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MOLECULAR PHARMACOLOGY
Vol. 72, No. 4
38331/3256817
Mol Pharmacol 72:1033–1044, 2007
Printed in U.S.A.
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The Small Molecule NS11021 Is a Potent and Specific Activator of Ca^{2+}-Activated Big-Conductance K^+ Channels

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Received May 21, 2007; accepted July 16, 2007

ABSTRACT

Large-conductance Ca^{2+}- and voltage-activated K^+ channels (Kca1.1/BK/MaxiK) are widely expressed ion channels. They provide a Ca^{2+}-dependent feedback mechanism for the regulation of various body functions such as blood flow, neurotransmitter release, uresis, and immunity. In addition, a mitochondrial K^+ channel with KCa1.1-resembling properties has been found in the heart, where it may be involved in regulation of energy consumption. In the present study, the effect of a novel NeuroSearch compound, 1-(3,5-bis-trifluoromethyl-phenyl)-3-[4-bromo-2-(1*H*-tetrazol-5-yl)-phenyl]-thiourea (NS11021), was investigated on cloned KCa1.1 expressed in *Xenopus laevis* oocytes and mammalian cells using electrophysiological methods. NS11021 at concentrations above 0.3 μM activated KCa1.1 in a concentration-dependent manner by parallel-shifting the channel activation curves to more negative potentials. Single-channel analysis revealed that NS11021 increased the open probability of the channel by altering gating kinetics without affecting the single-channel conductance. NS11021 (10 μM) influenced neither a number of cloned Kv channels nor endogenous Na^+ and Ca^{2+} channels (L- and T-type) in guinea pig cardiac myocytes. In conclusion, NS11021 is a novel KCa1.1 channel activator with better specificity and a 10 times higher potency compared with the most broadly applied KCa1.1 opener, NS1619. Thus, NS11021 might be a valuable tool compound when addressing the physiological and pathophysiological roles of KCa1.1 channels.

Large-conductance Ca^{2+}-activated K^+ channels (KCa1.1, BK, MaxiK, hSlo, and KCNMA1 channels) are unique among the family of K^+-selective ion channels being activated by both membrane depolarization and intracellular Ca^{2+}. The sensitivity of KCa1.1 to Ca^{2+} makes it an important negative-feedback system for Ca^{2+} entry in many cell types. KCa1.1 channels are distributed in both excitable and nonexcitable cells and are important for many cellular functions such as neuronal excitability, action potential repolarization, neurotransmitter and hormone release, tuning of cochlear hair cells, innate immunity, transepithelial transport, and regulation of the tone of vascular, uterine, gastrointestinal, airway, and bladder smooth muscle tissue (for review, see Ghatta et al., 2006; Lu et al., 2006). However, predicting the role of KCa1.1 channels in different tissues is difficult. This is probably because of the complex regulation of KCa1.1 channels, which in addition to Ca^{2+} and voltage, also include factors such as phosphorylation state, pH, shear stress, alternative splice variants of the *KCNMA1α* subunit, and the presence or absence of β subunits (Salkoff et al., 2006). In addition, the cellular localization plays a role for the physiological function of KCa1.1 channels. This is exemplified by the KCa1.1-resembling channels located in the mitochondrial inner membrane of cardiomyocytes that are suggested to be involved in cardioprotection (Xu et al., 2002).

Even though the exact function of Kca1.1 can be difficult to foresee, the physiological importance of KCa1.1 has incontrovertibly been emphasized by the KCa1.1 knockout mouse. The KCa1.1-deficient mouse demonstrates incontinency, bladder overactivity, and erectile dysfunction (Meredith et al., 2004; Werner et al., 2005). In addition, a gain-of-function

The work was supported by The Danish National Research Foundation and by a grant from Danish Cardiovascular Research Academy (B.H.B.).
Article, publication date, and citation information can be found at http://molpharm.aspetjournals.org.
doi:10.1124/mol.107.038331.

ABBREVIATIONS: NS1619, 1,3-dihydro-1-[2-hydroxy-5-(trifluoromethyl)phenyl]-5-(trifluoromethyl)-2*H*-benzimidazol-2-one; NS1608, *N*-(3-(trifluoromethyl)phenyl)-*N'*-(2-hydroxy-5-chlorophenyl)urea; BMS-204352, (3*S*)-3-(5-chloro-2-methoxyphenyl)-3-fluoro-6-(trifluoromethyl)-1,3-dihydro-2*H*-indol-2-one; NS11021, 1-(3,5-bis-trifluoromethyl-phenyl)-3-[4-bromo-2-(1*H*-tetrazol-5-yl)-phenyl]-thiourea; HEK, human embryonic kidney; DMSO, dimethyl sulfoxide; KB130015, 2-methyl-3-(3,5-diiodo-4-carboxymethoxybenzyl)benzofuran; diCl-DHAA, 12,14-dichlorodehydroabietic acid; NS-8, 2-amino-5-(fluoro-phenyl)-4-methyl-1*H*-pyrrole-3-carbonitrile.

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mutation in the KCa1.1 gene *KCNMA1* has been reported in humans to result in a syndrome of coexistent generalized epilepsy and paroxysmal dyskinesia (Du et al., 2005).

Because of the profound physiological role of KCa1.1, these channels are appealing as therapeutic targets. Both naturally occurring and chemically synthesized modulatory agents have been identified. Among the inhibitory molecules, the peptide toxin iberiotoxin is regarded as the most specific blocker of KCa1.1 channels. Compounds that activate the KCa1.1 channel would be expected to hyperpolarize the cell membrane potential by enhancing the efflux of K^+ ions and thereby reduce excitability or cause relaxation of smooth muscles (for review, see Ghatta et al., 2006). Nevertheless, activation of KCa1.1 channels has been reported to increase neuronal excitability resulting in epilepsy as demonstrated by Du et al. (2005). This seemingly counterintuitive result is explained by the fact that KCa1.1 channel activation results in faster repolarization and thereby reduced neuronal refractory period. This emphasizes the difficulties in predicting the function of the KCa1.1 channel.

Numerous KCa1.1 channel activators have been identified, including the synthetic benzimidazolone derivatives (e.g., NS1619), the biarylureas (e.g., NS1608), the aryloxindoles (e.g., BMS-204352), the natural modulators (e.g., dihydrosoyasaponin-1) (for review see, Ghatta et al., 2006). Although these agents activate KCa1.1 channels, they also display additional nonspecific effects that may obscure the results when investigating the effect of KCa1.1 channel activation. This is also the case for NS1619, the most frequently used tool compound for studying KCa1.1 channel function, which at higher concentrations also directly inhibits some Ca^{2+} currents and voltage-activated K^+ and Na^+ channels (Olesen et al., 1994; Holland et al., 1996). However, the pioneer KCa1.1 activators have been invaluable for dissecting the physiological roles of KCa1.1 channels.

In the present study, we investigated the effect of a novel KCa1.1 channel activator NS11021 on cloned KCa1.1 channels expressed in *Xenopus laevis* oocytes and HEK293 cells using two-electrode voltage-clamp and patch-clamp techniques. NS11021 activated KCa1.1 channels in a concentration-dependent manner at micromolar concentrations by shifting the conductance-voltage relationship to more hyperpolarized potentials. NS11021 activated the KCa1.1 channel when applied from either side of the membrane and had the ability to bind to the channel in both open and closed conformation. NS11021 parallel-shifted the activation curves toward negative potentials at all internal Ca^{2+} concentrations ranging from "Ca^{2+}-free" up to 100 μM. The compound did not modulate a number of Kv (except Kv7.4), Na^+, and Ca^{2+} currents. Finally, from single-channel measurements NS11021 was found to increase the open probability of the channel by altering the open and closed time constants without affecting single-channel conductance.

In conclusion, NS11021 is demonstrated to activate KCa1.1 channels with approximately 10 times higher potency compared with the most broadly applied KCa1.1 opener NS1619. In addition, NS11021 is more selective compared with NS1619 and might therefore constitute a valuable pharmacological tool to address physiological functions of KCa1.1 channels.

Materials and Methods

Expression in *X. laevis* Oocytes

Female *X. laevis* frogs were anesthetized (2 g/l Tricaine; Sigma, St. Louis, MO) and ovarian lobes cut off through a small abdominal incision. All procedures were done in accordance with Danish National Committee for Animal Studies guidelines. After manual dissection of the oocytes into smaller groups, the oocytes were defolliculated by enzymatic treatment with collagenase (C0130; Sigma-Aldrich, Vallensbaek Strand, Denmark) for 1 h. Oocytes were then kept in Kulori solution (90 mM NaCl, 4 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, and 5 mM HEPES, pH 7.4) at 19°C for 24 h before injection of cRNA. cRNA for injection was prepared from linearized wild-type hKCa1.1, Kv7.1-4, Kir2.1-3, Kv1.4-5, Kv4.3, and Kv11.1 using the T7 mMessage mMachine kit (Ambion, Austin, TX) according to the manufacturer's instructions. RNA concentrations were quantified by UV spectroscopy, and RNA quality was checked by gel electrophoresis. cRNA (50.6 nl, 5–50 ng) was injected using a Nanoject microinjector (Drummond Scientific, Broomell, PA). Oocytes were kept in Kulori solution at 19°C, which was changed daily, and currents were recorded after 2 to 5 days.

