Molecular Pharmacology Fast Forward. Published on July 16, 2007 as doi: 10.1124/mol.107.038331
This article has not been copyedited and formatted. The final version may differ from this version.

Title page

The small molecule NS11021 is a potent and specific activator of Ca^{2+}-activated Big Conductance K^+ channels

Bo Hjorth Bentzen, Antonio Nardi, Kirstine Calloe, Lars Siim Madsen, Søren-Peter Olesen & Morten Grunnet

The Danish National Research Foundation Centre for Cardiac Arrhythmia, Department of Biomedical Sciences, University of Copenhagen, The Panum Institute, 3 Blegdamsvej, DK-2200 Copenhagen N, Denmark (BHB, KC, SPO, MG)

NeuroSearch A/S, Pederstrupvej 93, DK-2750 Ballerup, Denmark (AN, LSM, SPO, MG)

Molecular Pharmacology Fast Forward. Published on July 16, 2007 as DOI: 10.1124/mol.107.038331
This article has not been copyedited and formatted. The final version may differ from this version.

Running title page

NS11021 is a potent and specific activator of BK channels

Corresponding author: Morten Grunnet

NeuroSearch A/S

Pederstrupvej 93

DK-2750 Ballerup

Denmark

Phone: +4544608706. Fax: +4544608080. E-mail: mgr@neurosearch.dk

Number of pages: 37

Number of figures: 11

Number of tables: 0

Number of references: 22

Abstract: 200 words

Introduction: 748 words

Discussion: 1124 words

Abbreviations: Tetraethylammonium (TEA)

Molecular Pharmacology Fast Forward. Published on July 16, 2007 as DOI: 10.1124/mol.107.038331
This article has not been copyedited and formatted. The final version may differ from this version.

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, 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 activates 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 did neither influence 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 to 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.

Molecular Pharmacology Fast Forward. Published on July 16, 2007 as DOI: 10.1124/mol.107.038331
This article has not been copyedited and formatted. The final version may differ from this version.

Large-conductance Ca^{2+}-activated K^+ channels (KCa1.1, BK, MaxiK, hSLO, 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 non-excitable 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, gastro-intestinal, airway and bladder smooth muscle tissue (for review see (Ghatta et al., 2006; Lu et al., 2006). Accordingly, KCa1.1 channels are broadly expressed and are implicated in diverse physiological functions. However, predicting the role of KCa1.1 channels in different tissues is difficult. This is likely due to 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 *KCNMA1*α-subunit, and the presence or absence of β-subunits (Salkoff et al., 2006). Also 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, physiological importance of KCa1.1 has incontrovertibly been emphasized by the KCa1.1 knock-out 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 mutation in the KCa1.1 gene *KCNMA1* has been reported in humans to result in

Molecular Pharmacology Fast Forward. Published on July 16, 2007 as DOI: 10.1124/mol.107.038331
This article has not been copyedited and formatted. The final version may differ from this version.

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. (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 KCa1.1 channel.

Numerous KCa1.1 channel activators have been identified, including the synthetic benzimidazolone derivatives (NS1619), the biarylureas (NS1608), the aryloxindoles BMS-204352, the natural modulators dihydrosoyasaponin-1 (for review see (Ghatta et al., 2006). Although these agents activate KCa1.1 channels they also display additional non-specific 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 (Holland

et al., 1996; Olesen et al., 1994). Yet, 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 1-(3,5-Bis-trifluoromethyl-phenyl)-3-[4-bromo-2-(1H-tetrazol-5-yl)-phenyl]-urea (NS11021) on cloned KCa1.1 channels expressed in *Xenopus laevis* oocytes and HEK293 cells using two-electrode voltage-clamp and patch-clamp techniques. NS11021 activates KCa1.1 channels in a concentration-dependent manner at micromolar concentrations by shifting the conductance-voltage relationship to more hyperpolarized potentials. NS11021 activates the KCa1.1 channel when applied from either side of the membrane, and has the ability to bind to the channel in both open and closed confirmation. NS11021 parallel-shifted the activation curves towards 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 to the most broadly applied KCa1.1 opener NS1619. In addition, NS11021 is more selective compared to NS1619 and might therefore constitute a valuable pharmacological tool to address physiological functions of KCa1.1 channels.

Molecular Pharmacology Fast Forward. Published on July 16, 2007 as DOI: 10.1124/mol.107.038331
This article has not been copyedited and formatted. The final version may differ from this version.

Materials and methods

Expression in *Xenopus laevis* oocytes

Female *Xenopus laevis* frogs were anaesthetized (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. Following manual dissection of the oocytes into smaller groups, the oocytes were defolliculated by enzymatic treatment with collagenase (C0130, Sigma-Aldrich, Vallensbaek Strand, Denmark) for one hour. Oocytes were then kept in Kulori solution (90 mM NaCl, 4 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 5 mM HEPES, pH 7.4) at 19 ^oC for 24 hours 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 m-Message Machine kit (Ambion, Austin, TX, USA) according to the manufacturer's instructions. RNA concentrations were quantified by UV spectroscopy and RNA quality was checked by gel electrophoresis. 50.6 nl cRNA (5-50 ng) was injected using a Nanoject microinjector (Drummond, Broomell, PA, USA). Oocytes were kept in Kulori at 19 ^oC which was changed daily and currents were recorded after 2-5 days.

Cell cultures

Monoclonal HEK-293 cells stably expressing hKCa1.1 were grown in Dulbecco´s Modified Eagle Medium (DMEM), 10% fetal calf serum (Invitrogen, USA) with glutamax supplemented (Substrate Department, the Panum Institute, Denmark) and incubated at 37^o C in 5% CO_2. On the day of patch clamp experiments the cells were

Molecular Pharmacology Fast Forward. Published on July 16, 2007 as DOI: 10.1124/mol.107.038331
This article has not been copyedited and formatted. The final version may differ from this version.

transferred to glass coverslips coated with poly-L-lysin (3.5 mm diameter) and allowed to attach to the coverslip for 1 hour.

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, USA). 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 *Xenopus 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 (OD/ID 1.5/0.75 mm) (WPI, Sarasota, USA) and had a resistance of around 5 MΩ. A system for rapid solution changes allowed application of drugs in the close vicinity of the patch (SmartSquirt, AutoMate Scientific, Berkeley, USA). Currents were recorded using a HEKA EPC9 amplifier (HEKA, Lambrecht/Pfalz,

Molecular Pharmacology Fast Forward. Published on July 16, 2007 as DOI: 10.1124/mol.107.038331
This article has not been copyedited and formatted. The final version may differ from this version.

Germany) low pass filtered at 2 kHz using an 8 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 4 channels. Transitions between open and closed state were determined by setting the threshold at half the unitary current amplitude. Single-channel unitary current 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 binned and a square root transformation of the ordinate (events) was used. The distributions were fitted with exponential density function by the method of maximum likelihood with either two or three exponentials as suggested by Sigworth and Sine (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 free Ca^{2+} calculated (EqCal, Biosoft, Cambridge, U.K.) 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, Lambrecht/Pfalz, Germany). 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

Molecular Pharmacology Fast Forward. Published on July 16, 2007 as DOI: 10.1124/mol.107.038331
This article has not been copyedited and formatted. The final version may differ from this version.

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 free calculated calcium were used for inside-out measurements: 140 mM KCl, 10 mM EGTA and 10 mM HEPES pH 7.2. $CaCl_2$ and $MgCl_2$ were added in concentrations calculated (EqCal; BioSoft, Cambridge, UK) to give a free Ca^{2+} concentration of ~0, 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 multi-barrel system connected to the flow system or perfusion system from AutoMate Scientific USA.

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; Bio soft, Cambridge, UK). 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.

Molecular Pharmacology Fast Forward. Published on July 16, 2007 as DOI: 10.1124/mol.107.038331
This article has not been copyedited and formatted. The final version may differ from this version.

Contaminating $I_{Ca,L}$ was blocked by nisoldipine (1 µM) (Sigma-Aldrich, Vallensbaek Strand, Denmark). 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, NY, USA).

