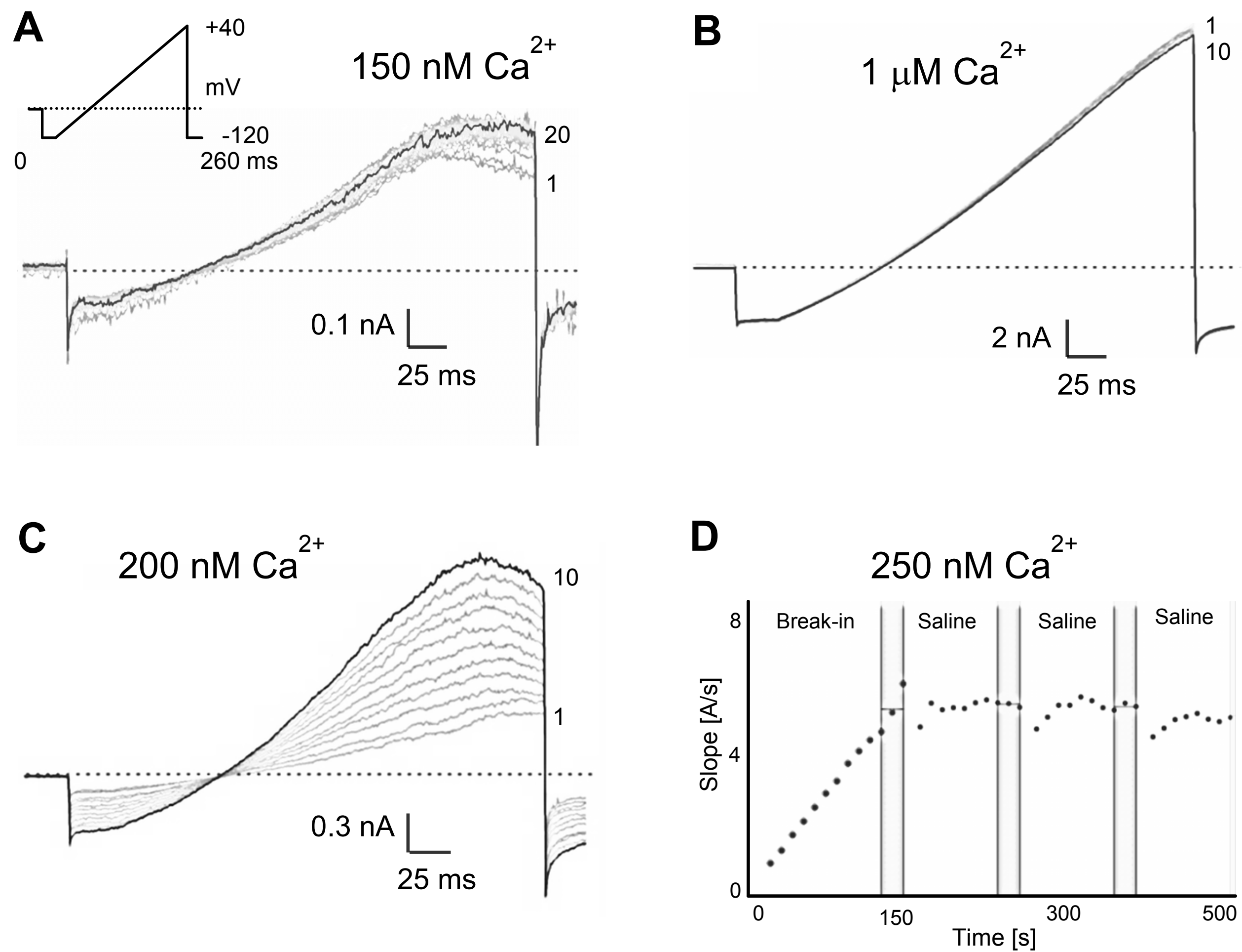


**Fig. 1** KCa3.1 current amplitudes depend on the amount of free $Ca^{2+}$ in the internal solution. **(A)** Current development following break-in with 150 nM free $Ca^{2+}$. **(B)** Much more rapid development of larger currents with 1 µM internal $Ca^{2+}$. **(C)** Current development following break-in with 200 nM free $Ca^{2+}$. **(D)** Plot of slope conductance versus time following the first 500 s after break-in with 250 nM of free $Ca^{2+}$. Vertical bars mark the division between two liquid application periods.