Cell Cultures

Monoclonal HEK293 cells stably expressing hKCa1.1 were grown in Dulbecco's modified Eagle's medium and 10% fetal calf serum (Invitrogen, Carlsbad, CA) supplemented with Glutamax (Substrate Department, the Panum Institute, Copenhagen, Denmark) and incubated at 37°C in 5% CO_2. On the day of patch-clamp experiments, the cells were transferred to glass coverslips coated with poly-L-lysine (3.5-mm diameter) and allowed to attach to the coverslip for 1 h.

Electrophysiological Recordings

Two-Electrode Voltage Clamp. The recordings were done at room temperature in Kulori solution using a two-electrode voltage-clamp amplifier (Dagan CA-1B; Chicago, IL). The oocytes were placed in homemade perfusion chambers connected to a continuous flow system and impaled with a current electrode and a voltage-clamp electrode pulled from borosilicate glass (Module Ohm, Herlev, Denmark) on a DMZ-Universal Puller (Zeitz Instruments, Munich, Germany). Recording electrodes were filled with 2 M KCl and had a resistance of 0.5 to 2.5 MΩ. Kulori solution was used for bath solution.

Macroscopic currents of expressed channels were activated by depolarized potentials. The exact voltage protocols are indicated in respective figures.

Single-Channel Recordings. Single-channel currents were recorded from *X. laevis* oocytes expressing hKCa1.1 using the inside-out configuration of the patch-clamp technique. The vitelline membrane was removed with forceps just before the experiments. Pipettes were fabricated from thick-walled borosilicate glass (o.d./i.d. 1.5/0.75 mm) (WPI, Sarasota, FL) and had a resistance of around 5 MΩ. A system for rapid solution changes allowed the application of drugs in the close vicinity of the patch (SmartSquirt; AutoMate Scientific, Berkeley, CA). Currents were recorded using a HEKA EPC9 amplifier (HEKA, Lambrecht/Pfalz, Germany) low pass-filtered at 2 kHz using an eight-pole Bessel filter and sampled at 20 kHz. Single-channel analysis was performed using ClampFit 9 (Molecular Devices, Sunnyvale, CA) on current recordings from patches containing less than four channels. Transitions between open and closed state were determined by setting the threshold at half the unitary current amplitude. Single-channel unitary currents were measured by using all-point histograms fitted with Gaussian distributions of the current records. Single-channel conductance was determined as the slope on unitary current-voltage plots. Mean open probability was corrected for the number of channels in the patch.

Distributions of the open and closed times were logarithmically

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binned, and a square-root transformation of the ordinate (events) was used. The distributions were fitted with an exponential density function by the method of maximum likelihood with either two or three exponentials as suggested by Sigworth and Sine (1987) using Clampfit 9.0. Recordings were performed at room temperature in symmetrical solutions containing 140 mM KCl, 10 mM EGTA, 10 mM HEPES, and 300 nM calculated free Ca^{2+} (EqCal, Biosoft, Cambridge, UK), pH 7.2.

Whole-Cell and Inside-Out Recordings in Mammalian Cells. All experiments were performed at room temperature with an EPC-9 amplifier (HEKA). Pipettes were pulled from borosilicate glass (Module Ohm, Herlev, Denmark) with a resistance between 1.5 and 2.5 MΩ. Coverslips with HEK293 cells stably expressing KCa1.1 channels were transferred to a homemade perfusion chamber mounted on the stage of an inverted microscope. The extracellular solution used consisted of 140 mM NaCl, 4 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, and 10 mM HEPES, pH 7.4. An intracellular solution with 100 nM free calcium was used for whole-cell measurements, whereas intracellular solutions with three different concentrations of calculated free calcium were used for inside-out measurements: 140 mM KCl,

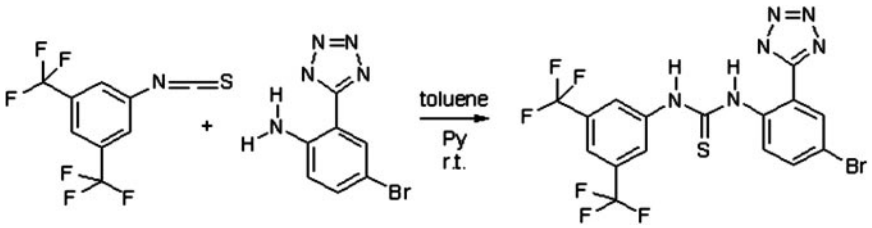

Fig. 1. Synthesis of NS11021. Chemical synthesis of NS11021 involves reaction between commercial 3,5-bis(trifluoromethyl)phenylisothiocyanate and 4-bromo-2-(1*H*-tetrazol-5-yl)-phenylamine (Postovskii and Golomolzin, 1970).

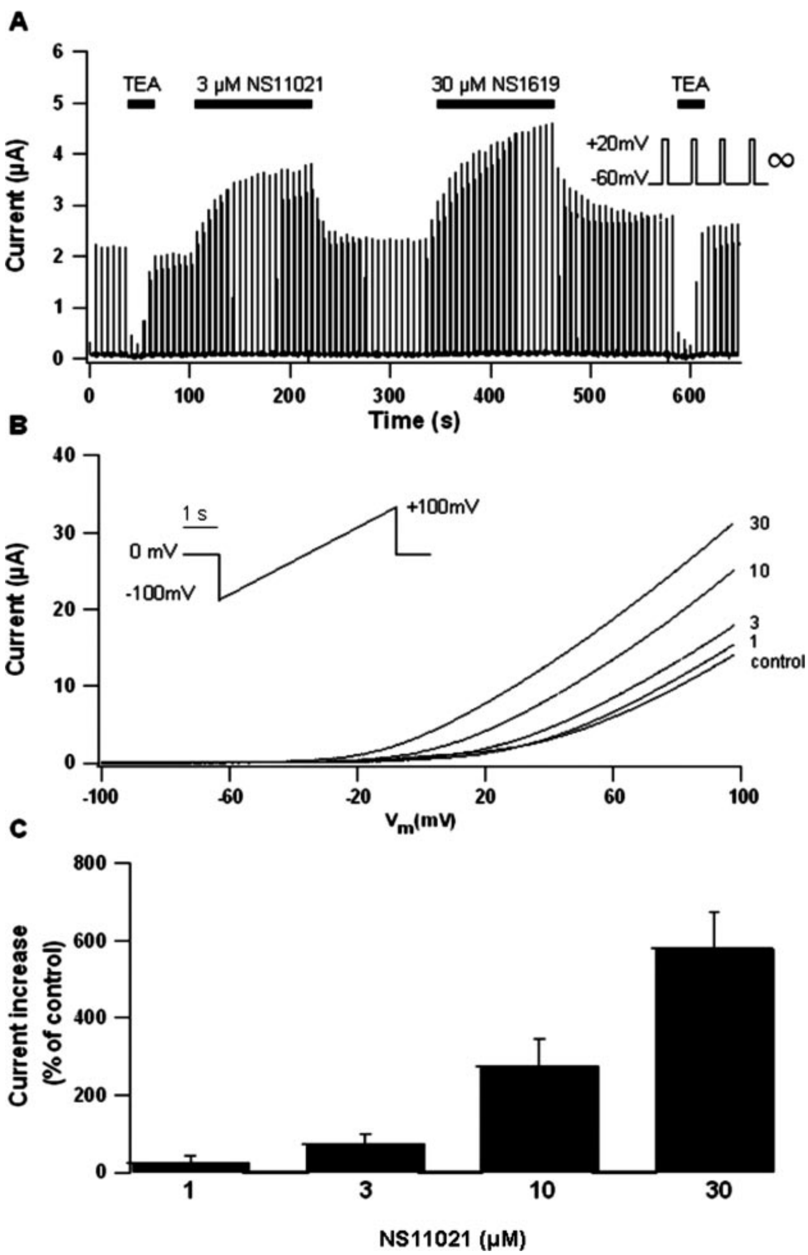

Fig. 2. NS11021 activates hKCa1.1 currents in *X. laevis* oocytes. A, two-electrode voltage-clamp current traces of KCa1.1 channels expressed in *X. laevis* oocytes before and after the application of 3 μM NS11021. Currents were elicited by repeatedly stepping from −60 mV for 5 s to +20 mV for 1 s. The effect of NS11021 was compared with 30 μM of another KCa1.1 channel activator, NS1619. Tetraethylammonium (5 mM) was applied before and after the addition of activators to confirm KCa1.1 currents. B, voltage dependence of different concentrations of NS11021 was addressed by ramp protocols from −100 to +100 mV. C, summarized effects of NS11021 on activation of KCa1.1 channels. Current increase was determined using a voltage protocol as shown in A. The depolarizing voltage step was adjusted to give a baseline KCa1.1 current of 1 μA. Current increase at 1 μM NS11021 was 25 ± 16%; at 3 μM, 74 ± 24%; at 10 μM, 273 ± 59%; and at 30 μM, 581 ± 94% (n = 8).