Isolation of Single Ventricular Cardiomyocytes

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

Calculations

Data was acquired using Pulse software (HEKA, Munich, Germany) and analyzed using Igor Pro 4.04 software (WaveMetrics, OR, USA) ClampFit 9.0 or Graph Pad Prism (GraphPad Software Inc, CA, USA). All values are shown as means ± SEM. 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

$$\text{I/Imax (V)} = \frac{1}{1+\exp[(V-V_{0.5})/k]}$$ where I/Imax 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 experiments 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 (1-(3,5-Bis-trifluoromethyl-phenyl)-3-[4-bromo-2-(1H-tetrazol-5-yl)-phenyl]-thiourea) were synthesised at NeuroSearch A/S, Ballerup, Denmark. Chemical synthesis of NS11021 is outlined in Figure 1 and involves reaction between commercial 3,5-cis(trifluoromethyl)phenylisothiocyanate and 4-bromo-2-(1H-tetrazol-5-yl)-phenylamine (Postovskii and Golomolzin, 1970).

Molecular Pharmacology Fast Forward. Published on July 16, 2007 as DOI: 10.1124/mol.107.038331
This article has not been copyedited and formatted. The final version may differ from this version.

Results

1-(3,5-Bis-trifluoromethyl-phenyl)-3-[4-bromo-2-(1H-tetrazol-5-yl)-phenyl]-urea (NS11021) activates hKCa1.1 channels expressed in *Xenopus 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 *Xenopus 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 (figure 2A). 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 about 4.5 µA. Before and after application of KCa1.1 activator, current specificity was confirmed by adding 5 mM of the pore blocker tetraethylammonium (TEA) resulting in an easily revertible inhibition of the 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 mV to +100mV lasting for 5 s. As seen in figure 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 figure 2C. The voltage

Molecular Pharmacology Fast Forward. Published on July 16, 2007 as DOI: 10.1124/mol.107.038331
This article has not been copyedited and formatted. The final version may differ from this version.

protocol applied in these experiments is shown in figure 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 can bind to the channel in both open and closed confirmation

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 fifth second from a holding potential of -80 mV. A representative recording is demonstrated in figure 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 figure 3A by a 120 s clamp at -80 mV. NS11021 was added when the channel was clamped at -80mV. Increase in current amplitude was recorded at the first depolarizing step to +20 mV and compared to baseline level before the 120 s clamp to -80 mV. A representative example is depicted in figure 3B. Summarized data for both opened/closed channels and channels kept in closed conformation during application of NS11021 is depicted in figure 3C. 3 µM NS11021 increased hKCa1.1 current by 171 ± 31% for channels in repeatedly shifted between opened and closed conformation. In comparison, 3 µM NS11021 increased hKCa1.1 current by 187 ± 27% for channels kept

Molecular Pharmacology Fast Forward. Published on July 16, 2007 as DOI: 10.1124/mol.107.038331
This article has not been copyedited and formatted. The final version may differ from this version.

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

NS11021 enhances hKCa1.1 currents in HEK293 cells

To exclude that 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 were 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 fifth second by 500 ms voltage ramps (-120 mV to +50 mV). Addition of NS11021 increased the current amplitude measured at 50 mV, and caused a leftward shift in the threshold for current activation (figure 4A). The time dependent effect of NS11021 is displayed in figure 4B. Data show the current amplitude from the same experiment as in figure 4A as a function of time. Application of NS11021 to the extracellular side of the membrane significantly increased the current amplitude already at 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).

Molecular Pharmacology Fast Forward. Published on July 16, 2007 as DOI: 10.1124/mol.107.038331
This article has not been copyedited and formatted. The final version may differ from this version.

Effect of NS11021 on hKCa1.1 current-voltage relationship and voltage dependence of activation.

The current traces in figure 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-dependency of steady-state channel activation as is depicted in figure 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 mV 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 around +70 mV under control situation (figure 5A) and at +30 mV after application of 1μM NS11021 (figure 5B). At potentials between +30 mV and +150 mV NS11021 augments the steady-state current amplitude, whereas no additional effect on the amplitude is seen at +150 mV to +190 mV**.** Figure 5C summarizes the current-voltage (I-V) relationship in 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 figure 5D illustrates that addition of NS11021 increased the peak

Molecular Pharmacology Fast Forward. Published on July 16, 2007 as DOI: 10.1124/mol.107.038331
This article has not been copyedited and formatted. The final version may differ from this version.

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 figure 5 it appears 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 100nM. Patches expressing hKCa1.1 were clamped for 75 ms at potentials between -150 mV 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. 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 (figure 6).

NS11021 can activate hKCa1.1 channels independently of the free intracellular Ca^{2+} concentration

Since Ca^{2+} modulates the activity of KCa1.1 channels, it was investigated whether the effect of NS11021 was calcium-dependent (figure 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

Molecular Pharmacology Fast Forward. Published on July 16, 2007 as DOI: 10.1124/mol.107.038331
This article has not been copyedited and formatted. The final version may differ from this version.

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, figure 6) the $V_{0.5}$ is shifted to more negative potentials compared to Ca^{2+} free conditions. However, application of NS11021 shifts the $V_{0.5}$ (129.9±3.5 mV in the absence 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 to 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→36.8±2.9 mV in the absence and $\Delta V_{0.5}$= -23.1 mV in the presence of 10 μM NS11021). Since the degree of activation of KCa1.1 channels is different in the absence of Ca^{2+} compared to 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

In order 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 macro-patches excised from *Xenopus laevis* oocytes expressing hKCa1.1. The bathing (intracellular) 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

Molecular Pharmacology Fast Forward. Published on July 16, 2007 as DOI: 10.1124/mol.107.038331
This article has not been copyedited and formatted. The final version may differ from this version.

application of 3 μM NS11021. Representative current traces are shown in figure 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 is depicted in figure 8B. From the current traces in figure 8A and 8B and from the summary of similar experiments in figure 8C it is clearly shown that 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 application of 3μM NS11021, in symmetric potassium concentrations. Single-channel slope conductance obtained from pooled unitary current-voltage relationships (figure 8D) were 224 ± 7 pS before and 223 ± 6 pS during application of 3μM NS11021. Thus KCa1.1 channel conductance values were not significantly altered by application of 3 μM NS11021.

From a single recording at 80 mV, the open and closed times were studied (figure 9). Open times were best fitted to a double exponential function and 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 application of NS11021 3 μM clearly shifted the distribution of the three time constants. The slowest component was completely abolished and the relative contribution of the fast and intermediate were shifted towards the fastest components.

Selectivity towards other ion channels

The selectivity of NS11021 toward other ion channels was also characterized. Kv7.1-4, Kv1.4-5, Kv4.3, Kir2.1-3 and Kv11.1 were expressed in *Xenopus laevis* oocytes and

Molecular Pharmacology Fast Forward. Published on July 16, 2007 as DOI: 10.1124/mol.107.038331
This article has not been copyedited and formatted. The final version may differ from this version.

studied using two-electrode voltage-clamp. Channel activation was obtained by voltage step protocols as indicated in figure 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 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, Kv11.1 and Kir2.2 currents were observed after 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 acethylcholine 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 min 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 pA/pF to -15.6±2.5 pA/pF for $I_{Ca,L}$ and -14.4±3.5 pA/pF to -13.2±3.0 pA/pF for I_{Na} n=6 and 4). Moreover, NS11021 did not affect $I_{Ca,T}$ as seen by similar

Molecular Pharmacology Fast Forward. Published on July 16, 2007 as DOI: 10.1124/mol.107.038331
This article has not been copyedited and formatted. The final version may differ from this version.

appearance of the shoulder (seen from -40 to -20 mV) on the current-voltage relationships before and after application of 30 μM NS11021 (figure 11).

Molecular Pharmacology Fast Forward. Published on July 16, 2007 as DOI: 10.1124/mol.107.038331
This article has not been copyedited and formatted. The final version may differ from this version.

Discussion

Selective molecules that block or activate ion channels are important biological tools since 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 thio-urea 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 single-channel level this is accomplished by as 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 imply 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 that the increase in open probability cannot simply be explained by an increased affinity for Ca^{2+}. Also the mechanism of action for NS11021 must involve the voltage dependent gating of the channel. In the opposite situation when all high affinity

Molecular Pharmacology Fast Forward. Published on July 16, 2007 as DOI: 10.1124/mol.107.038331
This article has not been copyedited and formatted. The final version may differ from this version.

sites for Ca^{2+} are occupied (100μM free Ca^{2+}) 10μM 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 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 overlap 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 BMS204352, when whole-cell current amplitudes were examined (Gribkoff et al., 2001). NS11021 belongs to the group of KCa1.1 channel openers, which include NS1608 (Strobaek 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).