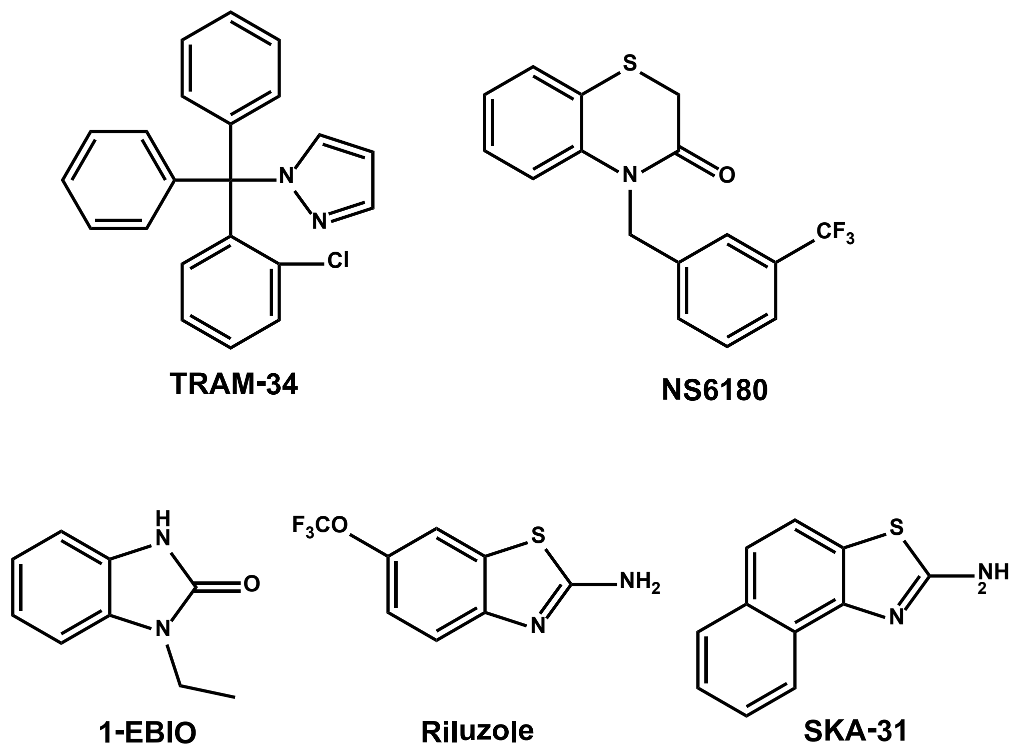


**Fig. 2.** Chemical structures of small molecule KCa3.1 blockers and activators.

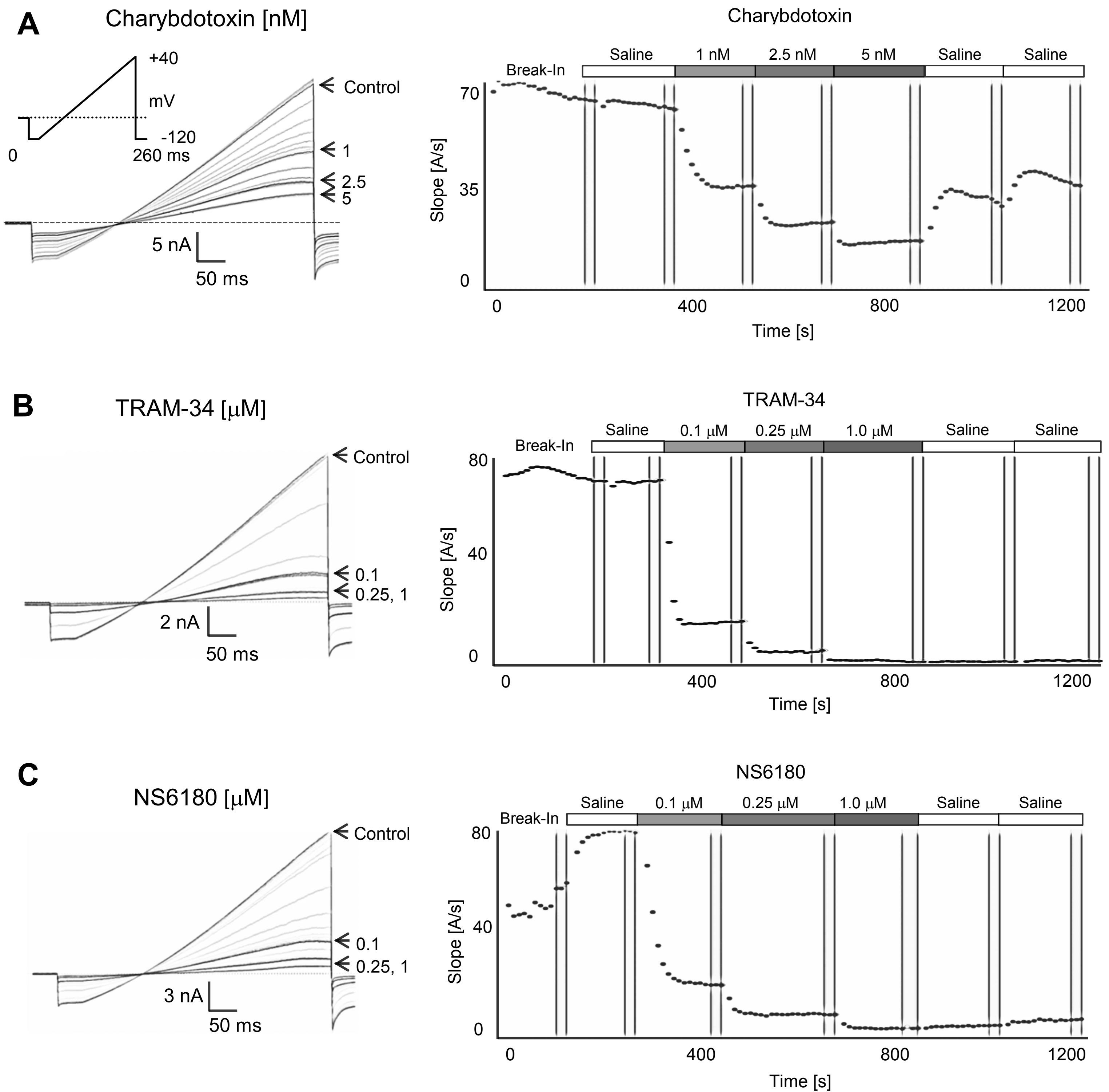


**Fig. 3.** KCa3.1 blocker testing with 1 μM of free $Ca^{2+}$ in the internal solution. **(A)** Raw current traces and plot of slope conductance versus time for KCa3.1 block by charybdotoxin (1, 2.5 and 5 nM). **(B)** Effect of TRAM-34 (0.1, 0.25 and 1 μM). **(C)** Effect of NS6180 (0.1, 0.25 and 1 μM). Vertical bars mark the division between two liquid application periods.