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10 mM EGTA, and 10 mM HEPES, pH 7.2. $CaCl_2$ and $MgCl_2$ were added in concentrations calculated (EqCal) to give free Ca^{2+} concentrations of ~0 and 100 nM and 100 μM. For whole-cell recordings, cell capacitance and series resistance were updated before each pulse application. Series resistance values were between 2.5 and 8.0 MΩ, and only experiments where the resistance remained constant during the experiments were analyzed.

Current signals were low pass-filtered at 3 kHz and acquired using PULSE software (HEKA). Drug delivery was performed using a homemade multibarrel system connected to the flow system or perfusion system from AutoMate Scientific.

Native Cardiomyocytes for Recordings of Ca^{2+} and Na^+ Current. Currents were measured with patch-clamp method as described by Christ et al. (2005). The pipette solution had the following composition: 90 mM cesium methanesulfonate, 20 mM CsCl, 10 mM HEPES, 4 mM Mg-ATP, 0.4 mM Tris-GTP, 10 mM EGTA, and 3 mM $CaCl_2$, pH 7.2, with a calculated free Ca^{2+} concentration of ~60 nM (EqCal). Ca^{2+} currents were measured with the following Na^+-free bath solution: 120 mM tetraethylammonium chloride, 10 mM CsCl, 10 mM HEPES, 2 mM $CaCl_2$, 1 mM $MgCl_2$, and 20 mM glucose, pH 7.4 (adjusted with CsOH). For measuring I_{Na}, NaCl (5 mM) was added to the bath solution, and $CaCl_2$ was reduced to 0.5 mM. Contaminating $I_{Ca,L}$ was blocked by nisoldipine (1 μM) (Sigma-Aldrich). All measurements were performed at 37°C. A system for rapid solution changes allowed application of drugs close to the cells (Cell Micro Controls; ALA Scientific Instruments, New York, NY).

Isolation of Single Ventricular Cardiomyocytes

Cells were isolated and incubated as described previously (Hansen et al., 2006).

Calculations

Data were acquired using Pulse software (HEKA) and analyzed using Igor Pro 4.04 software (WaveMetrics, Lake Oswego, OR), ClampFit 9.0 or Prism (GraphPad Software Inc., San Diego, CA). All values are shown as means ± S.E.M. The voltage dependence of activation was determined from tail current analysis using peak tail current measured immediately after stepping back to −120 mV from variable potentials. The tail current-voltage relationship was then fitted to Boltzmann equation: I/I_{max} $(V) = 1/1 + \exp[(V - V_{0.5})/k]$, where I/I_{max} is the normalized tail current amplitude, $V_{0.5}$ is the potential for half-maximal activation, and k is the slope factor.

Drugs

NS11021 and NS1619 were dissolved in DMSO to obtain concentrated stock solutions. On the day of electrophysiological experi-

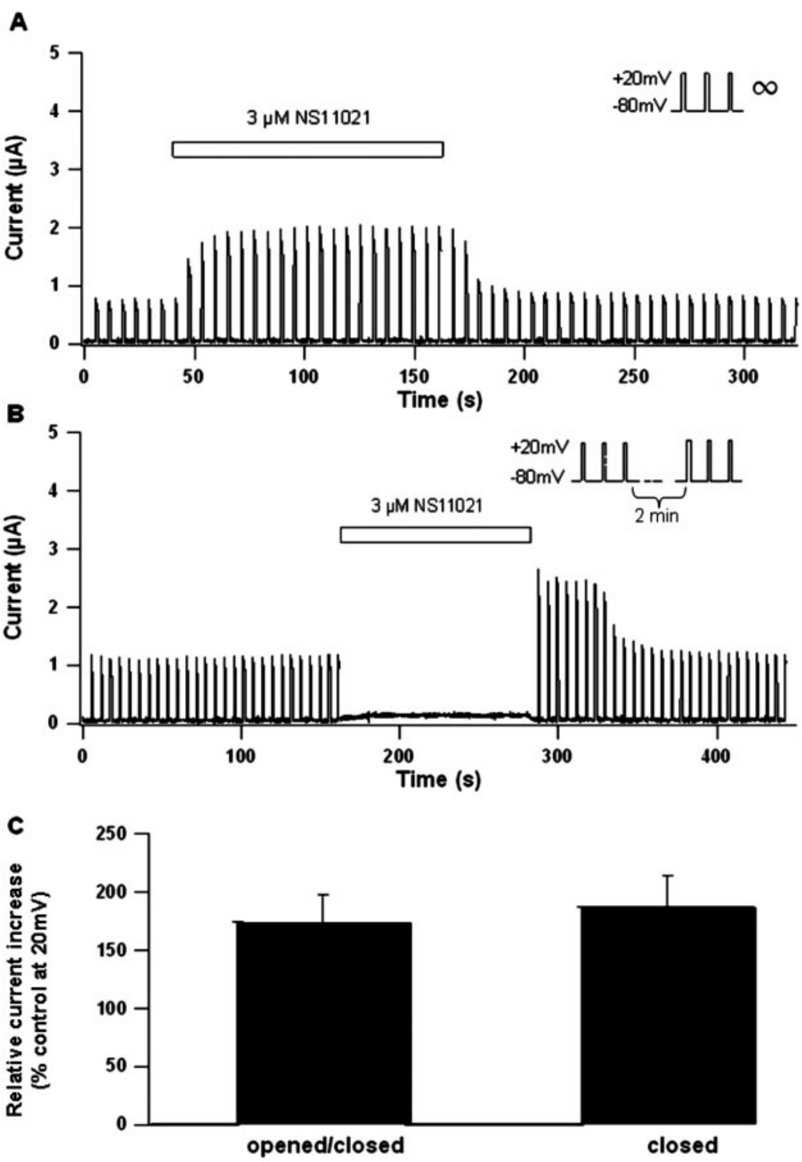

Fig. 3. NS11021 binds both to open and closed channels. Representative online recording from *X. laevis* oocytes expressing KCa1.1 channels recorded using the two-electrode voltage-clamp technique. A, the effect of 3 μM NS11021 when applied during continuous periods of depolarizing voltage steps to +20 mV for 1 s followed by 5 s at −80 mV. Compound was applied for approximately 120 s. B, the effect of 3 μM NS11021 when applied during voltage clamping at 80 mV for 120 s (closed channel). Summarized data for activation of KCa1.1 channels by 3 μM NS11021. Current increase for channels repeatedly shifted between opened and closed conformation was 171 ± 31%. In comparison, 3 μM NS11021 increased KCa1.1 current to a similar degree (187 ± 27%) for channels kept in closed conformation during the entire drug application (n = 6).

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ments, the stock solutions were thawed and diluted to their final concentrations. The final DMSO concentration in the used drug solutions never exceeded 0.1%. At this concentration, DMSO did not influence the electrical properties of the cells (data not shown). NS1619 and NS11021 were synthesized at NeuroSearch A/S, Ballerup, Denmark. Chemical synthesis of NS11021 is outlined in Fig. 1 and involves a reaction between commercial 3,5-cis(trifluoromethyl)phenylisothiocyanate and 4-bromo-2-(1*H*-tetrazol-5-yl)-phenylamine (Postovskii and Golomolzin, 1970).

