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Activation of HERG potassium channels by the diphenylurea NS1643

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Abrreviations:
HERG: human *ether-a-go-go* related gene, TdP: torsade de pointes, APD: action potential duration, EAD: early afterdepolarization, I_{Kr}: rapid delayed rectifier current, I_{Ks}: slow delayed rectifier current

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Abstract

The cardiac action potential is generated by a concerted action of different ion channels and transporters. Dysfunction of any of these membrane proteins can give rise to cardiac arrhythmias, which is particularly true for the repolarizing potassium channels. We suggest that an increased repolarization current could be a new anti-arrhythmic principle, since it possibly would attenuate after-depolarizations, ischemic leak currents and re-entry phenomena. Repolarization of the cardiac myocytes is crucially dependent on the I_{Kr} current conducted by ERG potassium channels. We have developed the diphenylurea compound NS1643 and tested whether this small organic molecule could increase the activity of human ERG (HERG) channels expressed heterologously. In *Xenopus laevis* oocytes NS1643 increased both steady-state and tail current at all voltages tested. The EC_{50} value for HERG channel activation was 10.5 μM. These results were reproduced on HERG channels expressed in mammalian HEK293 cells. In guinea pig cardiomyocytes, studied by patch-clamp, application of 10 μM NS1643 activated I_{Kr} and significantly decreased the action potential duration to 65 % of the control values. The effect could be reverted by application of 100 nM of the specific HERG channel inhibitor E-4031. Application of NS1643 also resulted in a prolonged post-repolarization refractory time. Finally, cardiomyocytes exposed to NS1643 resisted re-activation by small depolarising currents mimicking early after-depolarizations. In conclusion, HERG channel activation by small molecules such as NS1643 increases the repolarization reserve and presents an interesting new anti-arrhythmic approach.

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Introduction

The action potential initiates and controls the contraction of cardiac cells. The shape and duration of the action potential is the result of an ordered sequence of changes in membrane permeability to specific ions. The action potential duration (APD) and refractoriness are especially sensitive to the membrane permeability to potassium ions. Voltage-gated potassium channels are activated at different stages of the action potential including the early transient outward current (I_{to}) and ultra-rapid delayed rectifier current (I_{Kur}), as well as the late rapid delayed rectifier current (I_{Kr}) and slow delayed rectifier current (I_{Ks}) (Snyders, 1999). Most of the ion channels responsible for these currents will undergo inactivation during sustained depolarization. Release from inactivation is seen upon repolarization to the resting membrane potential. Inactivation is especially pronounced and fast for I_{Kr} current. This implies that I_{Kr} current contribution is almost negligible during the action potential plateau-phase, but very prominent during the repolarization and the start of the diastolic interval. The large I_{Kr} current at the early diastolic interval is a consequence of the slow deactivation kinetics of this channel (Hancox et al., 1998). I_{Kr} and I_{Ks} are well-characterized and can be separated based on their sensitivity towards organic and inorganic blockers (Sanguinetti and Jurkiewicz, 1990). It is now widely accepted that the I_{Kr} current is mediated by the potassium channel encoded by the human *ether-a-go-go* related gene (HERG) (Sanguinetti et al., 1995) whereas I_{Ks} is constituted by the KCNQ1 α-subunits together with KCNE1 (minK) β-subunits (Barhanin et al., 1996;Sanguinetti et al., 1996). It has been suggested that either KCNE1 or KCNE2 (MiRP1) β-subunits are obligatory components of native I_{Kr} (McDonald et al., 1997). This is however still controversial (Abbott et al., 1999;McDonald et al., 1997;Weerapura et al., 2002) . Together I_{Ks} and I_{Kr} currents define the late repolarization phase of the cardiac action potential, and are mainly responsible for terminating the plateau phase.

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Most arrhythmias originate from disturbances in the function of ion channels that generate the normal action potentials. Class III anti-arrhythmic drugs block cardiac potassium channels and prolong the duration of the action potential. As a result, a longer effective refractory period is obtained and the likelihood of re-entry is diminished. Anti-arrhythmic drugs that compromise HERG channel function without effect on Ca^{2+} channels such as dofetilide or d-sotalol introduce a risk of pro-arrhythmic events since they reduce the repolarization reserve and may therefore lead to increased susceptibility to ventricular arrhythmias and eventually to sudden cardiac death. Pharmacological block of HERG channels or loss-of-function mutations prolong APD, which is reflected on the ECG as a longer QT interval. Ventricular action potential prolongation can develop into Torsade-de-Pointes (TdP) arrhythmia and ventricular fibrillation ((Metzger and Friedman, 1993;Monahan et al., 1990;Pohjola-Sintonen et al., 1993;Sanguinetti and Jurkiewicz, 1990;Woosley et al., 1993).

Opening of HERG channels could thus present a novel anti-arryhythmic principle, and several genetic studies support this notion. It has been demonstrated that increasing I_{Kr} by over-expression of HERG, either by adenoviral transfer in rabbit (Nuss et al., 1999) and guinea pig (Hoppe et al., 2001) or by transgenic modification in mouse (Royer et al., 2005), significantly shortens APD, increases the refractory period of cardiac tissue, and suppresses electrical alternans in dog (Hua et al., 2004). These are all indications that point towards an increase of the HERG current as a beneficial anti-arrhythmic approach.

In a patent application submitted in 2003 we reported the first examples of small molecule HERG channel activators (Olesen S.P et al., 2005Priority date 04-11 2003). In the meantime, another such compound, has been shown to open HERG channels, shorten the QT interval, and increase T-wave amplitude (Kang et al., 2005).

In the present work we have applied a new pharmacological approach to this potential anti-arrhythmic treatment by identifying the bis-phenol NS1643 as a small molecule activator of the HERG channel. Our objective was to thoroughly examine the effect of this agent on heterologously expressed HERG channels and to test whether the drug has any effect on action potentials generated by native cardiomyocytes. The pharmacological impact of this compound supports the idea of HERG channel activation as a new anti-arrhythmic approach.

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Materials and Methods

Chemical synthesis

NS1643 (1,3-Bis-(2-hydroxy-5-trifluoromethyl-phenyl)-urea) was synthesized at *NeuroSearch A/S.* The symmetrical bis-phenol NS1643 was synthesized in two steps from 2-methoxy-5-trifluoromethylaniline as shown in Figure 1. Reaction of two equivalents of the aniline with phosgene provided the urea (1), which was treated with BBr_3 in dichloromethane to furnish NS1643 in 80 % overall yield.