Molecular Pharmacology Fast Forward. Published on July 16, 2007 as DOI: 10.1124/mol.107.038331
This article has not been copyedited and formatted. The final version may differ from this version.

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 to the small-conductance and intermediate-conductance Ca^{2+} activated potassium channels. Therefore we examined the effect of NS11021 on cloned voltage sensitive ions channels, with emphasis on voltage dependent potassium channels and other cardiac ion channels. In the present study it was shown that KCa1.1 current is significantly increased by NS11021 in as low concentrations as 0.3 μM. We found that 30 μM NS11021 did not affect native Ca^{2+} and Na^+ currents recording from isolated guinea pig cardiomyocytes or heterologous expressed Kv1.4-5, Kv4.3, Kv7.1, Kir2.2 and Kv11.1 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, 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 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. Except for the increase in Kv7.4 current and 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 (Schroder 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 non-excitable cells and play an essential role in the regulation of cell excitability and function. Considering the

Molecular Pharmacology Fast Forward. Published on July 16, 2007 as DOI: 10.1124/mol.107.038331
This article has not been copyedited and formatted. The final version may differ from this version.

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 (Douglas et al., 2006; Xu et al., 2002) 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 to the most broadly applied KCa1.1 opener NS1619. In addition, NS11021 is more selective compared to NS1619. We therefore believe that

Molecular Pharmacology Fast Forward. Published on July 16, 2007 as DOI: 10.1124/mol.107.038331
This article has not been copyedited and formatted. The final version may differ from this version.

NS11021 will be a valuable tool compound for addressing the physiological functions of KCa1.1 channels in the future.

Molecular Pharmacology Fast Forward. Published on July 16, 2007 as DOI: 10.1124/mol.107.038331
This article has not been copyedited and formatted. The final version may differ from this version.

Acknowledgements

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

References

Christ,T., Wettwer,E., and Ravens,U. (2005). Risperidone-induced action potential prolongation is attenuated by increased repolarization reserve due to concomitant block of I(Ca,L). *Naunyn Schmiedebergs Arch. Pharmacol.* **371**, 393-400.

Dick,G.M., Rossow,C.F., Smirnov,S., Horowitz,B., and Sanders,K.M. (2001). Tamoxifen activates smooth muscle BK channels through the regulatory beta 1 subunit. *J Biol. Chem.* **276**, 34594-34599.

Douglas,R.M., Lai,J.C., Bian,S., Cummins,L., Moczydlowski,E., and Haddad,G.G. (2006). The calcium-sensitive large-conductance potassium channel (BK/MAXI K) is present in the inner mitochondrial membrane of rat brain. *Neuroscience* **139**, 1249-1261.

Du,W., Bautista,J.F., Yang,H., ez-Sampedro,A., You,S.A., Wang,L., Kotagal,P., Luders,H.O., Shi,J., Cui,J., Richerson,G.B., and Wang,Q.K. (2005). Calcium-sensitive potassium channelopathy in human epilepsy and paroxysmal movement disorder. *Nat. Genet*. **37**, 733-738.

Gessner,G., Heller,R., Hoshi,T., and Heinemann,S.H. (2007). The amiodarone derivative 2-methyl-3-(3,5-diiodo-4-carboxymethoxybenzyl)benzofuran (KB130015) opens large-conductance Ca2+-activated K+ channels and relaxes vascular smooth muscle. *Eur. J Pharmacol.* **555**, 185-193.

Ghatta,S., Nimmagadda,D., Xu,X., and O'rourke,S.T. (2006). Large-conductance, calcium-activated potassium channels: structural and functional implications. *Pharmacol. Ther.* **110**, 103-116.

Gribkoff,V.K., Starrett,J.E., Jr., Dworetzky,S.I., Hewawasam,P., Boissard,C.G., Cook,D.A., Frantz,S.W., Heman,K., Hibbard,J.R., Huston,K., Johnson,G., Krishnan,B.S., Kinney,G.G., Lombardo,L.A., Meanwell,N.A., Molinoff,P.B., Myers,R.A., Moon,S.L., Ortiz,A., Pajor,L., Pieschl,R.L., Post-Munson,D.J., Signor,L.J., Srinivas,N., Taber,M.T., Thalody,G., Trojnacki,J.T., Wiener,H., Yeleswaram,K., and Yeola,S.W. (2001). Targeting acute ischemic stroke with a calcium-sensitive opener of maxi-K potassium channels. *Nat. Med* **7**, 471-477.

Hansen,R.S., Diness,T.G., Christ,T., Demnitz,J., Ravens,U., Olesen,S.P., and Grunnet,M. (2006). Activation of human ether-a-go-go-related gene potassium channels by the diphenylurea 1,3-bis-(2-hydroxy-5-trifluoromethyl-phenyl)-urea (NS1643). *Mol. Pharmacol.* **69**, 266-277.

Holland,M., Langton,P.D., Standen,N.B., and Boyle,J.P. (1996). Effects of the BKCa channel activator, NS1619, on rat cerebral artery smooth muscle. *Br. J Pharmacol.* **117**, 119-129.

Horrigan,F.T. and Aldrich,R.W. (2002). Coupling between voltage sensor activation, Ca2+ binding and channel opening in large conductance (BK) potassium channels. *J Gen. Physiol* **120**, 267-305.

Lu,R., Alioua,A., Kumar,Y., Eghbali,M., Stefani,E., and Toro,L. (2006). MaxiK channel partners: physiological impact. *J Physiol* **570**, 65-72.

Meredith,A.L., Thorneloe,K.S., Werner,M.E., Nelson,M.T., and Aldrich,R.W. (2004). Overactive bladder and incontinence in the absence of the BK large conductance Ca2+-activated K+ channel. *J Biol. Chem.* **279**, 36746-36752.

Olesen,S.P., Munch,E., Moldt,P., and Drejer,J. (1994). Selective activation of Ca(2+)-dependent K+ channels by novel benzimidazolone. *Eur. J Pharmacol.* **251**, 53-59.

Postovskii,I. and Golomolzin,B. (1970). Benzodiazines. XI. Covalent hydration in a series of benzosubstituted derivatives of tetrazolo[1,5-c]quinazoline. *Khimiya Geterotsiklicheskikh Soedinenii* **1**, 100-102.

Sakamoto,K., Nonomura,T., Ohya,S., Muraki,K., Ohwada,T., and Imaizumi,Y. (2006). Molecular mechanisms for large conductance Ca2+-activated K+ channel activation by a novel opener, 12,14-dichlorodehydroabietic acid. *J Pharmacol. Exp. Ther.* **316**, 144-153.

Salkoff,L., Butler,A., Ferreira,G., Santi,C., and Wei,A. (2006). High-conductance potassium channels of the SLO family. *Nat. Rev. Neurosci.* **7**, 921-931.

Schroder,R.L., Jespersen,T., Christophersen,P., Strobaek,D., Jensen,B.S., and Olesen,S.P. (2001). KCNQ4 channel activation by BMS-204352 and retigabine. *Neuropharmacology* **40**, 888-898.

Sigworth,F.J. and Sine,S.M. (1987). Data transformations for improved display and fitting of single-channel dwell time histograms. *Biophys. J* **52**, 1047-1054.

Strobaek,D., Christophersen,P., Holm,N.R., Moldt,P., Ahring,P.K., Johansen,T.E., and Olesen,S.P. (1996). Modulation of the Ca(2+)-dependent K+ channel, hslo, by the substituted diphenylurea NS 1608, paxilline and internal Ca2+. *Neuropharmacology* **35**, 903-914.

Werner,M.E., Zvara,P., Meredith,A.L., Aldrich,R.W., and Nelson,M.T. (2005). Erectile dysfunction in mice lacking the large-conductance calcium-activated potassium (BK) channel. *J Physiol* **567**, 545-556.