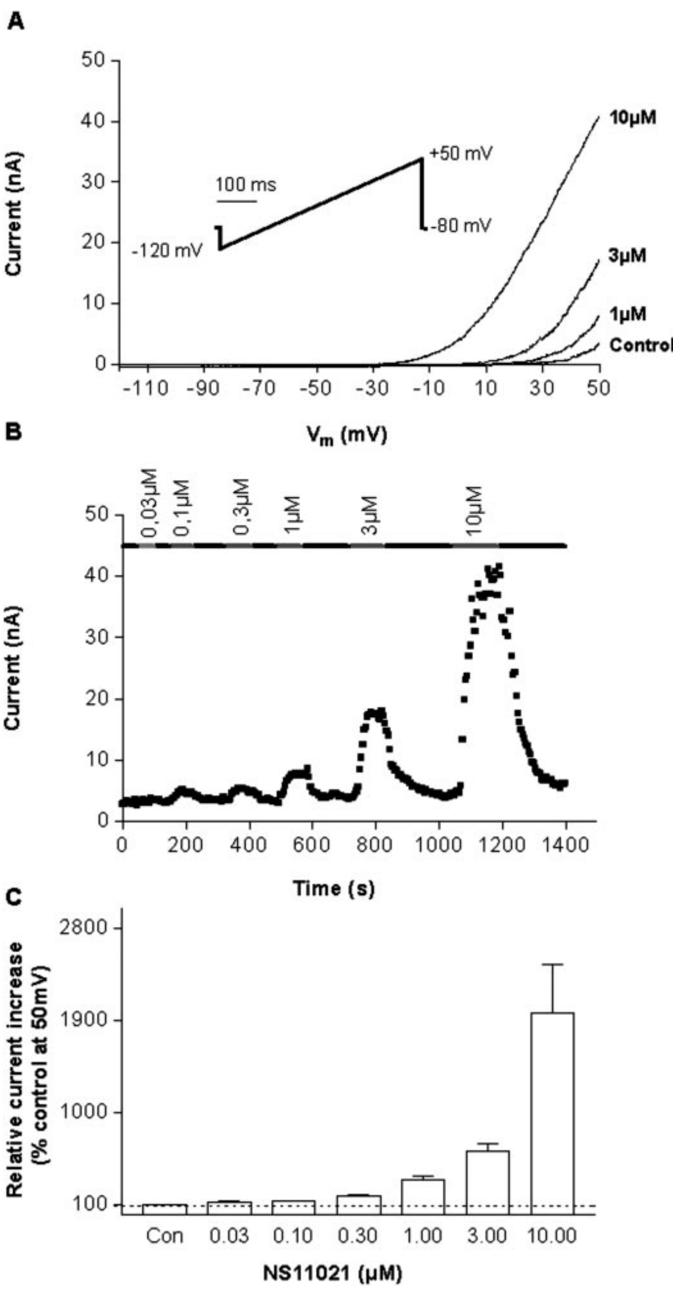

Fig. 4. NS11021 activates hKCa1.1 currents in HEK293 cells. A, whole-cell recordings from HEK293 cells expressing hKCa1.1 channels. Currents were elicited by the voltage ramp shown in inset before and after the application of 1, 3, and 10 μM NS11021. B, time course of the effect of NS11021 at different concentrations. The effect was investigated by repeating the voltage ramp in A and measuring the current at +50 mV. C, bar chart showing the mean relative increase in current after application of NS11021 at different concentrations from 6 to 11 cells measured at 50 mV. Bars represent S.E.M.

Results

NS11021 Activated hKCa1.1 Channels Expressed in *X. laevis* Oocytes. A number of natural occurring and synthetic compounds have been found to activate hKCa1.1 channels. Figure 1 depicts the structure of a novel compound, NS11021, with such properties.

To obtain information about the impact of NS11021 on hKCa1.1 channel activity, two-electrode voltage-clamp experiments were performed. Figure 2 demonstrates the effect of NS11021 on hKCa1.1 expressed in *X. laevis* oocytes. In initial experiments, currents were elicited by a continuously repeated step protocol with depolarizing voltage steps to +20 mV, lasting for 1 s. In between depolarizing steps, cells were clamped at −60 mV for 5 s (Fig. 2A). The application of 3 μM NS11021 increased the hKCa1.1 current from approximately 2 μA to approximately 4 μA. The current amplitude could be reverted to baseline condition after washing. For comparison, the oocyte was subsequently exposed to 30 μM of the well described KCa1.1 channel opener NS1619, resulting in a current increase to approximately 4.5 μA. Before and after the application of KCa1.1 activator, current specificity was confirmed by adding 5 mM tetraethylammonium, a pore blocker, resulting in an easily revertible inhibition of the KCa1.1 current. The potency of NS11021 was determined by adding increasing concentration of compound to oocytes expressing hKCa1.1 channels. Current was elicited by continuously voltage ramps from −100 to +100 mV, lasting for 5 s. As seen in Fig. 2B, NS11021 increased the hKCa1.1 current at concentrations from 1 to 30 μM. The ability of NS11021 to increase hKCa1.1 current in oocytes is summarized in Fig. 2C. The voltage protocol applied in these experiments is shown in Fig. 2A. The depolarizing voltage step was chosen to give a basal hKCa1.1 current of 1 μA, and between each application of NS11021, the current amplitude was reverted to baseline by washing (data not shown). Current increase at 1 μM was 25 ± 16%; at 3 μM, 74 ± 24%; at 10 μM, 273 ± 59%; and at 30 μM, 581 ± 94% (n = 8).

NS11021 Could Bind to the Channel in Both Open and Closed Conformations. To further characterize the effect of NS11021 on hKCa1.1 channels expressed in oocytes, we determined whether NS11021 activated hKCa1.1 channels equally well in the closed and opened conformation. These experiments were conducted by repeatedly applying depolarizing voltage steps to +20 mV for 1 s every 5 s from a holding potential of −80 mV. A representative recording is demonstrated in Fig. 3A. NS11021 was added for approximately 120 s and caused an increase in current amplitude. The ability of NS11021 to interact with a closed channel was addressed by interrupting the repeated voltage steps in Fig. 3A by a 120-s clamp at −80 mV. NS11021 was added when the channel was clamped at −80 mV. Increase in current amplitude was recorded at the first depolarizing step to +20 mV and compared with baseline level before the 120-s clamp to −80 mV. A representative example is depicted in Fig. 3B. Summarized data for both opened/closed channels and channels kept in closed conformation during application of NS11021 are depicted in Fig. 3C. NS11021 (3 μM) increased the hKCa1.1 current by 171 ± 31% for channels that repeatedly shifted between opened and closed conformation. In comparison, 3 μM NS11021 increased the hKCa1.1 current by 187 ± 27% for channels kept in closed conformation dur-

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ing the entire drug application ($n = 6$). These results demonstrate that NS11021 can equally well affect opened and closed channels.

NS11021 Enhanced hKCa1.1 Currents in HEK293 Cells. To exclude whether the ability of NS11021 to activate hKCa1.1 channels was a unique property of the oocyte expression system, the effect of NS11021 on hKCa1.1 whole-cell currents was measured in HEK293 cells expressing hKCa1.1 channels. Figure 4 illustrates the effect of NS11021 at different concentrations on whole-cell currents when administered to the extracellular side (100 nM free calcium in the pipette solution). Currents were repeatedly elicited every 5 s by 500-ms voltage ramps (−120 to +50 mV). The addition of NS11021 increased the current amplitude measured at 50 mV and caused a leftward shift in the threshold for current activation (Fig. 4A). The time-dependent effect of NS11021 is displayed in Fig. 4B. Data show the current amplitude from the same experiment as in Fig. 4A as a function of time. The application of NS11021 to the extracellular side of the membrane significantly increased the current amplitude at already low concentrations (0.1 μM), and the augmentation of current increased with increasing concentrations of NS11021. Upon termination of application of NS11021, the current amplitude was restored to pretreatment levels. Figure 4C summarizes the concentration-dependent facilitation of hKCa1.1 currents ($n = 6–11$ for each data point).

Effect of NS11021 on hKCa1.1 Current-Voltage Relationship and Voltage Dependence of Activation. The current traces in Fig. 4A revealed that NS11021 produced a negative shift in the threshold for current activation. This was further investigated by looking at the current-voltage relationship and the voltage dependence of steady-state channel activation as is depicted in Fig. 5. Using the inside-out configuration, patches from HEK cells stably expressing hKCa1.1 were examined. From a holding potential of −80 mV, the patches were clamped for 75 ms at potentials ranging from −150 to +190 mV in 20-mV increments. The current amplitudes were measured at steady state. Tail currents were measured when stepped back to −120 mV and plotted as a function of the preceding potential. Steady state and tail current were measured in the presence and absence of 1 μM NS11021. The free internal Ca^{2+} concentration was calculated to 100 nM. Current traces from patches containing hKCa1.1 channels revealed a fast activating and deactivating current activating at approximately +70 mV under control situation (Fig. 5A) and at +30 mV after the application of 1 μM NS11021 (Fig. 5B). At potentials between + 30 and +150 mV NS11021 augments the steady-state current amplitude, whereas no additional effect on the amplitude is seen at +150 to +190 mV. Figure 5C summarizes the current-voltage relationship in the absence (black squares) or presence (open squares) of NS11021. In all experiments, the channels were initially activated by the voltage protocol in the absence of compound and subsequently in the presence of NS11021, thereby functioning as their own controls. The voltage dependence of activation depicted in Fig. 5D illustrates that the addition of NS11021 increased the peak tail current amplitude and shifted the voltage dependence of activation to more hyperpolarized potentials.

hKCa1.1 Activation by NS11021 at Constant Ca^{2+} Concentration. From Fig. 5, it seems that the ability of NS11021 to activate KCa1.1 channels is better investigated by addressing the shift in the voltage dependence of activation rather than the augmentation of steady-state current amplitude. Using the inside-out configuration, concentration-response experiments were performed with a constant intracellular free calcium concentration of 100 nM. Patches expressing hKCa1.1 were clamped for 75 ms at potentials between −150 and +290 mV in 20-mV increments, and tail currents were measured when stepped back to −120 mV,