Molecular biology

cDNA encoding HERG (KCNH2, Kv11.1) channels was introduced into the custom-made vector pXOOM, which is optimized for expression in both *Xenopus laevis* oocytes and mammalian cells (Jespersen et al., 2002).cRNA preparation and capping were performed by *in vitro* transcription using the mCAP mRNA capping kit (Stratagene, La Jolla, CA, USA) or Ambion T7 m-Message Machine kit (Ambion, Woodward Austin, TX, USA) according to the manufacturer's instructions. mRNA was phenol/chloroform extracted, ethanol precipitated and dissolved in TE buffer to approximate concentrations of 1 µg/µl. For proof of purity and integrity, mRNA was inspected by gel electrophoresis and concentrations were determined photometrically. mRNA was stored at –80 ^{o}C until injection.

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Expression in Xenopus laevis oocytes and mammalian HEK293 cells

Xenopus laevis surgery and oocyte treatment were done as previously described (Grunnet et al., 2001). Oocytes were collected under anaesthesia (Tricain 2 g/l, Sigma A-5040) at guidelines approved by the Danish National Committee for Animal Studies. Before injection of 50 nl mRNA (approximately 50 ng), oocytes were kept for 24 hours at 19 ^{o}C in Kulori medium consisting of (in mM) 90 NaCl, 1 KCl, 1 $MgCl_2$, 1 $CaCl_2$, 5 HEPES, pH 7.4 with NaOH. Injection of mRNA was accomplished using a Nanoject microinjector from Drummond (Drummond Scientific, Broomall, PA, USA). Oocytes were kept at 19 ^{o}C for 2-5 days before measurements were performed.

Herg/MinK expression in HEK293 cells

HEK293 cells stably transfected with the Herg/MinK complex were used for patch clamp experiments. The cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) with Ultraglutamin 1 (BioWhittaker, Walkersville, MD) supplemented with 10 % foetal calf serum (FCS) at 37° C in 5 % CO_2.

Isolation of single ventricular cardiomyocytes

Ventricular cardiomyocytes were isolated using a method slightly modified from (Mitra and Morad, 1985). Perfusion velocity was for all solutions 10-15 ml/min and all solutions heated to 37 ^{o}C and filtered before use. All solutions were filtered before use. Guinea pigs were anaesthetized with intra-peritoneal injection of pentobarbital (50-75 mg/kg). In addition, 1 ml/kg of heparin (1000 i.e./ml) was injected in vena femoralis. Respiration was maintained by artificial ventilation through a cannula in trachea (volume 12 ml/kg, rate 60 strokes/min). Upon thoracotomy, a perfusion cannula was inserted and fixed in aorta for retrograde perfusion in a simplified Langendorff set-up. To release intra cardiac

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pressure a small incision was made in the pulmonary artery. The heart was surgically removed from the thorax and perfused for 5 min with oxygenated (100 % O_2) Tyrodes solution (with Ca^{2+}) consisting of (in mM) 135 NaCl, 4 KCl, 1 $MgCl_2$, 0.33 NaH_2PO_4, 10 HEPES, 10 Glucose, 2 $CaCl_2$, pH 7.4 with NaOH. This was followed by 5 min perfusion with oxygenated (100 % O_2) no-Ca^{2+} Tyrodes. Perfusion was continued for 5 min with an oxygenated K^+ gluconate solution consisting of (in mM) 120 K^+gluconate, 20 NaCl, 1 $MgCl_2$, 10 HEPES, 10 Glucose, pH 7.4 with KOH. Glucose and K^+gluconate (28.10 g/l) were added just before use. Finally, enzymatic digestion was performed by perfusion with oxygenated (100 % O_2) K^+ gluconate solution containing 0.5 mg/ml collagenase (Type CLS-2, Medinova Scientific No LS004176) and 50 µM $CaCl_2$. The first 25 ml of this solution was discarded. The remaining solution was continuously recycled until the heart appeared swelled and glassy and perfusion velocity increased (typically obtained within 7-10 min.). Ventricular muscles were minced with scissors and dispersed with gentle agitation in oxygenated (100 % O_2) K^+ gluconate buffer containing collagenase and $CaCl_2$. Cells were filtered through an 80 mesh metal grid**,** centrifuged 400 rpm for 2 min and gently resuspended in K^+gluconate solution without collagenase and $CaCl_2$. Cells were stored at room temperature until use.

Electrophysiological recordings

Oocytes

Current through expressed HERG channels was monitored using a two-electrode voltage-clamp amplifier (Dagan CA-1B, Minneapolis, MN, USA). Electrodes were pulled from borosilicate glass capillaries on a horizontal patch electrode puller (DMZ universal puller, Zeitz instruments, München, Germany) and had tip resistance between 0.3 and 2.5 MΩ when filled with 1 M KCl. During the

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experiments oocytes were placed in a small chamber (volume: 200 µl) connected to a continuous flow system (flow: 3 ml/min.). HERG channels were activated by membrane depolarization and channel activity was measured in Kulori solution. The exact voltage protocols are indicated in respective figures. All experiments were performed at room temperature. The condition of each single oocyte was controlled before measurements by recording membrane potentials. Only oocytes with membrane potentials below –30 mV were used for current recordings.

HEK293 cells

All experiments were performed in whole-cell configuration, voltage clamp mode, at room temperature with an EPC-9 amplifier (HEKA Electronics, Lambrecht, Germany). Pipettes were pulled from thin walled borosilicate glass (ModelOhm, Copenhagen, Denmark) and had a resistance between 1.5 and 2.5 MΩ. A custom-made perfusion chamber (volume 15 µl) with a fixed AgCl-Ag pellet electrode was mounted on the stage of an inverted microscope. A coverslip with HERG transfected HEK293 cells was transferred to the perfusion chamber and superfused with physiological solution (low K^+) (in mM) 150 NaCl, 4 KCl, 2 $CaCl_2$, 1 $MgCl_2$ and 10 HEPES, pH 7.4 with NaOH. Pipettes were filled with solution consisting of (in mM) 144 KCl, 10 EGTA, 10 HEPES, 4 ATP, pH 7.2 with KOH. $CaCl_2$ and $MgCl_2$ were added in concentrations calculated (EqCal, BioSoft, Cambridge, UK) to give a free Mg^{2+} concentration of 1 mM and a free Ca^{2+} concentration of 100 nM. No zero current or leak current subtraction was performed during the experiments. Cell capacitance and series resistance was updated before each pulse application. Series resistance values were between 2.5 and 10.0 MΩ and only experiments where the resistance remained constant during the experiments were analyzed. Current

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signals were low-pass filtered at 3 kHz and acquired using Pulse software (HEKA Electronics, Lambrecht, Germany).