Xu,W., Liu,Y., Wang,S., McDonald,T., Van Eyk,J.E., Sidor,A., and O'Rourke,B. (2002). Cytoprotective role of Ca2+- activated K+ channels in the cardiac inner mitochondrial membrane. *Science* **298**, 1029-1033.

Zakharov,S.I., Morrow,J.P., Liu,G., Yang,L., and Marx,S.O. (2005). Activation of the BK (SLO1) potassium channel by mallotoxin. *J Biol. Chem.* **280**, 30882-30887.

Molecular Pharmacology Fast Forward. Published on July 16, 2007 as DOI: 10.1124/mol.107.038331
This article has not been copyedited and formatted. The final version may differ from this version.

Footnotes

The work was supported by The Danish National Research Foundation and by a grant from Danish Cardiovascular Research Academy (BHB).

Molecular Pharmacology Fast Forward. Published on July 16, 2007 as DOI: 10.1124/mol.107.038331
This article has not been copyedited and formatted. The final version may differ from this version.

Legends for figures

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

Figure 2. NS11021 activates hKCa1.1 currents in *Xenopus laevis* oocytes. (A) Two-electrode voltage-clamp current traces of KCa1.1 channels expressed in *Xenopus laevis* oocytes before and after 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. 5 mM TEA was applied before and after addition of activators to confirm KCa1.1 currents. (B) Voltage dependency of different concentrations of NS11021 was addressed by ramp protocols from -100 mV 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 1μA. 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).

Figure 3. NS11021 binds both to open and closed channels. Representative online recording from *Xenopus laevis* oocytes expressing KCa1.1 channels recorded using the two-electrode voltage-clamp technique. (A) The effect of 3 μM NS11021 when applied

Molecular Pharmacology Fast Forward. Published on July 16, 2007 as DOI: 10.1124/mol.107.038331
This article has not been copyedited and formatted. The final version may differ from this version.

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 -80mV 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).

Figure 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 insert before and after 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 6-11 cells measured at 50 mV. Bars represent SEM

Figure 5. Effect of NS11021 1μM on hKCa1.1 current-voltage relationship and voltage dependence of activation. Representative current traces elicited by the voltage protocol showed in the insert panel, before (A) and after application of NS11021 1 μM (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 100nM. (C) Summarized current voltage relationship of similar

Molecular Pharmacology Fast Forward. Published on July 16, 2007 as DOI: 10.1124/mol.107.038331
This article has not been copyedited and formatted. The final version may differ from this version.

experiments as A and B. Currents measured at steady-state were plotted against test potential in the absence (black squares) and presence of NS11021 1 μM (open square)**.** (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 ± SEM and n=7

Figure 6. Concentration-response relationship of NS11021. Using the inside-out configuration concentration-response experiments were performed with a constant intracellular free calcium concentration of 100nM. 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 mV 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).

Figure 7. NS11021 can activate KCa1.1 channels independently of the free intracellular Ca^{2+} concentration. Current-voltage relation obtained as in figure 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

Molecular Pharmacology Fast Forward. Published on July 16, 2007 as DOI: 10.1124/mol.107.038331
This article has not been copyedited and formatted. The final version may differ from this version.

in brackets are mean shift in half-activation potential $V_{0.5}$ ± standard deviation calculated using a Boltzmann fit. Bars represent SEM and n=6-10.

Figure 8. NS11021 increases the single-channel open probability and has no effect on single-channel conductance. Single hKCa1.1 currents recorded in in-side-out macropatch configuration from *Xenopus laevis* oocytes at +80, +60, +40, +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 ± SEM) 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 ± SEM and n=5. The slope conductance of KCa1.1 was found to be 224±7 pS before and 223±6 pS after application of 3 μM NS11021.

Figure 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.

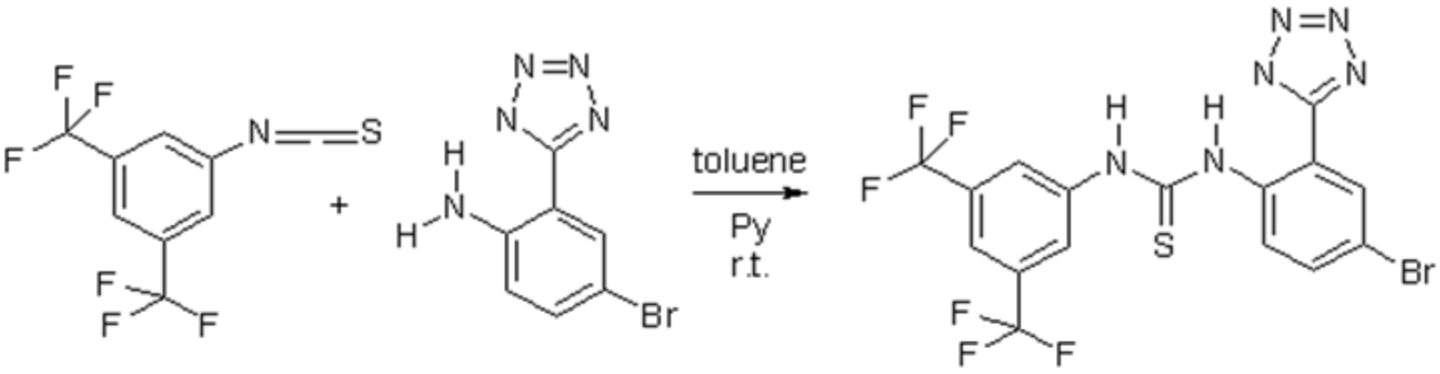
Molecular Pharmacology Fast Forward. Published on July 16, 2007 as DOI: 10.1124/mol.107.038331
This article has not been copyedited and formatted. The final version may differ from this version.

Figure 10. Specificity of NS11021. The activity of 30 μM NS11021 towards 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 +50mV was used to elicit Kir currents. Black lines represent control current traces whereas gray are after application of 30 μM NS11021. n=4-8.

Figure 11. Specificity of NS11021 on native currents. The effect of 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 addition of 30 μM NS11021.On the I-V curve a small shoulder is seen between -40mV and -20mV representing the $I_{Ca,T}$; the remaining current (peak at +5mV) 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 application of 30 μM NS11021 is depicted in (C) and the time course for peak I_{Na} in (D). Inserts 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 ± SEM and n=6.

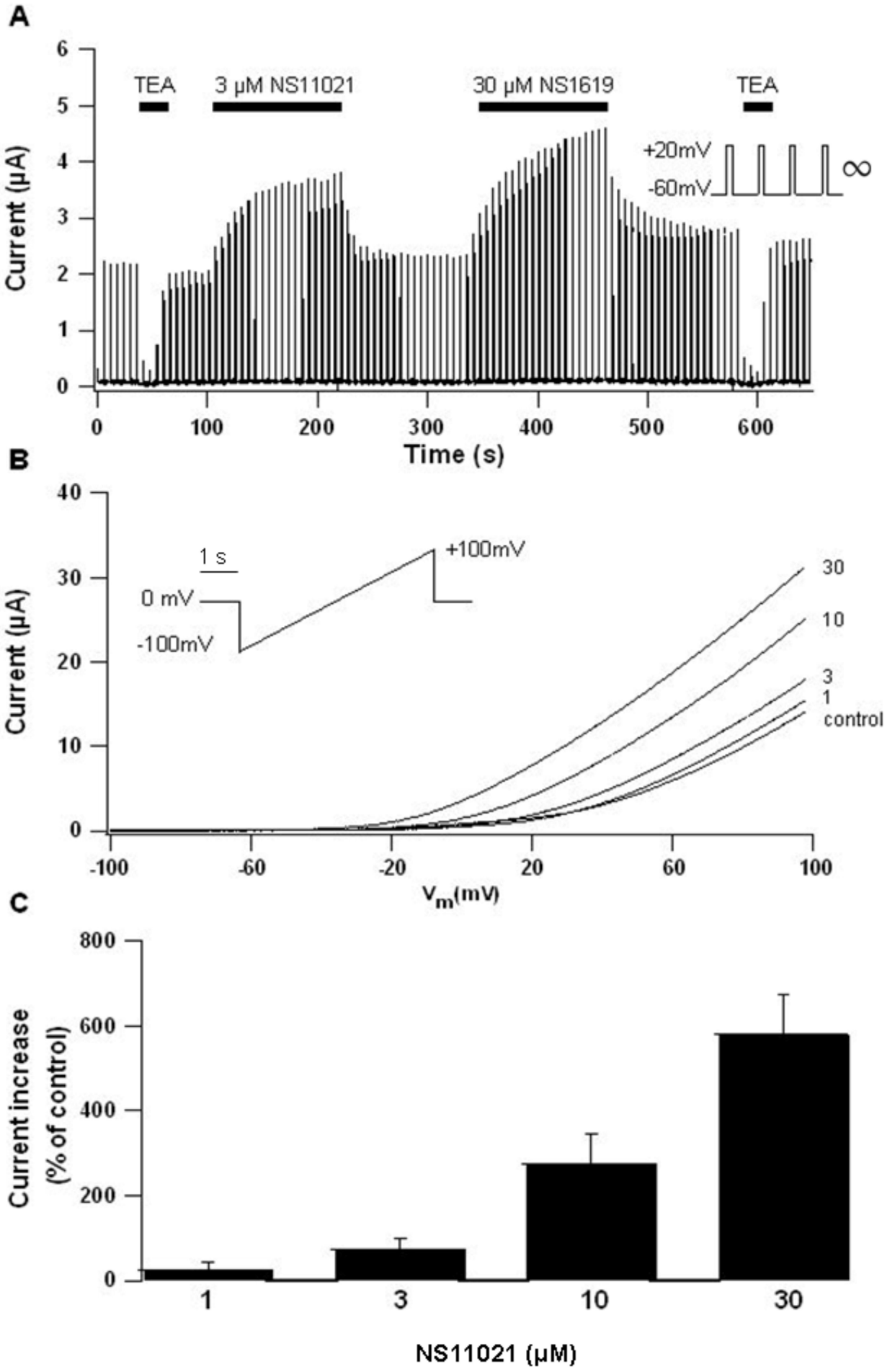
Molecular Pharmacology Fast Forward. Published on July 16, 2007 as DOI: 10.1124/mol.107.038331
This article has not been copyedited and formatted. The final version may differ from this version.