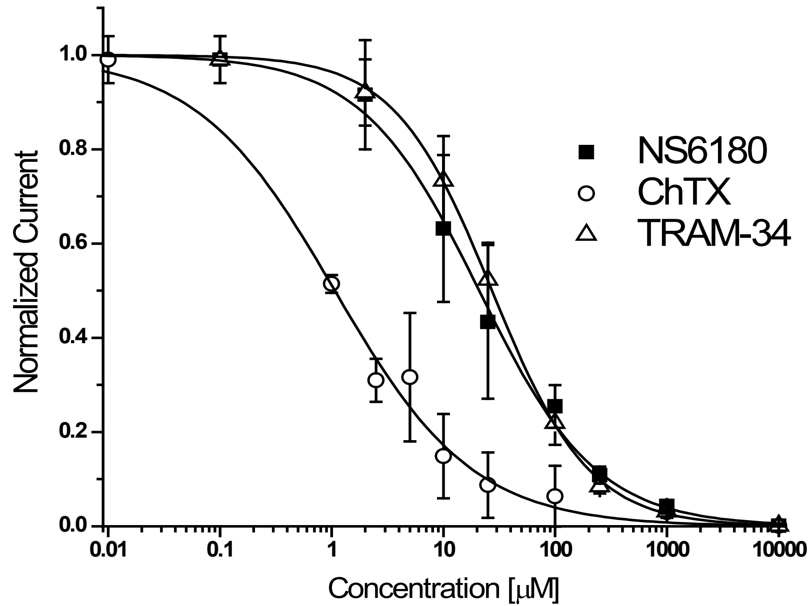


**Fig. 4.** Concentration response curves for KCa3.1 blockers. Plotted is normalized current inhibition measured as reduction of slope conductance. ChTX: $IC_{50}$ = 1.1 ± 0.2 nM, $n_H$ = 0.7, (n = 3 per concentration point); TRAM-34: $IC_{50}$ = 27.2 ± 0.6 nM, $n_H$ = 1.0 (n = 5 per concentration point); NS6180: $IC_{50}$ = 20.9 ± 1.7 nM, $n_H$ = 0.83 (n = 5 per concentration point). Data are given as mean ± SD.