Native cardiomyocytes

Cells in suspension were transferred to cover slips and left for 15 min before recordings. Before performing patch clamp experiments, cells were transferred to the perfusion chamber and superfused for 5 minutes with K^+gluconate solution (in mM) 120 K^+gluconate, 20 NaCl, 1 $MgCl_2$, 10 HEPES, 10 Glucose, pH 7.4 with KOH before changing to Tyrodes solution (in mM) 135 NaCl, 4 KCl, 1 $MgCl_2$, 0.33 NaH_2PO_4, 10 HEPES, 10 Glucose, 2 $CaCl_2$, pH 7.4 with NaOH. The drugs were also added in this solution. Pipettes were filled with solutions identical to the one used for HEK293. Pipettes had a resistance between 1.5 and 2.5 MΩ. When the on-cell configuration was established, cells were held in the current clamp mode and current was injected until the whole cell configuration was obtained. Cells with a measured membrane potential, which deviated more than ±15 mV from –80 mV, were discarded. Initially current steps (lasting 2 ms) from 1000 to 5000 pA were applied in 500 pA increments. Having identified an appropriate amount of current injection necessary for evoking action potentials, all further action potentials were initiated by a pulse of 1.2 times the rheobase value. To mimic early and late afterdepolarizations repetitive current injections of approximately 0.5 times rheobase values were applied at an appropriate time after evocation of the first action potential. To assess the refractory period, a sequence of premature currents was injected as close to the repolarizing action potential as possible without prolonging action potential duration. Voltage signals were low-pass filtered at 3 kHz and acquired using Pulse software (HEKA Electronics, Lambrecht, Germany).

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Native cardiomyocytes for recordings of Ca^{2+} and Na^{+} current

Currents were measured with the single electrode voltage clamp method as described in detail elsewhere (Christ et al., 2005).

The pipette solution had the following composition (in mM), 90 Caesium methanesulfonate, 20 CsCl, 10 HEPES, 4 MgATP, 0.4 Tris-GTP, 10 EGTA and 3 $CaCl_2$, with calculated free Ca^{2+} concentration of ~60 nM (computer program EQCAL, Bio soft, Cambridge, UK; pH 7.2). Ca^{2+} currents were measured with Na^{+}-free superfusion solution (in mM), 120 Tetraethylammonium chloride, 10 CsCl, 10 HEPES, 2 $CaCl_2$, 1 $MgCl_2$ and 20 Glucose, pH 7.4 (adjusted with CsOH). $I_{Ca,L}$ was measured from a holding potential of -80 mV with test steps (200 ms) between –70 and +65 mV in 5 mV increments at 37°C. For measuring I_{Na} NaCl 5 mM was added to the superfusion solution and $CaCl_2$ was reduced to 0.5 mM. Contaminating $I_{Ca,L}$ was blocked by nisoldipine (1 µM). I_{Na} was measured at room temperature from a holding potential of -100 mV, with test steps (100 ms) between –80 and +5 mV in 5 mV increments. $I_{Ca,L}$ and I_{Na} amplitudes were determined as the difference between peak inward current and current at the end of the depolarizing step. A system for rapid solution changes allowed application of drugs in the close vicinity of the cells (Cell Micro Controls, Virginia Beach, VA; ALA Scientific Instruments, Long Island, NY, USA).

Drugs and solutions

Unless otherwise mentioned all chemicals were of analytical grade and were obtained from Sigma (St. Louis, MO, USA). Nisoldipine was a gift of Baver AG (Wuppertal, Germany). Drugs were dissolved in dimethylsulfoxide (DMSO) as concentrated stock solutions and diluted directly into the superfusion

solution to yield the final concentration. DMSO concentration never exceeded 0.1 % in final solutions. At this concentration no influence on any measurements were observed (*data not shown*).

Analysis of data

Data analysis and drawings were performed using IGOR software (WaveMetrics, Lake Oswego, OR, USA) or GraphPad Prism software (GraphPad Software, San Diego, CA, USA). All deviations of calculated mean averages are given as standard error of the mean (SEM) values. Significance was calculated as paired t-test or by ANOVA analysis.

Inactivation data were calculated as normalized peak current data as a function of the previous membrane potential. Data were normalized and fitted to a Boltzmann sigmoidal function: $I = I_{max} / (1 + \exp[(V_t - V_{50})/k])$, where I is the current, V_{50} is the voltage required for half activation, V_t is the test membrane potential, and k is the slope factor. Deactivation was calculated by fitting to the double exponential function: $I_{tail} = K_0 + K_{fast}*\exp-(t/\tau_{fast}) + K_{slow}*\exp(t/\tau_{slow})$, where t is time in seconds and fast and slow deactivation constants are determined by τ_{fast} and τ_{slow}, respectively.

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Results

A number of drugs and chemical compounds inhibit current flow through HERG (*Human Ether a-go-go* Related Gene) potassium channels, and it is generally agreed that these compounds may potentially evoke lethal cardiac arrhythmias. It has recently proven possible to synthesize compounds having the opposite effect i.e. compounds that increase HERG channel activity. Figure 1 shows the structure of such a compound, named NS1643, and the present work was devoted to a thorough *in vitro* characterization of its ability to open HERG channels.

To obtain information about the impact of 30 μM NS1643 on HERG channel activity, heterologous expression of HERG in *Xenopus laevis* oocytes was performed. Channel activity was initiated by different voltage clamp protocols. Figure 2A is a representative example of a HERG channel activated by 2 s voltage steps from a holding potential of -80 mV to potentials ranging from -80 to +60 mV. Increment between steps was +20 mV and tail current was recorded at -60 mV for 5 s. As expected, HERG channels responded to this protocol with a voltage-dependent activation followed by strong inactivation. As a consequence of the inactivation the IV curve recorded during the step protocol from –80 to +60 mV had a bell-shaped appearance with maximal amplitude at 0 mV (figure 2C). Release from inactivation is revealed by the instant large tail current observed upon repolarization to –60 mV. Summarized tail current data are shown in figure 2E. When HERG expressing oocytes were challenged by similar voltage protocols in the presence of 30 μM NS1643 an increase in current was obvious in both steady-state current and tail current (figure 2B, D and F). Bolzmann fits of activation revealed a half-maximal activation of -10.0 ± 2 mV for control experiments and -10.0 ± 3 mV for recordings in the presence of NS1643. Inactivation properties in the presence and absence of NS1643 were investigated by a complete activation and inactivation of the channels by clamping the