FIGURE 1



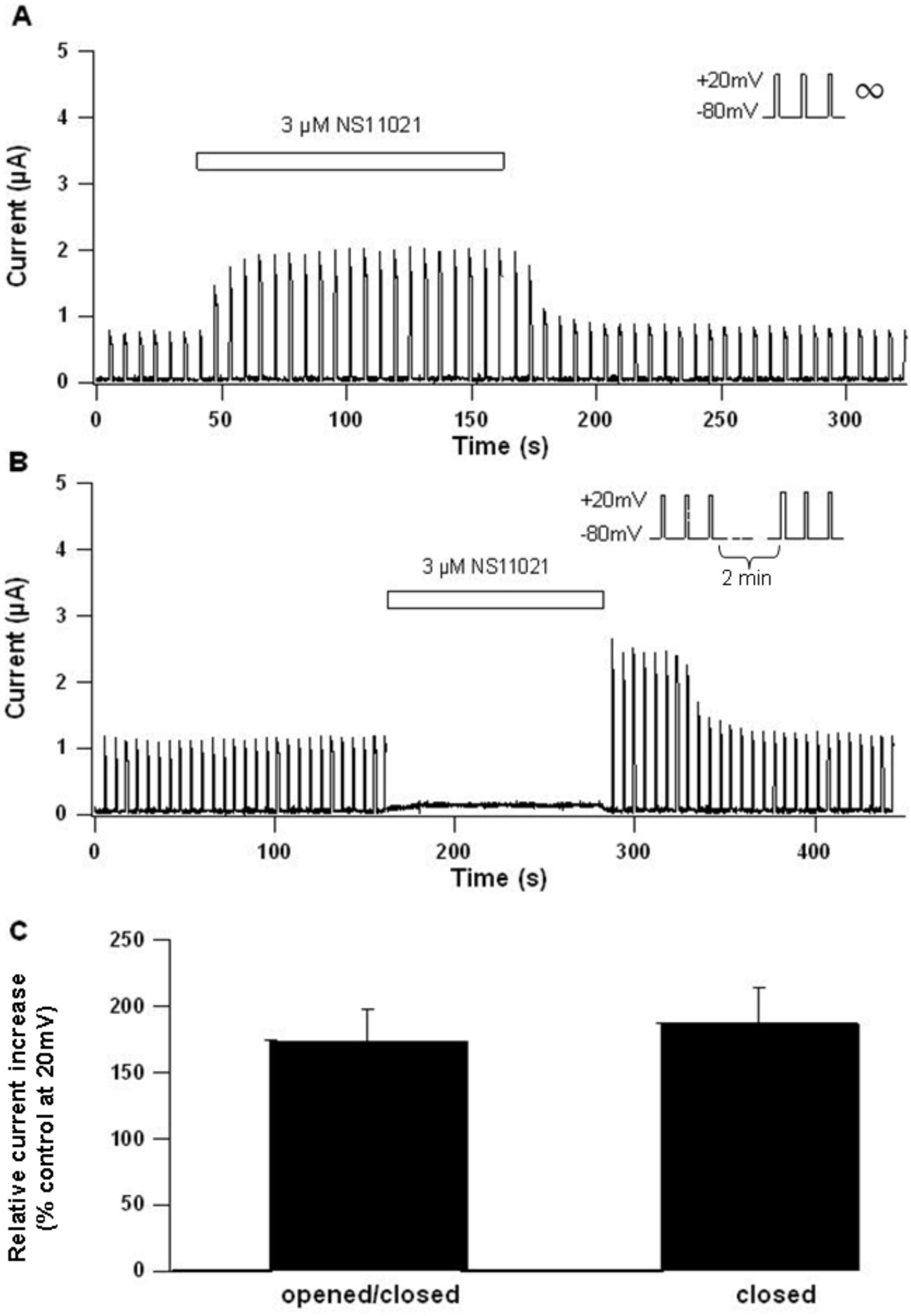
Molecular Pharmacology Fast Forward. Published on July 16, 2007 as DOI: 10.1124/mol.107.038331
This article has not been copyedited and formatted. The final version may differ from this version.

FIGURE 2



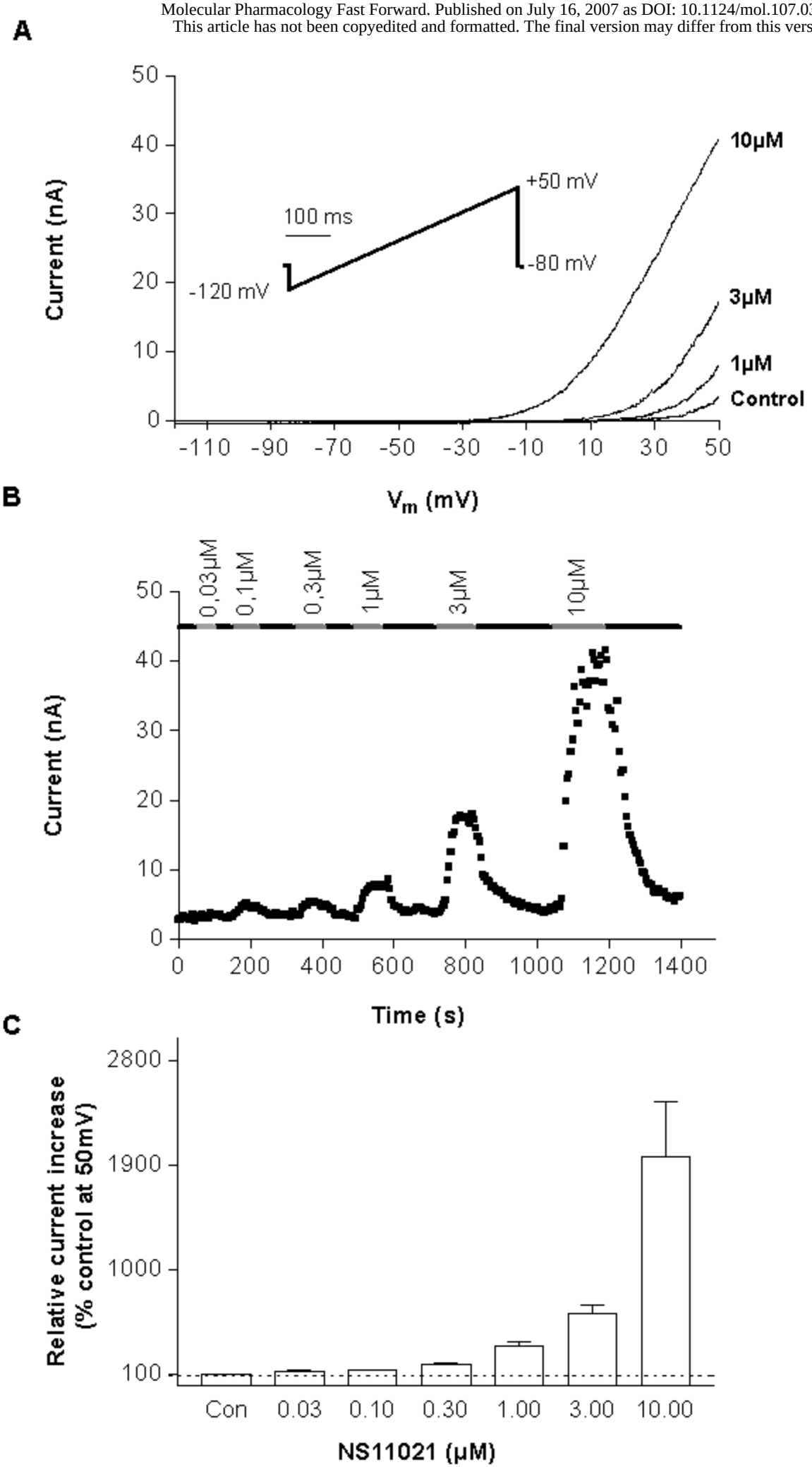
Molecular Pharmacology Fast Forward. Published on July 16, 2007 as DOI: 10.1124/mol.107.038331
This article has not been copyedited and formatted. The final version may differ from this version.