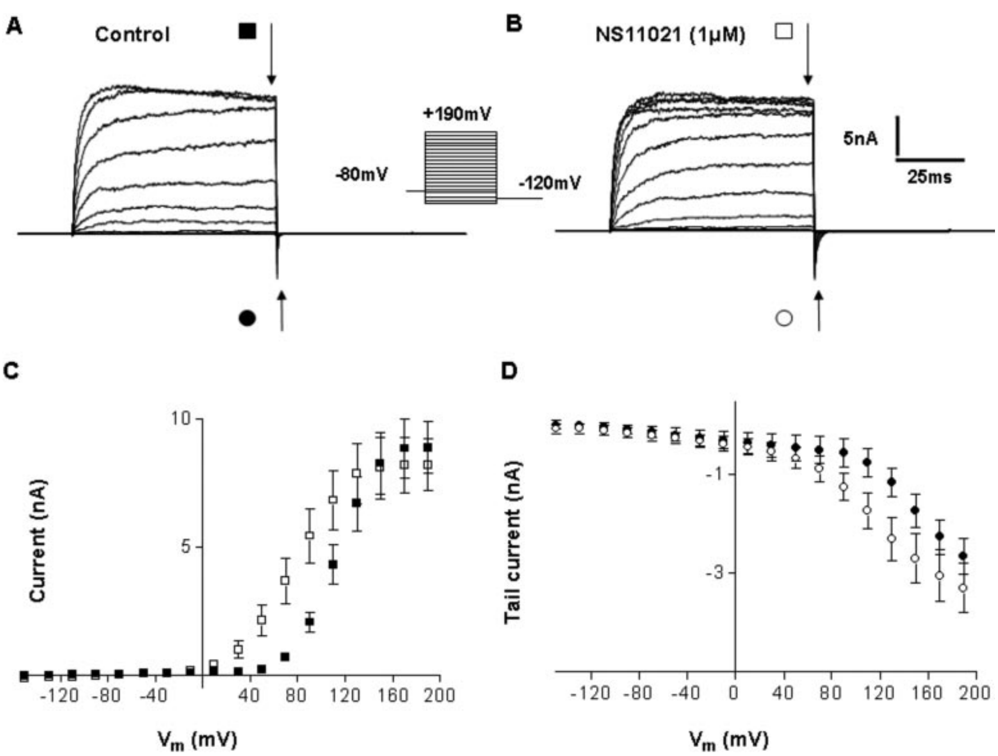

Fig. 5. Effect of 1 μM NS11021 on hKCa1.1 current-voltage relationship and voltage dependence of activation. Representative current traces elicited by the voltage protocol showed in the inset, before (A) and after the application of 1 μM NS11021 (B). Currents were recorded using the inside-out configuration from patches excised from hKCa1.1 expressing HEK293 cells, with an intracellular bath solution containing calculated free Ca^{2+} of 100 nM. C, summarized current-voltage relationship of similar experiments as A and B. Currents measured at steady state were plotted against test potential in the absence (■) and presence of 1 μM NS11021 (□). D, tail current-voltage relationships of similar experiments as A and B. The voltage dependence of activation was investigated by plotting the tail current amplitude measured immediately after stepping back to −120 mV against the preceding step potential. Each point represents the mean ± S.E.M., and $n = 7$.

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normalized, and plotted as a function of the preceding potential in the absence or presence of increasing concentrations of NS11021. From Boltzmann fits, the potential of half-maximal activation ($V_{0.5}$) was found for each applied concentration of NS11021. The potency of NS11021 was subsequently determined by depicting $V_{0.5}$ values as a function of compound concentrations. Using this approach, the EC_{50} for NS11021 was determined to be 0.4 μM (Fig. 6).

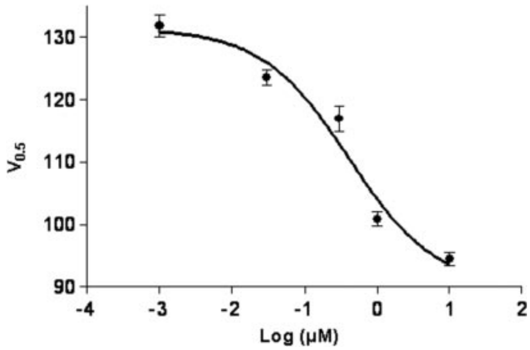

Fig. 6. Concentration-response relationship of NS11021. Using the inside-out configuration, concentration-response experiments were performed with a constant intracellular free calcium concentration of 100 nM. For each compound concentration tested, a full tail-current analysis was performed to determine $V_{0.5}$. Patches expressing hKCa1.1 were clamped for 75 ms at potentials between −150 and +290 mV in 20-mV increments, and tail currents were measured when stepped back to −120 mV, normalized, and plotted as a function of the preceding potential in the absence or presence of increasing concentrations of NS11021. The EC_{50} for NS11021 was determined to be 0.4 μM (n = 4–8).

NS11021 Activated hKCa1.1 Channels Independently of the Free Intracellular Ca^{2+} Concentration. Because Ca^{2+} modulates the activity of KCa1.1 channels, it was investigated whether the effect of NS11021 was calcium-dependent (Fig. 7). Using the inside-out patch-clamp configuration, three different concentrations of intracellular Ca^{2+} were examined: Ca^{2+}-free, 100 nM free Ca^{2+}, and 100 μM free Ca^{2+}. In the absence of Ca^{2+}, the KCa1.1 channel activated at extreme potentials with a $V_{0.5}$ of 157.0 ± 1.6 mV found from Boltzmann fits of tail currents measured at −120 mV. Despite the lack of Ca^{2+}, 10 μM NS11021 still shifted the voltage dependence of activation to more hyperpolarized potentials ($V_{0.5}$ = 94.6 ± 1.5 mV; $\Delta V_{0.5}$ = −62.4 mV). At more physiological intracellular free Ca^{2+} concentrations (100 nM, Fig. 6), the $V_{0.5}$ is shifted to more negative potentials compared with Ca^{2+}-free conditions. However, the application of NS11021 shifts the $V_{0.5}$ (129.9 ± 3.5 mV in the absence 10 μM NS11021 to 94.5 ± 2.5 mV in the presence of 10 μM NS11021; $\Delta V_{0.5}$ = −35.4 mV) to a lesser extend compared

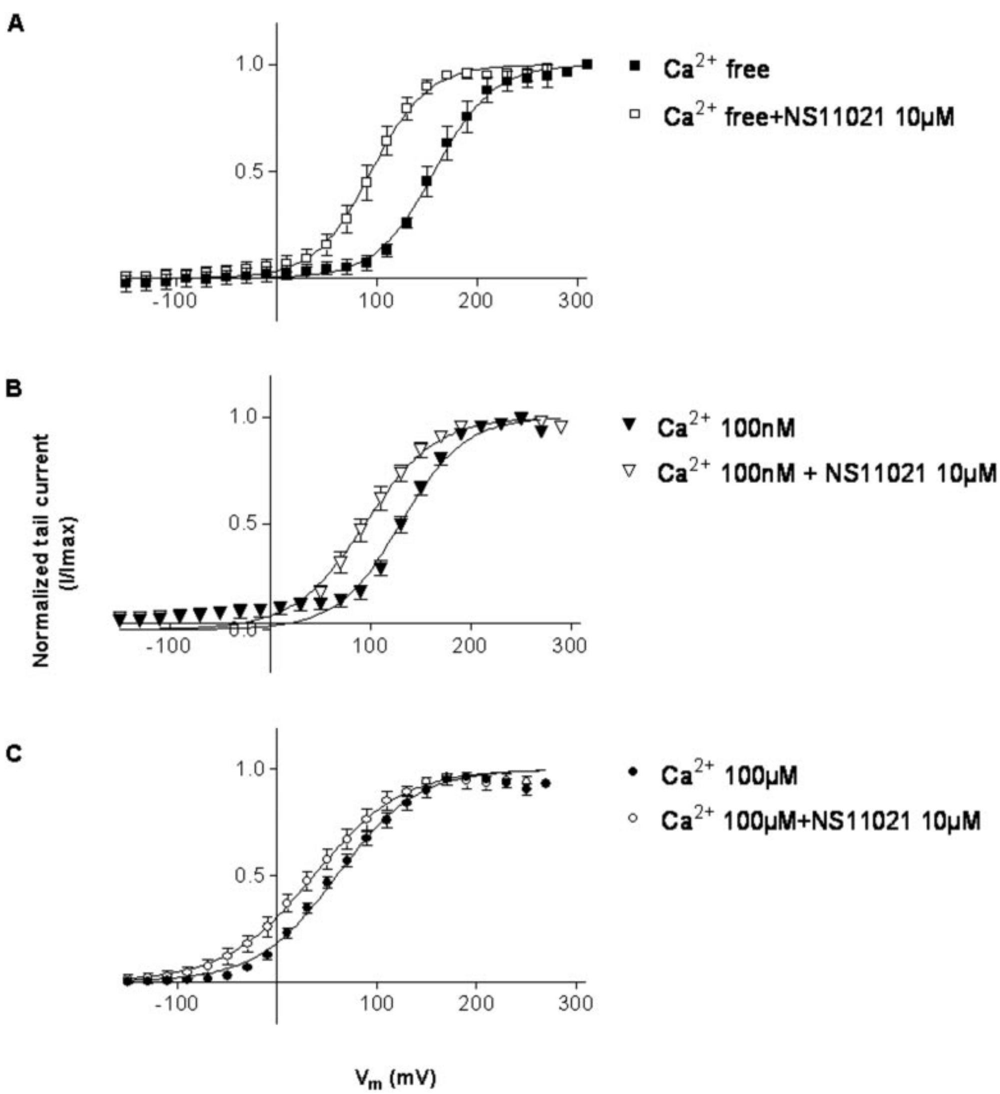

Fig. 7. NS11021 can activate KCa1.1 channels independently of the free intracellular Ca^{2+} concentration. Current-voltage relation obtained as in Fig. 6 from patches exposed to different concentrations of calculated free Ca^{2+} [free (A), 100 nM (B), and 100 μM (C)] before (black) and after (open) application of 10 μM NS11021. Ca^{2+}-free conditions ($V_{0.5}$: 157.0 ± 1.6 mV→94.6 ± 1.5 mV), 100 nM free Ca^{2+} ($V_{0.5}$: 129.9 ± 3.5 mV→94.5 ± 2.5 mV), and 100 μM free Ca^{2+} ($V_{0.5}$: 59.9 ± 2.2 mV→36.8 ± 2.9 mV). Values in brackets are mean shift in half-activation potential $V_{0.5}$ ± S.D. calculated using a Boltzmann fit. Bars represent S.E.M., and n = 6–10.