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membrane potential to +20 mV for 1 s. This was followed by brief (10 ms) hyperpolarized steps from -150 mV to +20 mV, before the potential was again clamped for 1 s at +20 mV. The 10 ms hyperpolarized step results in a release from inactivation, and is sufficiently short to prevent initiation of deactivation. From this protocol the voltage dependency of recovery from inactivation was determined by plotting the peak current recorded, at the second clamp to +20 mV, as function of the previous potential (figure 2G and 2H). Data were normalized and fitted to a Boltzmann function. In control oocytes V_{50} was -72.7 ± 2 mV and the slope factor 15.9 ± 2 mV. For oocytes exposed to NS1643 V_{50} was –63.7 ± 3 mV and the slope factor 16.8 ± 3 mV. Summarized data represent n = 6. Since it has been reported that compounds can have a different profile when tested in different expression systems, experiments were also conducted with HERG channels expressed in a mammalian cell system (figure 2I and 2J). The effect of NS1643 as a HERG channel activator was investigated in HEK293 cells stably expressing HERG channels together with the β-subunit KCNE1 (MinK). In initial experiments application of 30 μM NS1643 was performed, however, long lasting whole-cell recordings were difficult to obtain since seals were unstable in the presence of this compound concentration. Experiments were therefore performed at lower compound concentration than was applied in oocyte experiments. When stable control currents were obtained, 10 μM NS1643 was added to the bath. Application of the drug to the cells led to an increase of the peak tail current of 45.1 ± 10.1 % (n = 3), figure 2J. To confirm expression of HERG channels and to determine the magnitude of leak current experiments were completed by adding 100 nM of the specific HERG channel blocker E-4031, which in mammalian cells is sufficient to obtain a complete inhibition of HERG current (*data not shown*).

The effect of NS1643 on the tail current and deactivation kinetics at different voltages was monitored by a protocol whereby oocytes were fully activated and inactivated by clamping to +40 mV

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for 1 s, followed by steps from -130 to +40 mV lasting for 4 s. Increment between steps was 10 mV. Between steps oocytes were clamped for 3 s at –80 mV. Representative results are depicted in figure 3. Fast activation and inactivation was similar both in the presence and absence of 30 µM NS1643. In contrast, an increase in peak tail current amplitude was observed in the presence of NS1643 compared to controls. In addition, a right-ward shift was observed for maximal peak current amplitude in the presence of NS1643. In control situations the largest tail current could be recorded at -50 mV, while results obtained in the presence of NS1643 revealed a maximal tail current at -40 mV. Summarized data are plotted as IV curves (n = 6). The deactivation-kinetics for tail currents was calculated by fitting to a double exponential function. Summarized numbers for τ_{fast} and τ_{slow} values at preceding membrane potentials from –130 to +40 mV are depicted in figure 3E and 3F. No significant difference in deactivation kinetic could be observed after application of NS1643 even though there was a tendency towards decreasing τ_{fast} and τ_{slow} values in the presence of NS1643.

In an attempt to reveal the time-course for HERG activation by NS1643, oocytes were continuously challenged by a voltage protocol starting at +20 mV for 1 s followed by 3 s at –60 mV. Cells were clamped for 3 s at –80 mV between steps. Figure 4A demonstrates how HERG channels responded to a single pulse of this protocol. Current recorded at +20 mV was slightly increased in the presence of NS1643 compared to control. Tail currents recorded at –60 mV in the presence of NS1643 revealed a robust increase in current amplitude during the entire time-course of the deactivation. An online analysis of this protocol repeated continuously in depicted in figure 4B. Peak tail current as a function of time reveals how addition of 30 µM of NS1643 increases HERG current approximately 100 % within a time scale of roughly 200 s. Washing for 400 s only reduced the current level around 50 %. To confirm specificity, the experiment was finalized by addition of 10 µM of the HERG channel

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inhibitor haloperidol. A rapid decrease in current amplitude that could be reverted by washing was observed. In non-injected oocytes the applied voltage protocol only resulted in insignificant endogenous current activation of less than 200 nA in maximal amplitude.

The protocol applied in time-course experiments was also used in defining the EC_{50} value for HERG activation by NS1643. These studies were conducted with oocytes exposed to only a single concentration of NS1643 in the range from 1 to 300 µM. Results were monitored as increase in peak tail current. Data are summarized in figure 5A. As seen, saturation in increment of HERG current was observed at concentrations equal to or above 100 µM. Specificity of current was confirmed by application of 10 µM haloperidol or the more specific HERG channel inhibitor E-4031. This compound has both high specificity and affinity when applied in mammalian expression systems with complete block observed with less than 100 nM E-4031. The affinity for HE RG channels expressed in *Xenopus laevis* oocytes is however severely compromised. 100 nM E-4031 was not able to reduce HERG current and a 100-fold increase in concentration only reduced the current to 35 % of control value. Such change in affinity of E-4031 between expression systems has been reported before (Sanguinetti et al., 1995). The EC_{50} value for NS1643 activation of HERG channels was calculated to 10.5 ± 1.5 µM.

Since KCNQ1 channels and HERG channels are both major players in repolarization of the cardiac action potential as part of the I_{Ks} and I_{Kr} complexes, respectively, it is obvious to investigate if NS1643 could have any impact on KCNQ1 current. For this purpose, oocytes expressing KCNQ1 channels were activated by voltage ramps from –100 to +60 mV in 20 mV increments followed by tail recordings at –30 mV. As seen in figure 6A, characteristic slowly activating KCNQ1 currents were obtained. Application of 30 µM NS1643 resulted in a reduction in current amplitude without any obvious effect on kinetic parameters (Figure 6B). On average, application of 30 µM NS1643 reduced

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KCNQ1 current to 64.7 ± 9 % of control level (n = 5). Since the cardiac I_{Ks} current is composed of a channel complex containing both KCNQ1 and KCNE1 the ability of NS1643 to inhibit this current was also investigated. As can be seen in figure 6D, 6E and 6F a pronounced current inhibition of KCNQ1/KCNE1 current was observed. On average, application 30 µM NS1643 reduced KCNQ1/KCNE1 current to 34.5 ± 28.4 % of control level (n = 5). To further investigate the effect of NS1643 by simultaneously application to both KCNQ1 and HERG channels co-expression studies were performed in *Xenopus laevis* oocytes. Channel activation was achieved by a voltage protocol starting with 3 s at –80 mV, followed by 1 s at +10 mV and finalized with 3 s at –60 mV. With this protocol KCNQ1 contribution to the summarized current will be most prominent at +10 mV, due to the strong inactivation of HERG current at this potential, while the HERG current contribution will play have a large impact at –60 mV as a consequence of the release from inactivation. When NS1643 was applied to co-expressing oocytes a current reduction was observed at +10 mV while the tail current recorded at –60 was increased (figure 6G). The corresponding on-line analysis recorded at +10 mV and –60 mV respectively, are shown in figure 6H and 6I. These results demonstrate that the overall effect of NS1643 on action potentials recorded from native cells is likely to depend on the relative contribution of HERG and KCNQ1 to the repolarizing phase 3 current.