FIGURE 3



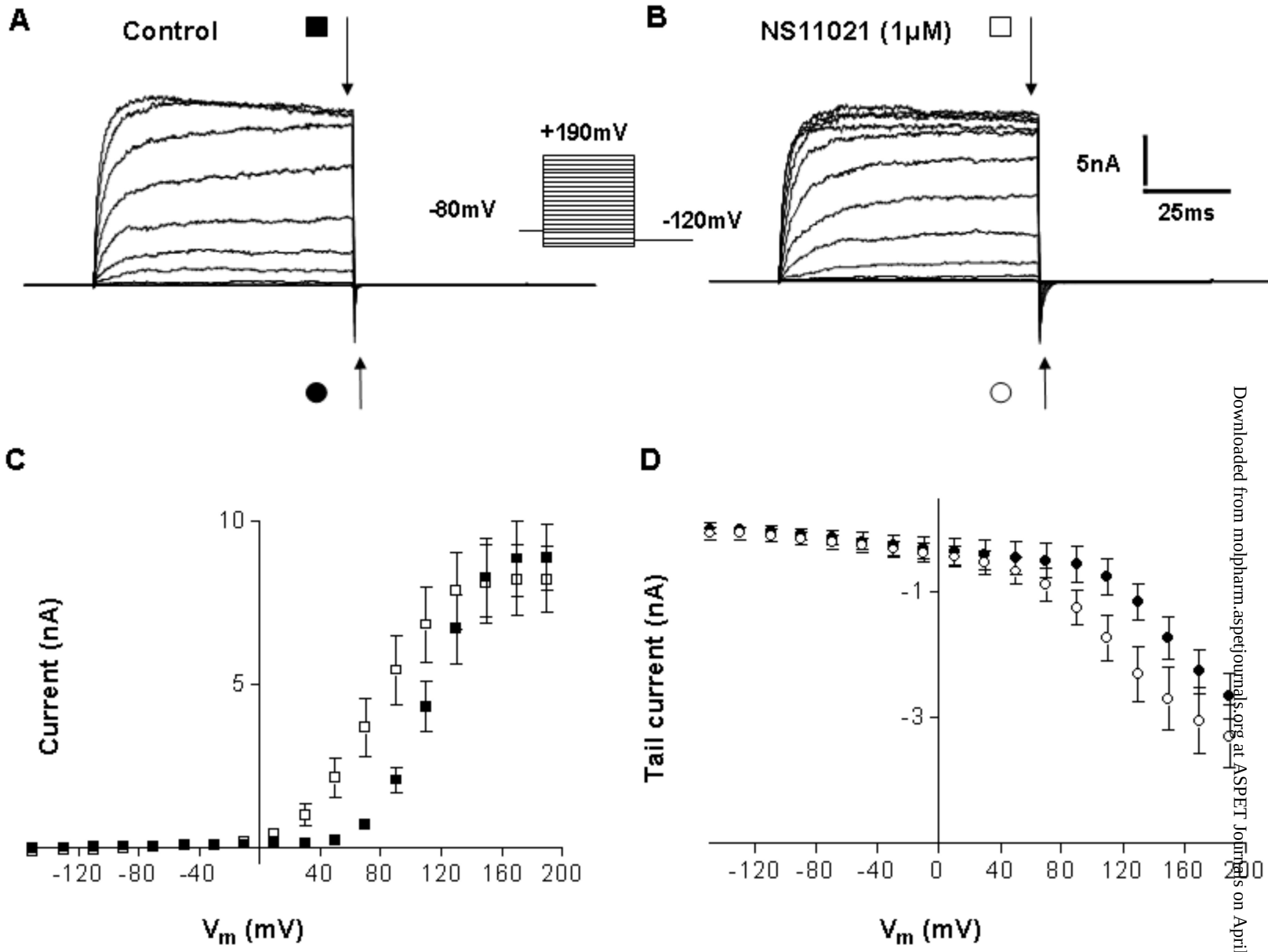
Molecular Pharmacology Fast Forward. Published on July 16, 2007 as DOI: 10.1124/mol.107.038331
This article has not been copyedited and formatted. The final version may differ from this version.

FIGURE 4



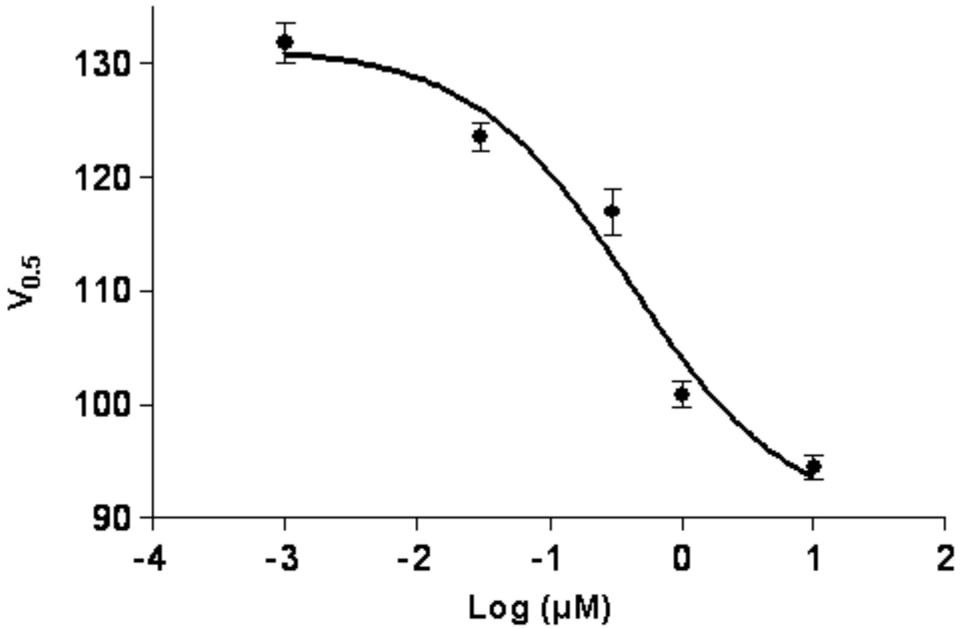
Molecular Pharmacology Fast Forward. Published on July 16, 2007 as DOI: 10.1124/mol.107.038331
This article has not been copyedited and formatted. The final version may differ from this version.