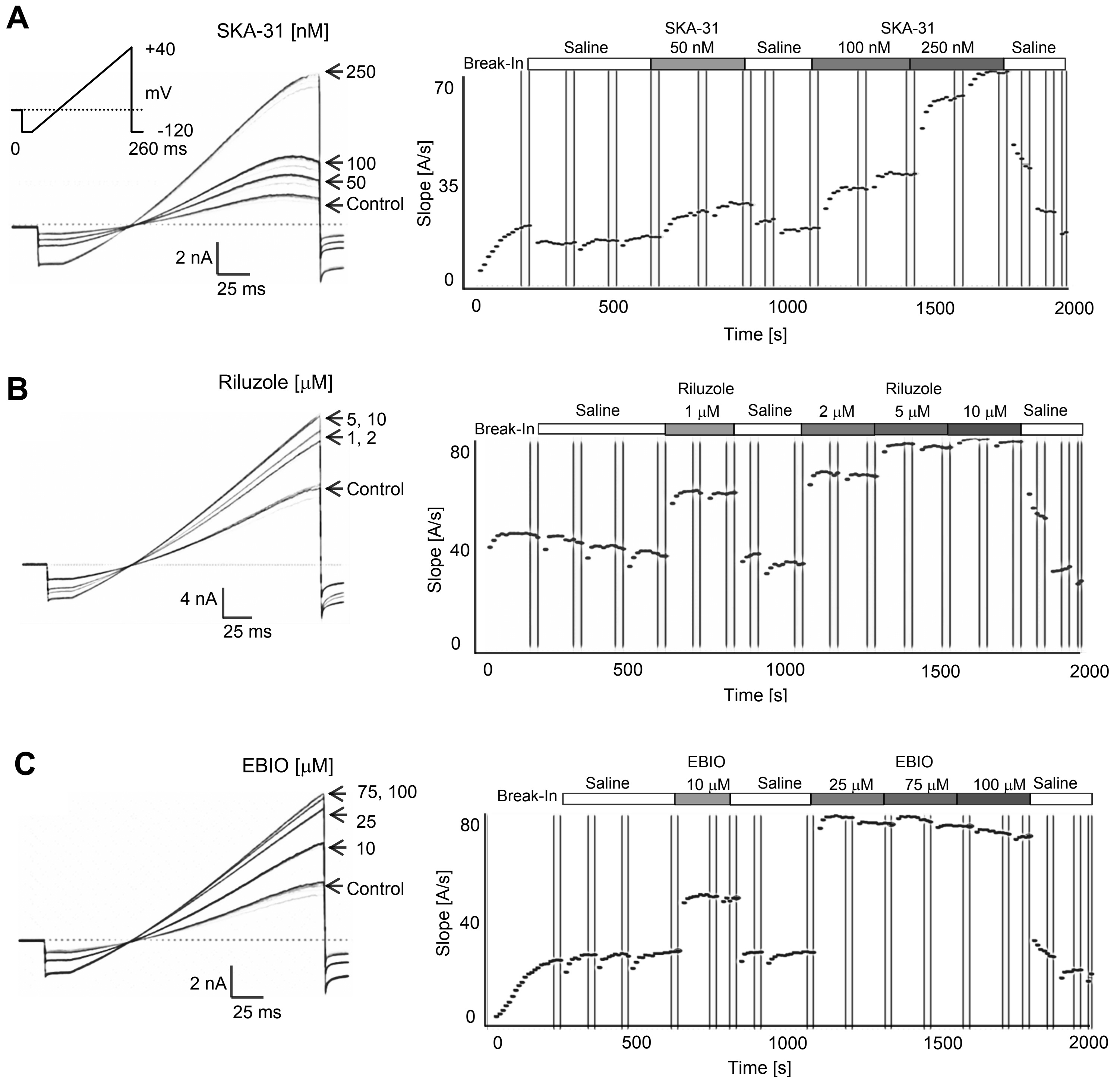


**Fig. 5.** KCa3.1 activator testing with 250 nM of free $Ca^{2+}$ and 1 mM ATP in the internal solution. **(A)** Raw current traces and plot of slope conductance versus time for KCa3.1 activation by SKA-31 (50, 100 and 250 nM). **(B)** Effect of riluzole (1, 2, 5 and 10 μM). **(C)** Effect of EBIO (10, 25, 75 and 100 μM). Vertical bars mark the division between two liquid application periods.

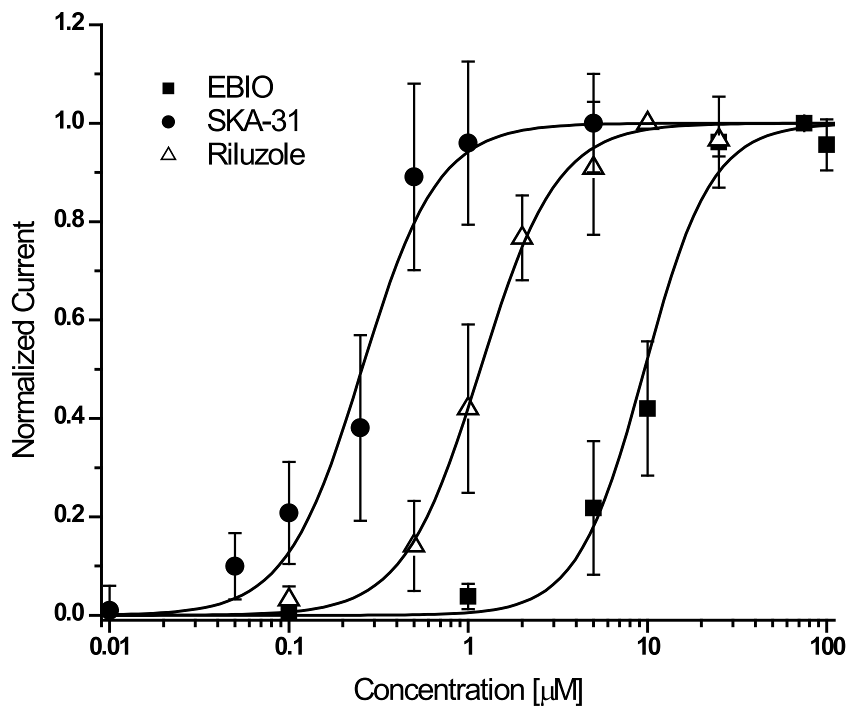


**Fig. 6.** Concentration response curves for KCa3.1 activators. Plotted is normalized increase in slope conductance. SKA-31: $EC_{50}$ = 226 ± 31 nM, $n_H$ = 2.1, (n = 6 per concentration point); riluzole: $EC_{50}$ = 1.2 ± 0.4 μM, $n_H$ = 2.0 (n = 5 per concentration point); EBIO: $EC_{50}$ = 9.6 ± 2.0 μM, $n_H$ = 1.9 (n = 6 per concentration point). Data are given as mean ± SD.