The selectivity of NS1643 towards other important cardiac potassium currents was also characterized. Kv4.3 potassium channels (representing I_{to}) and Kv1.5 potassium channels (representing I_{Kur}) were studied by expression of the human cloned genes in *Xenopus laevis* oocytes. Channel activation was obtained by voltage step protocols from –80 mV to +40 mV. 30 µM NS1643 inhibited Kv4.3 current to 84.6 ± 8 % of control levels when current was recorded at +40 mV (Figure 7A, 7B and 7C, n = 4). In contrast, no effect was observed on Kv1.5 currents (figure 7D, 7E and 7F, n = 3).

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The effect of NS1643 on L- and T-type calcium currents ($I_{Ca,L}$, $I_{Ca,T}$) as well as sodium currents (I_{Na}) was examined in experiments performed with native guinea pig ventricular cardiomyocytes. Five minutes after establishing access by whole cell patch clamping, the cells were exposed to 10 µM NS1643. $I_{Ca,L}$ exhibited a substantial and variable run-down over time when exposed the drug, however, this decline was not different compared to the spontaneous run-down in time-matched controls (i. e. from 12.9 ± 1.5 pA/pF to 10.8 ± 1.4, n = 11). $I_{Ca,L}$ density was 13.1 ± 2.1 pA/pF before and 10.7 ± 2.1 pA/pF after drug exposure ($P < 0.05$, n = 13). Moreover, NS1643 also did not affect $I_{Ca,T}$ (see figure 7G and 7H). Furthermore, we examined the effect of NS1643 on native sodium currents. I_{Na} was stable over time and the current density two minutes after exposure to NS1643 was not different from the respective control values (25.8 ± 3.9 pA/pF vs. 25.3 ± 4.1 pA/pF, n = 7) (figure 7I and 7J).

Since NS1643 was observed to activate HERG in both expression systems it was assumed that the drug would also affect the I_{Kr} of native cardiomyocytes. To test this hypothesis a series of patch clamps studies were performed on isolated guinea pig cardiomyocytes. Cells were held in current clamp mode and stimulated for 2 ms with 1-5 nA (1.2 times rheobase value) in order to induce action potentials. Upon application of 10 µM NS1643, action potential duration at 90 % repolarization (APD_{90}) was shortened significantly to 66 ± 8 % (n = 3) (figure 8) relative to control. This effect was reversed by application of 100 nM E-4031 (*data not shown*). Furthermore, it was observed that the shape of the action potential was not altered. From these results we concluded that the I_{Kr} of the native cardiomyocytes was activated by NS1643 resulting in a decrease in action potential duration, without any obvious effect on other ion channels involved in the action potential. Moreover, it was observed that the variability of the action potential duration was much more pronounced in control action

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potentials than in action potentials elicited after exposure to NS1643, indicating that action potentials are more uniform in the presence of 10 µM NS1643 (*data not shown*).

Due to the increase in repolarizing reserve we hypothesized that NS1643 would affect the refractory period of the guinea pig cardiomyocytes. To test whether this was true, action potentials were elicited as described earlier, and a second current of same magnitude was applied as close to the fully repolarized action potential as possible. The refractory period independently of APD was defined as the time-span from repolarization until it was again possible to elicit a new action potential as previously described (Nuss et al., 1999). As can be seen from figure 9, application of 10 µM NS1643 dramatically and significantly ($P < 0.01$) altered the refractoriness of the guinea pig cardiomyocyte from 19 ± 9 ms to 156 ± 36 ms ($n = 3$). Subsequent application of 100 nM E-4031 resulted in a prolonged action potential and a decrease in the post-repolarization refractory period, although the second stimulus was unable to elicit full size action potentials (fig. 9C).

Early after depolarizations (EADs) and triggered activity can be caused by reactivation of L-type Ca^{2+} channels and may occur when premature stimuli coincide with an action potential in the repolarizing phase. Together with a reduction of repolarizing current, increase of sodium-calcium exchanged currents and an increase in late sodium current, premature reactivation of L-type Ca^{2+} current is generally believed to be the one of the cellular mechanisms underlying initiation of Torsade de Pointes and related polymorphic tachycardias (Burashnikov and Antzelevitch, 1998;Nattel and Quantz, 1988;Patterson et al., 1997;Szabo et al., 1995). L-type Ca^{2+} channels are activated at membrane potentials around -30 mV, and since NS1643 was shown to hasten repolarization and increase refractoriness we hypothesized that application of NS1643 could counteract the effect of simulated EADs. To test whether this was true, cells were held in current clamp mode, action potentials

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were elicited and the cell was stimulated at APD_{90} with a sequence of 10-20 premature currents at 50 % of the stimulation current. The train of current injections was adjusted so it did not elicit an action potential when applied after the action potential had fully repolarized (figure 10A and 10C). As seen on figure 10B and 10E, a pronounced prolongation of the action potential was triggered in the control cells when the premature stimuli were applied at $APD_{90.}$ This effect was observed in all control experiments (n = 3). In contrast, when 10 µM NS1643 was applied, the action potential was only marginally affected by the premature sequence at APD_{90} (figs. 10D and 10E, n = 3), indicating that NS1643 stabilizes the action potential as an add-on to its prolongation of refractoriness. The effect seen with NS1643 was completely antagonized by application of E-4031 (*data not shown*).

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Discussion

In the present study the bis-phenol NS1643 was shown to activate cloned human ether-a-go-go related gene (HERG) channels and to abbreviate action potentials recorded from isolated cardiomyocytes. The reduction of the action potential duration was seen without other obvious changes in action potential shape, and the shortening was reversible upon application of the specific HERG blocking compound E-4031. These findings suggest that the molecular mechanism of action of NS1643 is to primarily activate the native HERG channels underlying the I_{Kr} of the cardiomyocytes, as was further supported by the selectivity data for the compound.