FIGURE 5



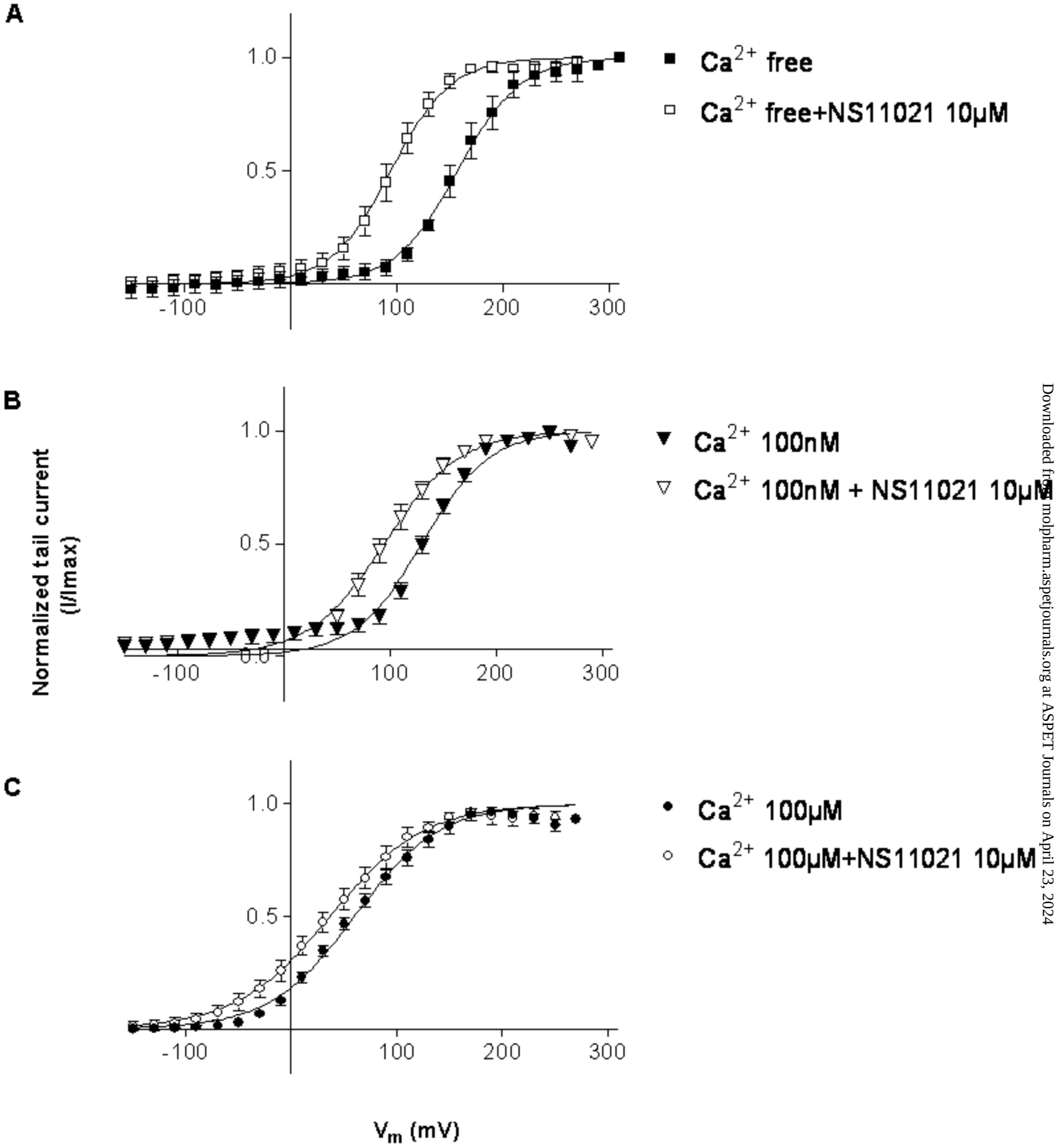
Molecular Pharmacology Fast Forward. Published on July 16, 2007 as DOI: 10.1124/mol.107.038331
This article has not been copyedited and formatted. The final version may differ from this version.

FIGURE 6



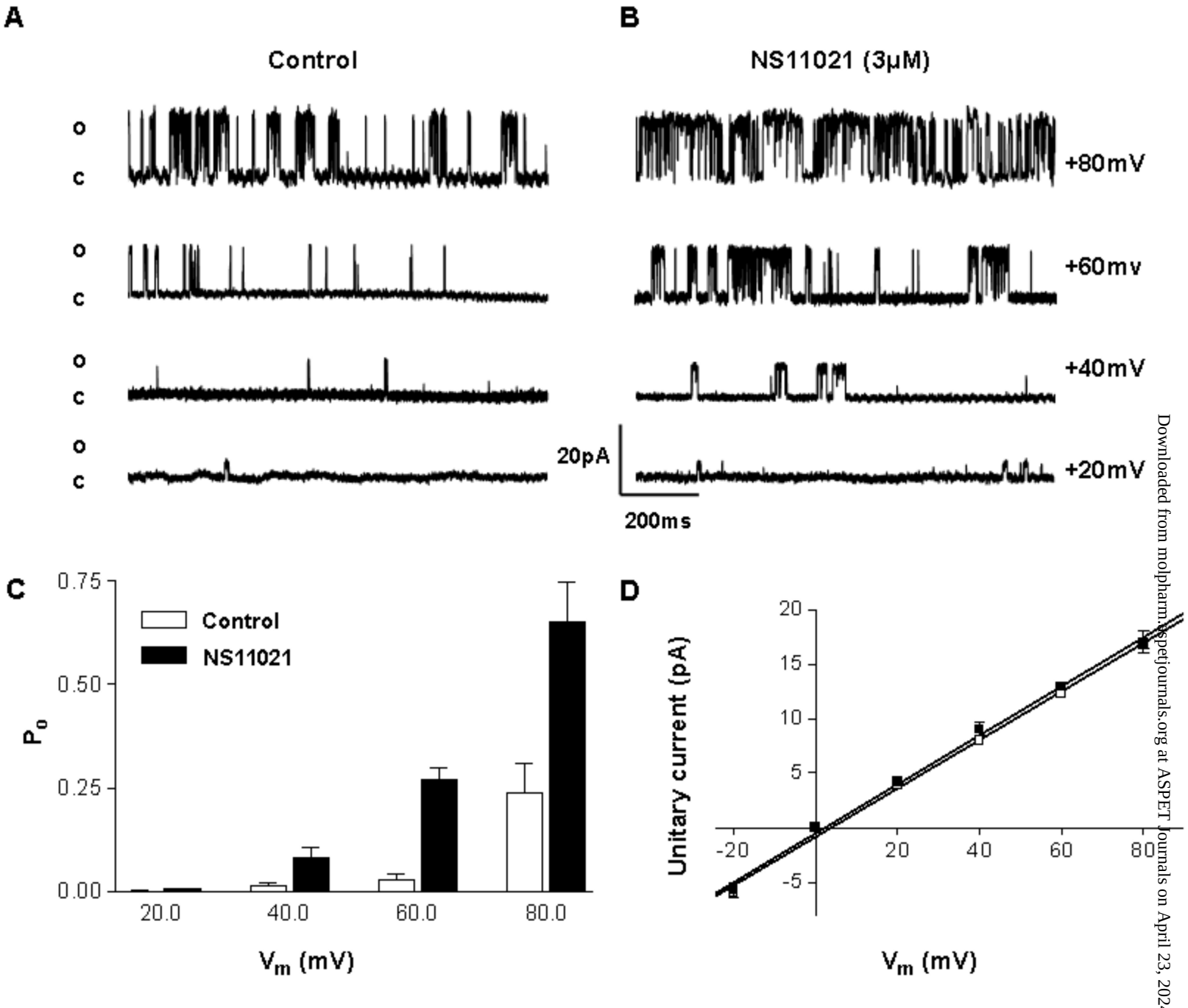
Molecular Pharmacology Fast Forward. Published on July 16, 2007 as DOI: 10.1124/mol.107.038331
This article has not been copyedited and formatted. The final version may differ from this version.