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with Ca^{2+}-free conditions. Finally, 100 μM free Ca^{2+} was used to approximate the Ca^{2+}-saturated condition for the high-affinity binding sites in KCa1.1 (Horrigan and Aldrich, 2002). Under these conditions, NS11021 was still capable of shifting the voltage dependence of activation ($V_{0.5}$: 59.9 ± 2.2 mV in the absence of 10 μM NS11021 to 36.8 ± 2.9 mV in the presence of NS11021; $\Delta V_{0.5} = -23.1$ mV). Because the degree of activation of KCa1.1 channels is different in the absence of Ca^{2+} compared with saturating concentrations of Ca^{2+}, it is likely that the mode of action for NS11021 involves both the Ca^{2+} and voltage-dependent activation mechanisms of KCa1.1 channels.

Effect of NS11021 on Single-Channel Currents. To establish whether the increase in macroscopic current following application of NS11021 was due to increased open probability or increased single-channel conductance, single-channel recordings were performed using macropatches excised from *X. laevis* oocytes expressing hKCa1.1. The bathing (in-

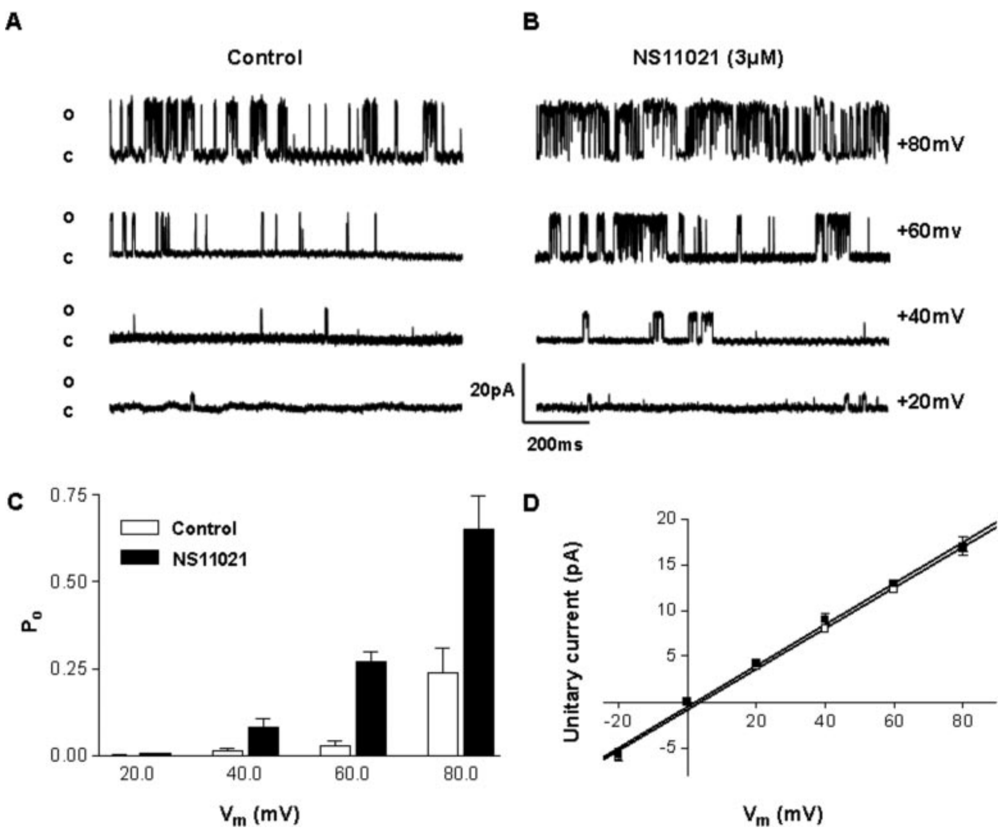

Fig. 8. NS11021 increases the single-channel open probability and has no effect on single-channel conductance. Single hKCa1.1 currents recorded in inside-out macropatch configuration from *X. laevis* oocytes at + 80, +60, +40, and +20 mV in symmetric 140 mM K^+ with a free Ca^{2+} of 300 nM, before (A) and after (B) application of 3 μM NS11021. C, bar chart representing mean opening probability (mean ± S.E.M.) at + 20, +40, +60, and +80 mV before and after 3 μM NS11021 (n = 5). D, effect of 3 μM NS11021 on single-channel conductance. The unitary current amplitude determined before and after NS11021 is plotted as a function of test voltages. Each point represents the mean ± S.E.M., and n = 5. The slope conductance of KCa1.1 was found to be 224 ± 7 pS before and 223 ± 6 pS after the application of 3 μM NS11021.

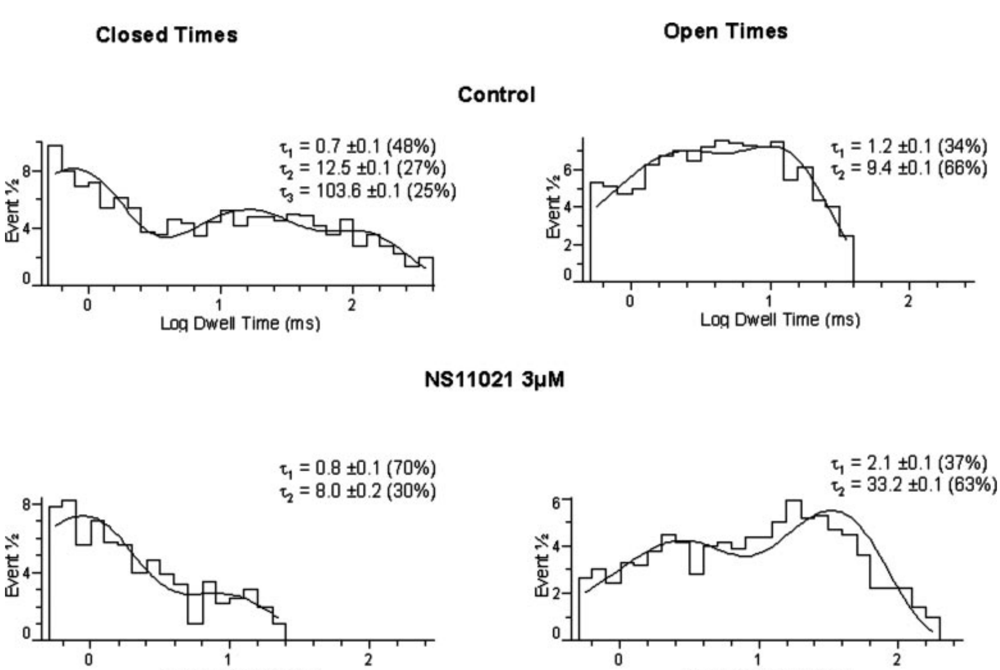

Fig. 9. NS11021 increases open times and decreases closed times. Closed time (left) and open time (right) distributions for hKCa1.1 single-channel activity recorded at +80 mV in symmetric 140 mM K^+ conditions with a free Ca^{2+} of 300 nM, for control situation (A) and after application of 3 μM NS11021 (B). Open time distributions were fitted to a two-exponential probability density function and close time distribution with a two- and three-exponential function for NS11021 and control situation, respectively. Fits are shown as smooth lines with the corresponding kinetic components and percentage distribution shown above each distribution.

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tracellular) and pipette solution contained symmetrical 140 mM K^+ and had a free Ca^{2+} concentration of 300 nM. Single-channel recordings were performed at different potentials before and 3 min after the application of 3 μM NS11021. Representative current traces are shown in Fig. 8A. The channel was barely open at +20 mV, and the opening of the channel was highly dependent on the potential. Corresponding traces in the presence of NS11021 are depicted in Fig. 8B. From the current traces in Fig. 8, A and B, and from the summary of similar experiments in Fig. 8C, it is clearly shown that the application of 3 μM NS11021 increased the open probability. To address whether NS11021 changes single-channel conductance, the current through single KCa1.1 channels was measured over a range of potentials before and during the application of 3 μM NS11021 in symmetric potassium concentrations. Single-channel slope conductance obtained from pooled unitary current-voltage relationships (Fig. 8D) were 224 ± 7 pS before and 223 ± 6 pS during the application of 3 μM NS11021. Thus, KCa1.1 channel conductance values were not significantly altered by the application of 3 μM NS11021.