During the course of the cardiac action potential, the HERG channels open at membrane depolarizations postitive to -40 mV. The channel open state is however transient, and the inactivation time-constant is very fast (Kiehn et al., 1999;Zhou et al., 1998). As a consequence of this mechanism HERG channels only provide a small steady-state current during the action potential plateau phase (phase 2). When the membrane potential repolarizes, the HERG channels are released from inactivation. As a result a large peak current with a long-lasting decay is seen reflecting a slow deactivation time-constant. Exposure to NS1643 increased the HERG channel steady-state as well as the peak currents in a concentration-dependent manner. Comparison of kinetic parameters revealed a right-ward shift of V_{50} for inactivation of 9 mV in the presence of NS1643. Activation of the HERG steady-state current during the plateau-phase of an action potential would be expected to affect the time course of the plateau-phase, which was not seen in the present experiments. Several reasons can account for this. Firstly, increase of the steady-state current may not be prominent enough to affect the plateau-phase. Another possibility may be that the steady-state current of the native HERG channel is not affected as strongly as seen in experiments performed with cloned channels, which may be due to

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the presence of native subunits and/or other intracellular modulators such as described by (Sanguinetti and Jurkiewicz, 1990). Finally, the compound was also found to block the KCNQ1 current and blocking of this slowly activating current during the plateau-phase may counteract the activation of the HERG steady-state current.

Recently, Kang et al. (2005) presented the first activator of the HERG channel, RPR260243, which mediated its action by an extreme slowing of the channel deactivation, while the compound did neither affect the steady-state nor the peak tail current. In native cardiomyocytes 30 μM RPR260243 shortened the action potential duration by 12 %, whereas no APD shortening effect was observed using lower concentrations. The deactivation of the cloned HERG channel was on the other hand slowed down by 10 μM of the compound. In the present work, we showed that 10 μM NS1643 increased both the peak tail current and the steady-state current. At the same concentration, the APD measured in cardiomyocytes was abbreviated by 34 %. These findings suggest that the HERG peak tail current strongly influences the duration of the action potential and represents a repolarization reserve, whereas an increase in deactivation time does not affect the action potential duration to a similar extend. An alternative explanation could simply be that the two compounds have different affinity for the HERG channel.

Anti-arrhythmic drugs have been developed to specifically increase the action potential duration and thereby the effective refractory period by blocking K^+ channels. Increase of the refractoriness of the cardiac tissue will tend to break re-entry loops and has proven effective in preventing some atrial arrhythmias (Nattel, 2002). The drugs are however pro-arrhythmic in other patients, which is probably caused by their reduction in the repolarization capacity and following hyperexcitability. The present HERG channel opener has the opposite effect in increasing the repolarizing reserve and reducing hyperexcitability. NS1643 also increases the post-repolarization refractory period. Whether the

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compound will increase or decrease the effective refractory period cannot be determined based on the cellular studies, since the absolute APD in cultured cardiac myocytes may differ from that measured in tissue and tend to be slightly longer. The key feature of the HERG channel openers is thus to stabilize the myocytes during repolarization and the following period, and since their mechanism is very different form that of the class III compounds they are not expected to be associated with the same pro-arrhythmic potential. In fact, it has been shown that over-expression of HERG channels in mouse led to a smaller susceptibility to atrial and ventricular fibrillation (Royer et al., 2005), and very likely, the same may be the case for a HERG channel-activating agent.

A common trigger of cardiac arrhythmias is EADs. EADs are caused by reactivation of L-type Ca^{2+} channels during the plateau and repolarization phase of the action potential. EADs probably underlie the initiation of TdP and related polymorphic ventricular tachycardias (January and Riddle, 1989). EADs can be rescued by increment of the repolarizing current, which results in a decrease in the sensitivity towards triggered activity, and HERG channels have been suggested to play a specific role in suppressing arrhythmias initiated by EADs and premature beats (Smith et al., 1996). For example, it has been shown that HERG over-expression in rabbit cardiomyocytes reduces the susceptibility to EADs in cultured cardiomyocytes. In studies by (Nuss et al., 1999) it was demonstrated that the amount of HERG channels and thereby the size of the I_{Kr} is determining the speed of repolarization and the propensity of the cells to go into arrhythmias triggered by EADs. Based on patch clamp experiments and computer modelling Lu et al. (Lu et al., 2001) suggested that I_{Kr} would probably oppose the reactivation of the L-type Ca^{2+} channels and suppress sodium channel activation, resulting in a normal refractoriness of the cardiac tissue. In the present study, we demonstrated that an increase in the normal I_{Kr} current of native cardiomyocytes by addition of NS1643 could counteract the effect of a train of stimuli resembling EADs, which otherwise elicited an abnormal long action potential probably due to

reactivation of L-type Ca^{2+} channels. We therefore believe that pharmacological compounds with a HERG opener profile such as NS1643 could be beneficial as anti-arrhythmic drug preventing EADs.

In conclusion, we have described the abilities of the bis-phenol compound NS1643 to activate cloned and native HERG channels. This compound represents a new investigational tool for the study of cloned HERG channels and of I_{Kr} in cardiomyocytes. NS1643 increase both the steady-state and the peak tail current of the cloned HERG channels, gives a right-ward shift in V_{50} for inactivation, and furthermore slowed the deactivation of the tail current. In the native cardiomyocytes, the drug abbreviates action potential duration, increase the post-repolarization refractoriness and suppress hyperexcitability. The present data support the concept of HERG channel activators as a novel anti-arrhythmic principle.

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Figure Legends

Fig 1. Synthesis of NS1643 1,3-Bis-(2-hydroxy-5-trifluoromethyl-phenyl)-urea

Fig 2. The current amplitude of HERG channels expressed in *Xenopus laevis* oocytes is increased in the presence of NS1643. HERG channel activation was obtained by application of a step protocol from –80 mV to +60 mV with 20 mV increments between steps. Tail current was recorded at –60 mV. Representative traces are shown in A and B. The steady state current revealed strong inactivation as seen by the bell shaped IV curve (open squares, C), while a saturation in tail current was observed at potentials more positive than 10 mV (closed circles, E). Application of 30 μM NS1643 gave a robust increase in current amplitude in both steady state current (D) and tail current (F). Inactivation properties were addressed by a voltage protocol starting at +20 mV for 1 s, followed by 10 ms hyperpolarizing steps from -150 mV to +20 mV, before again clamping the potential at +20 mV for 1 s. Data were normalized and fitted to a Boltzmann function (G and H). Analysis of inactivation data in control oocytes revealed a V_{50} of -72.7 ± 2 mV and a slope factor of 15.9 ± 2. For oocytes exposed to NS1643 V_{50} was –63.7 ± 3 mV and the slope factor 16.8 ± 3 mV. The difference in V_{50} is significant ($p = 0.02$). For all summarized experiments n = 6. I and J shows the effect of NS1643 on HEK293 cells stably expressing the HERG channel and the KCNE1 subunit. Voltage clamp recordings were performed applying protocol outlined in A. When stable control currents were obtained, 10 μM NS1643 was added to the cells. Application of the drug lead to a 45.1 ± 10.1 % increase of the tail peak current and a 20 ± 11.9% increment of the steady state current measured at 0 mV relative to the control (n = 3).