FIGURE 7



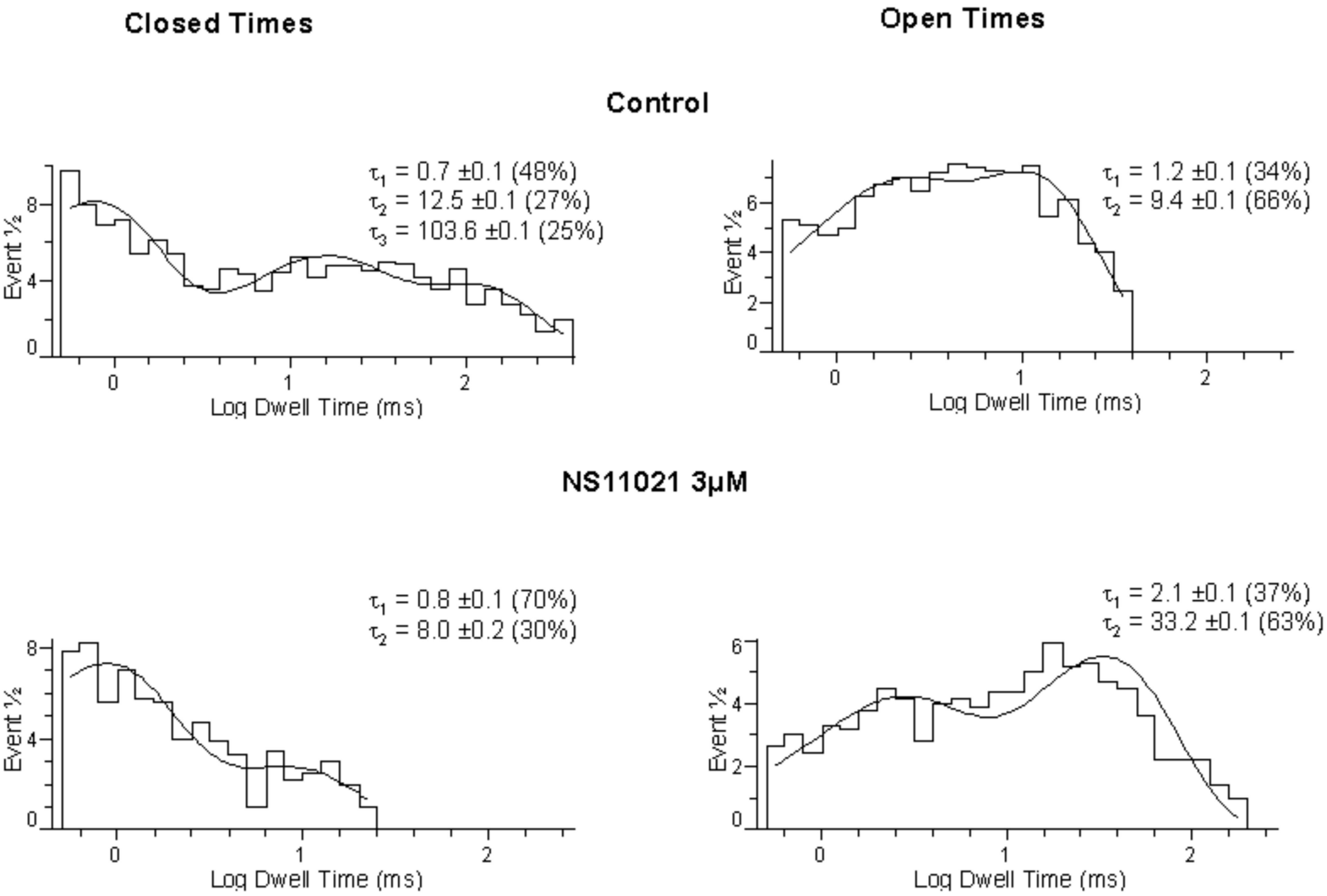
Molecular Pharmacology Fast Forward. Published on July 16, 2007 as DOI: 10.1124/mol.107.038331
This article has not been copyedited and formatted. The final version may differ from this version.

FIGURE 8



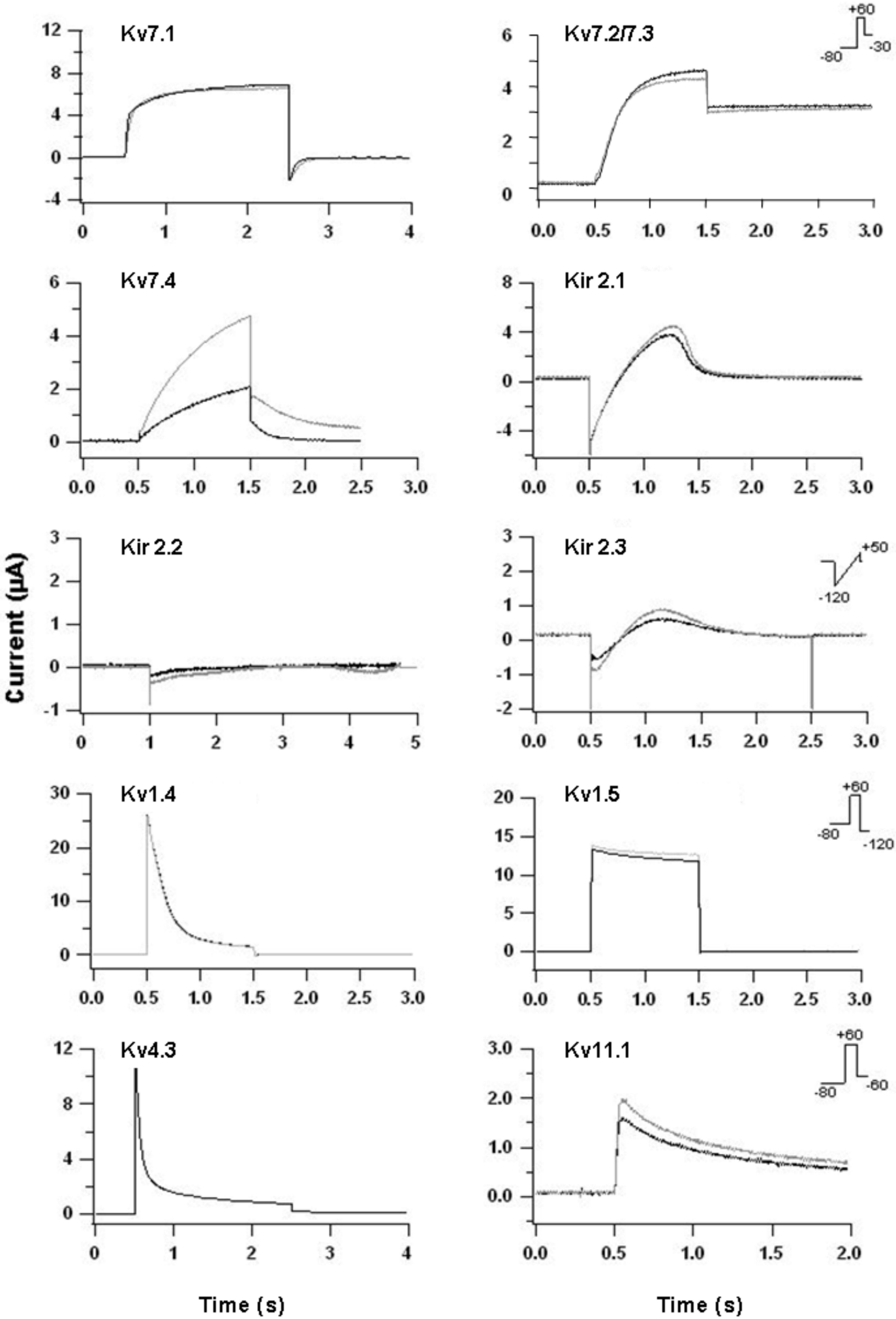
Molecular Pharmacology Fast Forward. Published on July 16, 2007 as DOI: 10.1124/mol.107.038331
This article has not been copyedited and formatted. The final version may differ from this version.

FIGURE 9



Molecular Pharmacology Fast Forward. Published on July 16, 2007 as DOI: 10.1124/mol.107.038331
This article has not been copyedited and formatted. The final version may differ from this version.

FIGURE 10



Molecular Pharmacology Fast Forward. Published on July 16, 2007 as DOI: 10.1124/mol.107.038331
This article has not been copyedited and formatted. The final version may differ from this version.

FIGURE 11