From a single recording at +80 mV, the open and closed times were studied (Fig. 9). Open times were best fitted to a double exponential function, and the application of 3 μM NS11021 clearly increased both the fast and slow time constant but did not alter their relative contribution. On the other hand, the closed times were best fitted with three exponentials, and the application of 3 μM NS11021 clearly shifted the distribution of the three time constants. The slowest component was completely abolished, and the relative contribution of the fast and intermediate was shifted toward the fastest components.

Selectivity toward Other Ion Channels. The selectivity of NS11021 toward other ion channels was also character-

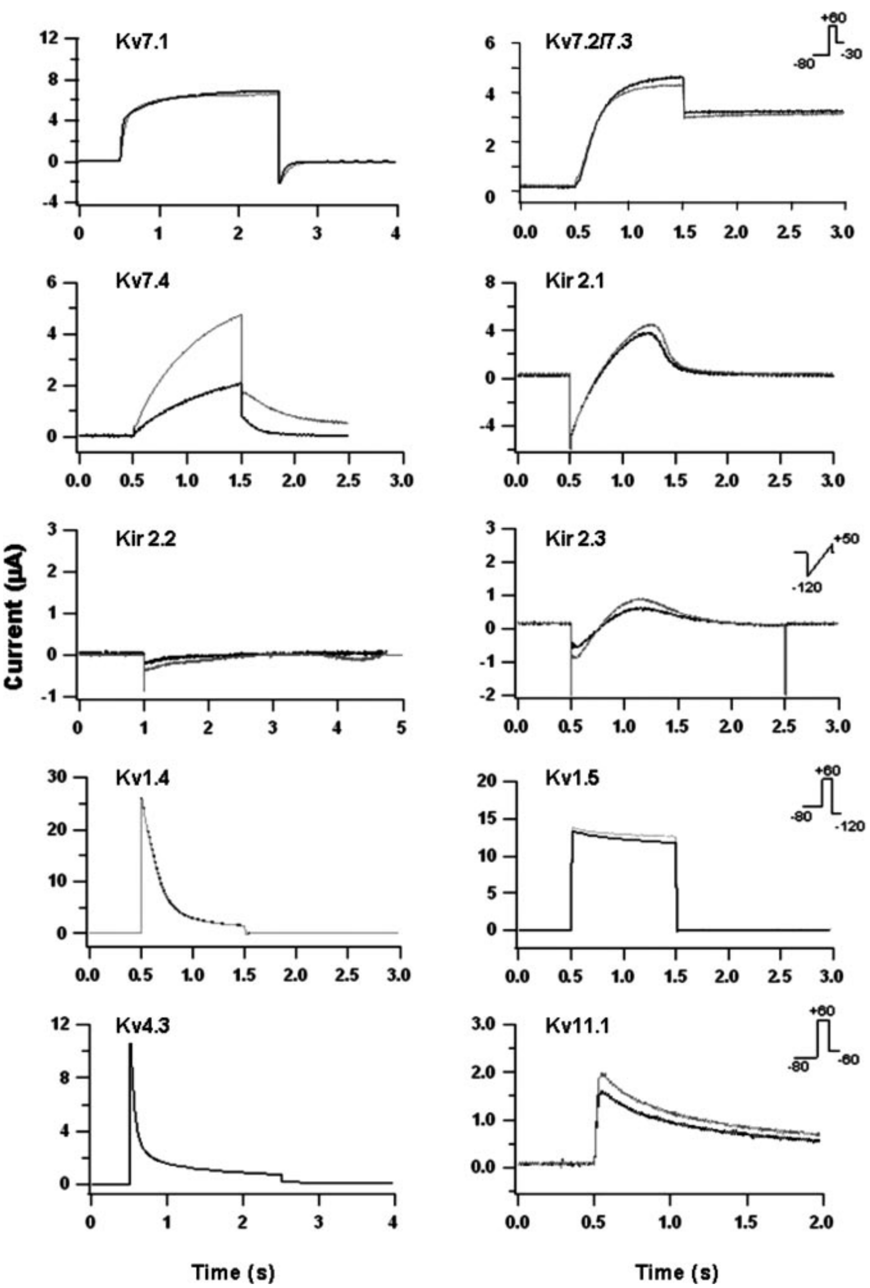

Fig. 10. Specificity of NS11021. The activity of 30 μM NS11021 toward a range of potassium channels expressed in oocytes was investigated. Kv7, Kv1.4-5, Kv4.3, and Kv11.1 currents were elicited by a depolarizing step to +60 mV from a holding potential of −80 mV followed by a step back to −30, −120, −120, and −60 mV, respectively, to measure the tail current. A voltage ramp spanning from −120 to +50 mV was used to elicit Kir currents. Black lines represent control current traces, whereas gray lines represent current traces after the application of 30 μM NS11021. n = 4–8.

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ized. Kv7.1-4, Kv1.4-5, Kv4.3, Kir2.1-3, and Kv11.1 were expressed in *X. laevis* oocytes and studied using a two-electrode voltage clamp. Channel activation was obtained by voltage-step protocols as indicated in Fig. 10. Control current traces are depicted in black and traces recorded in the presence of 30 μM NS11021 in gray. As demonstrated, 30 μM NS11021 increased the current through Kv7.4 significantly (by 96 ± 17%, n = 5), had a minor inhibitory effect on Kv7.2/7.3 channels, and had a minor activating effect on Kv11.1, Kir2.1, and Kir2.3 channels. It should be noted that the inhibitory effect of Kv7.2/7.3 channels was time- and voltage-dependent. Inhibition was only observed at potentials more positive than +10 mV after more than 2 s of depolarization, indicating that under physiological settings Kv7.2/7.3 channels will be unaffected by NS11021 (data not shown). No significant effect on Kv1.4, Kv1.5, Kv4.3, Kv7.1, Kv7.2/7.3, and Kir2.2 currents was observed after the application of 30 μM NS11021 (n = 4–8). At 10 μM NS11021, only the Kv7.4-activating effect was present (67 ± 31%, n = 4, data not shown). In addition, the effect of NS11021 was also tested on nicotine-sensitive acetylcholine receptors of the α7 type. NS11021 was a positive modulator with an EC_{50} value of 12 ± 1 μM (data not shown). Finally, NS11021 (20 μM) was also investigated for specificity against erythrocyte Cl^- channels and was found to be devoid of effect (data not shown).

The effect of NS11021 on L- and T-type calcium currents ($I_{Ca,L}$ and $I_{Ca,T}$) as well as I_{Na} was examined in native guinea pig ventricular cardiomyocytes. Five minutes after establishing the whole-cell patch-clamp configuration, the cells were exposed to 30 μM NS11021. $I_{Ca,L}$ and I_{Na} were both stable over time, and the current densities after 2 min of exposure to 30 μM NS11021 were not different from the respective control values (i.e., from −17.1 ± 2.4 to −15.6 ± 2.5 pA/pF for $I_{Ca,L}$ and −14.4 ± 3.5 to −13.2 ± 3.0 pA/pF for I_{Na}, n = 6 and 4, respectively). Moreover, NS11021 did not affect $I_{Ca,T}$ as seen by similar appearance of the shoulder (seen from −40 to −20 mV) on the current-voltage relationships before and after the application of 30 μM NS11021 (Fig. 11).

Discussion

Selective molecules that block or activate ion channels are important biological tools because they are useful for elucidating the physiological function of ion channels. In addition, they may also form effective drugs. In the present study, a novel selective KCa1.1 channel activator was characterized. The biphenyl thiourea NS11021 was found to activate cloned KCa1.1 in a concentration-dependent manner at low micromolar concentrations. NS11021 augments the KCa1.1 current by parallel-shifting the voltage dependence of activation to more negative potentials. On a single-channel level, this is accomplished by an increased open dwell time, decreased closed time, and an unaffected slope conductance.

NS11021 is effective when exposed to either side of the membrane and probably exerts its effect through direct binding to the KCa1.1 α subunit, indicated by the fast onset and the fact that the current activation was still present in the inside-out configuration where most of the intracellular signaling pathways are lost. In addition, the current facilitating effect was found not to be use-dependent.