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Fig 3. HERG tail current and deactivation kinetics in the presence of NS1643. HERG channels expressed in *Xenopus laevis* oocytes were activated and completely inactivated by holding at +40 mV for 1 s. Voltage-dependency of release from inactivation, in the presence and absence of NS1643, was studied by a tail protocol stepped from –130 to +40 mV. Current recorded at the peak of the tail resulted in a bell shaped IV curve with a maximum current at –50 mV for control experiments and -40 mV for recording in the presence of NS1643 (C and D). Application of 30 µM NS1643 gave approximately a current increase of 60 % at all voltage potentials (C and D). Deactivation was calculated by fitting to a double-exponential function. Summarized numbers for τ_{fast} and τ_{slow} values at preceding membrane potentials from –130 to +40 mV are depicted in E and F. No significant difference in deactivation kinetic could be observed after application of NS1643 even though there was a tendency towards decreasing τ_{fast} and τ_{slow} values in the presence of NS1643. (n = 6 for summarized data).

Fig 4. Time-course of HERG channel activation by NS1643. HERG channel expressed in *Xenopus laevis* oocytes were repeatedly activated by clamping 1 s at +20 mV followed by 3 s at –60 mV. Between steps cells were kept at –80 mV for 3 s. Current traces for a single step in the presence and absence of 30 µM NS1643 is demonstrated in A. The time-course for current recorded at the peak of the tail current (indicated by arrow), is depicted in B. From a stable level of around 4 µA, application of 30 µM NS1643 gave a current increase to about 8 µA. The effect of NS1643 could be partly reversed by washing. Specificity of HERG current was confirmed by application of 10 µM haloperidole.

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Fig 5. Concentration-response relationship of NS1643. Oocytes expressing HERG channels were activated by a continuously step protocol from +10mV to –60 mV and peak tail current recorded in the presence of different concentrations of NS1643. Only a single concentration of compound was applied to each single oocyte. HERG channel activity was in addition measured in the presence of the two HERG channel inhibitors haloperidole (10 µM) and E-4031 (100 nM and 10 µM) (A). Increase in HERG channel, activity as a function of the concentration of NS1643, was calculated to EC_{50} 10.5 ± 1. 5µM (B). Between 5 and 8 single oocytes were tested at every concentration of NS1643.

Fig 6. NS1643 inhibits KCNQ1 current. Oocytes expressing KCNQ1 channels were activated by step protocols from –100 mV to +60 mV. Current activity was measured in the absence (A) or in the presence (B) of 30 µM NS1643. IV curves are summarized in C. Using the same voltage protocol the effect of NS1643 on oocytes expressing both KCNQ1 and KCNE1 was addressed (D and E). IV curves are summarized in F. Dual effect of NS1643 on HERG and KCNQ1 current was investigated by co-expressing both channel types in oocytes. Current was activated by a repeated step protocol from +10 mV to –60 mV (G). Current trace in the absence of NS1643 is in black, while the current trace recorded in the presence of 30 µM NS1643 is labeled in gray. NS1643 inhibited current at +10 mV where KCNQ1 contributes the majority of the activity. In contrast, NS1643 amplified current recorded at –60 mV where HERG channels are mostly active. The activity recorded at +10 mV is demonstrated in H (open squares), while current recorded at –60 mV is depicted in I (closed circles).

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Fig 7. Specificity of NS1643. The activity of NS1643 towards Kv1.5 (representing I_{Kur}) and Kv4.3 (representing I_{to}) potassium channels was investigated after expression in oocytes. Currents were evoked by step protocols from –80 to +40 mV. Oocytes were clamped at –80 mV between steps and tail currents recorded at –60 mV. As demonstrated in A, B and C, NS1643 applied at 30 µM had a minor inhibitory effect on Kv4.3 current. The current amplitude was reduced to 84.6± 8 % of control levels. No effect on Kv1.5 current was observed after application of 30 µM NS1643 (D, E and F). The ability of NS1643 to affect L-type voltage gated Ca^{2+} channels and cardiac Na^{+} channels was addressed using guinea-pig ventricular myocytes. Current voltage relationship obtained by application of the depicted protocol for Ca^{2+} current recorded before and 2 minutes after addition of 10 µM NS 1643 (G). The small shoulder in the IV curve between -45 and -20 mV represents T-type Ca^{2+} current, the remaining current (peak at +5mV) is due to L-type current (Heubach et al., 2000). Time-course of peak $I_{Ca,L}$ is shown in H. The current voltage relationship for I_{Na} using the outlined protocol is demonstrated in I, and the time-course for peak I_{Na} in J. Figure H and J show original traces obtained at times marked by “a” and “b”, respectively. The horizontal bars indicate the time of drug exposure.

Fig 8. Action potential duration (APD) measured from acutely isolated ventricular guinea pig cardiomyocytes. Cells were held in the current clamp mode and stimulated to induce action potentials with injection of 120 % threshold current at 0.5 Hz. When stable action potentials were obtained, APD was measured at 90 % of repolarization (APD_{90}) (A). Addition of 10 µM NS1643 abbreviated APD_{90} significantly ($P < 0.01$) from 568.21 ± 60.1 ms to 373.5 ± 10.2 ms (n = 3) (B).

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Fig 9. Influence of NS1643 on the refractoriness of guinea pig cardiomyocytes. Action potentials were elicited as described in figure 8, APD was determined, and a 115 % thresold current was injected as close to the repolarizing action potential as possible, in order to determine the post-repolarization refractory time. The time period after repolarization to APD_{90}, in which it was not possible to elicit a new action potential, was determined and termed the post-repolarization refractory time (A). After application of 10 µM NS1643 this refractory time of the myocytes increased significantly ($P < 0.01$) from 19.3 ± 9.3 ms to 156.3 ± 36.0 ms ($n = 3$) (B). This increment could be completely reversed by 100 nM E-4031, which resulted in a refractory period of 19.0 ± 2.6 ms ($n = 3$) (C).

Fig 10. Response of cardiomyocytes to simulated early after depolarizations (EADs). After an elicited action potential a train of ten to twenty 50 % subthreshold stimuli were applied either at control situations after full repolarisation (A and C) or, to induce EADs, at APD_{90} (B and D). In the absence of NS1643 subthreshold stimuli applied at APD_{90} was able to induce EAD events every time the protocol was applied and prolongation of the action potential was continued even after cessation of current injection (B). With application of 10 µM NS1643 the premature train of stimuli was not able trigger any abnormal activity and application of the drug thus led to a stable action potential (D). Superimposed traces for subthreshold stimuli applied at APD_{90} in the absence or presence of NS1643 are shown in E.