As the name implies, KCa1.1 channels are not only regulated by voltage but also by intracellular Ca^{2+}. The inside-out configuration offers the possibility to control the intracellular milieu. In the absence of intracellular Ca^{2+}, the KCa1.1 channels are purely activated by voltage-gated mechanisms. Even under these conditions, 10 μM NS11021 was capable of shifting the potential of half-maximal activation ($V_{0.5}$) by −62.4 mV, indicating that the increase in open probability cannot simply be explained by an increased affin-

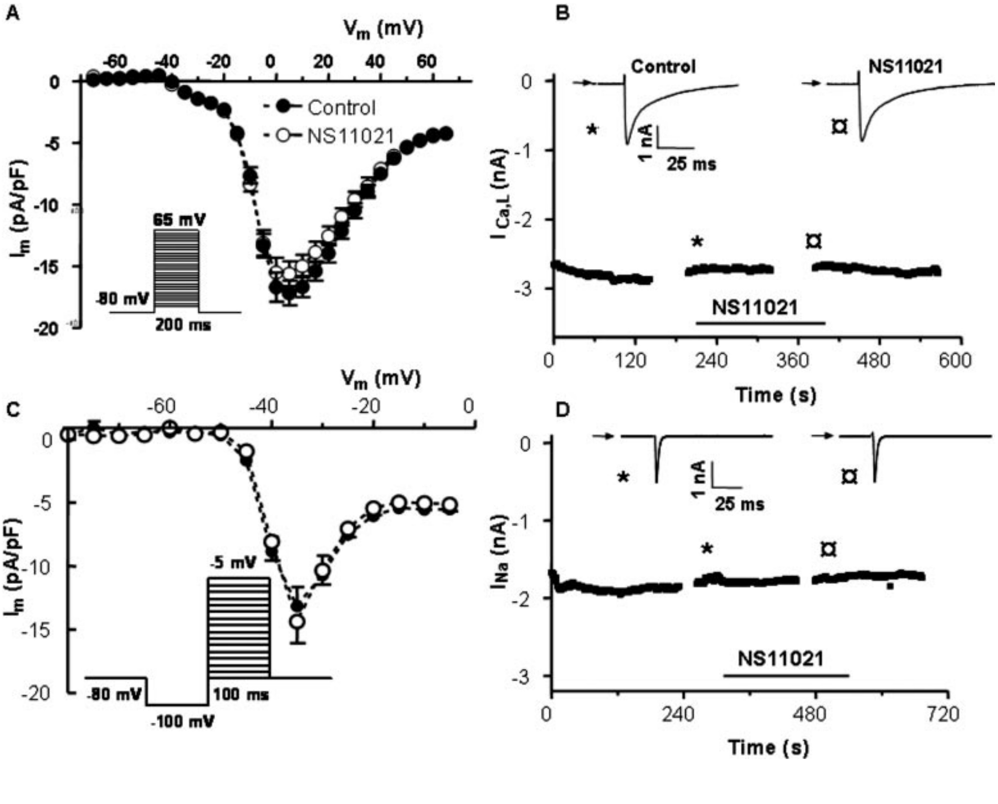

Fig. 11. Specificity of NS11021 on native currents. The effect of 30 μM NS11021 on L-type voltage-gated Ca^{2+} channels and cardiac Na^+ channels was addressed using guinea pig ventricular myocytes. A, current-voltage relationship obtained by application of the depicted protocol for Ca^{2+} current recorded before and 2 min after the addition of 30 μM NS11021. On the current-voltage curve, a small shoulder is seen between −40 and −20 mV representing the $I_{Ca,T}$; the remaining current (peak at +5 mV) is due to L-type current. Time course of peak $I_{Ca,L}$ is shown in B. The current-voltage relationship for I_{Na} using the outlined protocol recorded before and after the application of 30 μM NS11021 is depicted in C and the time course for peak I_{Na} in D. Insets in B and D show original traces obtained at times marked by I and II, respectively. The horizontal bars indicate the time of drug exposure. Each point represents the mean ± S.E.M., and n = 6.

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ity for Ca^{2+}. In addition, the mechanism of action for NS11021 must involve the voltage-dependent gating of the channel. In the opposite situation when all high-affinity sites for Ca^{2+} are occupied (100 μM free Ca^{2+}), 10 μM NS11021 caused a shift in $V_{0.5}$ by −23.1 mV. When looking collectively at the data obtained from inside-out experiments, including the effect of NS11021 measured at 100 nM Ca^{2+} ($\Delta V_{0.5}$ = −35.4 mV), it can be seen that the effect of NS11021 increases with decreasing concentrations of free Ca^{2+}, suggesting a competitive effect of NS11021 and Ca^{2+}. The disturbance in the Ca^{2+} sensitivity of the channel can also be appreciated by looking at the shift in $\Delta V_{0.5}$ from Ca^{2+}-free conditions to calcium-saturating conditions (100 μM) in a control situation ($\Delta V_{0.5}$ = −97.1 mV) and in the presence of NS11021 ($\Delta V_{0.5}$ = −57.8 mV). If no interference with the Ca^{2+} sensitivity of the channel was present, the $\Delta V_{0.5}$ should be equal. One could speculate that the interaction site of NS11021 is somehow related or overlaps with that of Ca^{2+}. This was, however, not investigated further. The Ca^{2+} dependence of other KCa1.1 channel activators differs. The fact that the potentiation of KCa1.1 current by NS11021 decreases with increasing concentrations of Ca^{2+} has also been found for another KCa1.1 activator, diCl-DHAA (Sakamoto et al., 2006), whereas the opposite holds true for BMS-204352, when whole-cell current amplitudes were examined (Gribkoff et al., 2001). NS11021 belongs to the group of KCa1.1 channel openers, which include NS1608 (Strøbaek et al., 1996), mallotoxin (Zakharov et al., 2005), and KB130015 (Gessner et al., 2007) that activates KCa1.1 channels in the absence of Ca^{2+}.

At 1 μM, NS11021 shifts the $V_{0.5}$ by approximately 30 mV in a hyperpolarizing direction, which resembles the effect of 1 μM tamoxifen (Dick et al., 2001), whereas NS1619 shifts $V_{0.5}$ −70 mV but at a 30-fold higher concentration (Olesen et al., 1994).

The KCa1.1 channel is genetically and in some aspects functionally, when considering the presence of a voltage sensor, more closely related to other voltage-dependent K^+ channels compared with the small-conductance and intermediate-conductance Ca^{2+}-activated potassium channels. Therefore, we examined the effect of NS11021 on cloned voltage-sensitive ion channels, with emphasis on voltage-dependent potassium channels and other cardiac ion channels. In the present study, it was shown that the KCa1.1 current is significantly increased by NS11021 in concentrations as low as 0.3 μM. We found that 30 μM NS11021 did not affect native Ca^{2+} and Na^+ current recording from isolated guinea pig cardiomyocytes or heterologously expressed Kv1.4-5, Kv4.3, Kv7.1, and Kir2.2, channels nor did it affect chloride currents, which are a common, unspecific effect of many KCa.1.1 activators. However, at 30 μM NS11021, an increase in current was seen for Kv7.4, Kv11.1, Kir2.1, and Kir2.3 channels, whereas a minor time- and voltage-dependent inhibition of Kv7.2/7.3 channels was observed. It should be noted that the inhibitory effect of Kv7.2/7.3 channels was only observed at potentials with positivity greater than +10 mV after more than 2 s of depolarization, indicating that under physiological settings, Kv7.2/7.3 channels will be unaffected by NS11021. Except for the increase in the Kv7.4 current, all of these effects where abolished when a lower concentration (10 μM) of NS11021 was applied. Augmentation of Kv7.4 channel activity by possible KCa1.1 channel activators is also known for another KCa1.1 opener, BMS-204352 (Schrøder et al., 2001). This comprehensive study on selectivity of a KCa1.1 activator will be helpful when interpreting future studies using NS11021 as a tool compound.

KCa1.1 channels are broadly expressed in both excitable and nonexcitable cells and play an essential role in the regulation of cell excitability and function. Considering the important role of KCa1.1 channels in mediating a negative-feedback mechanism for Ca^{2+} entry into cells, compounds that activate KCa1.1 channels could have important therapeutic potentials by enhancing an already existing physiological system for cell relaxation. Relaxation of smooth muscles by KCa1.1 channel activators could represent a new medical indication in a broad range of diseases such as hypertension, urinary incontinence, erectile dysfunction, chronic obstructive pulmonary disease, and others. In addition, the finding of KCa1.1-resembling channels in the inner mitochondrial membrane of the heart and brain (Xu et al., 2002; Douglas et al., 2006) might represent a new target for KCa1.1 activators with regard to protection against ischemia-reperfusion injuries. With respect to the role of KCa1.1 channels in neurons, one would expect KCa1.1 activators to hyperpolarize the membrane potential and reduce neuronal excitability. Therefore, KCa1.1 activators could be important in managing diseases such as epilepsy, neurodegeneration, and pain. The outcome of KCa.1.1 activators is however difficult to predict and will depend, among other things, on the subcellular localization of the channel in the neuron. Despite the numerous indications, no KCa1.1 channel opener has reached the market yet and the number of KCa1.1 openers under development is still somewhat limited. Two KCa1.1 openers, which were progressed to clinical development for overactive bladder (NS-8) and post-stroke neuroprotection (BMS-204352), have recently been discontinued.

In conclusion, NS11021 is demonstrated to activate KCa1.1 channels with at least 10 times higher potency compared with the most broadly applied KCa1.1 opener NS1619. In addition, NS11021 is more selective compared with NS1619. We therefore believe that NS11021 will be a valuable tool compound for addressing the physiological functions of KCa1.1 channels in the future.

Acknowledgments

We are grateful to Dr. med Torsten Christ (Dresden University of Technology, Dresden, Germany) for educational training in patch-clamping of guinea pig cardiac myocytes.

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