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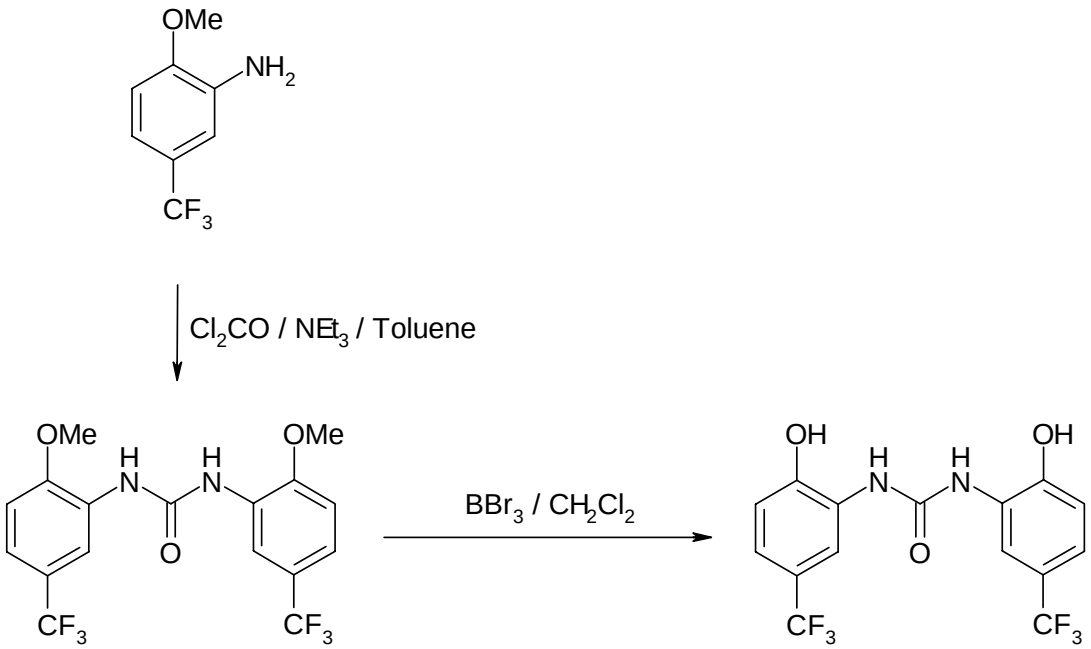
Figure 1



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NS1643
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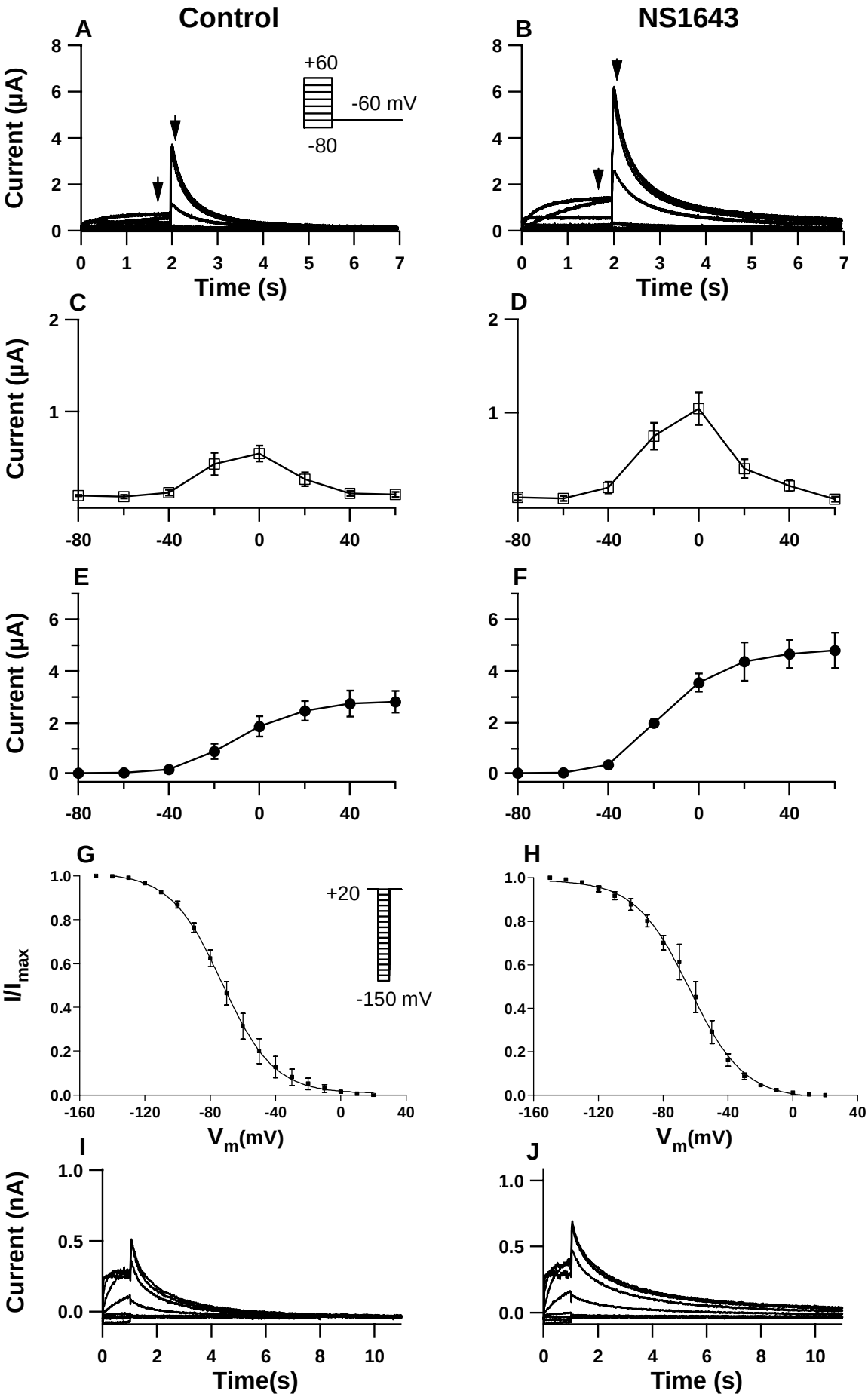

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Figure 3

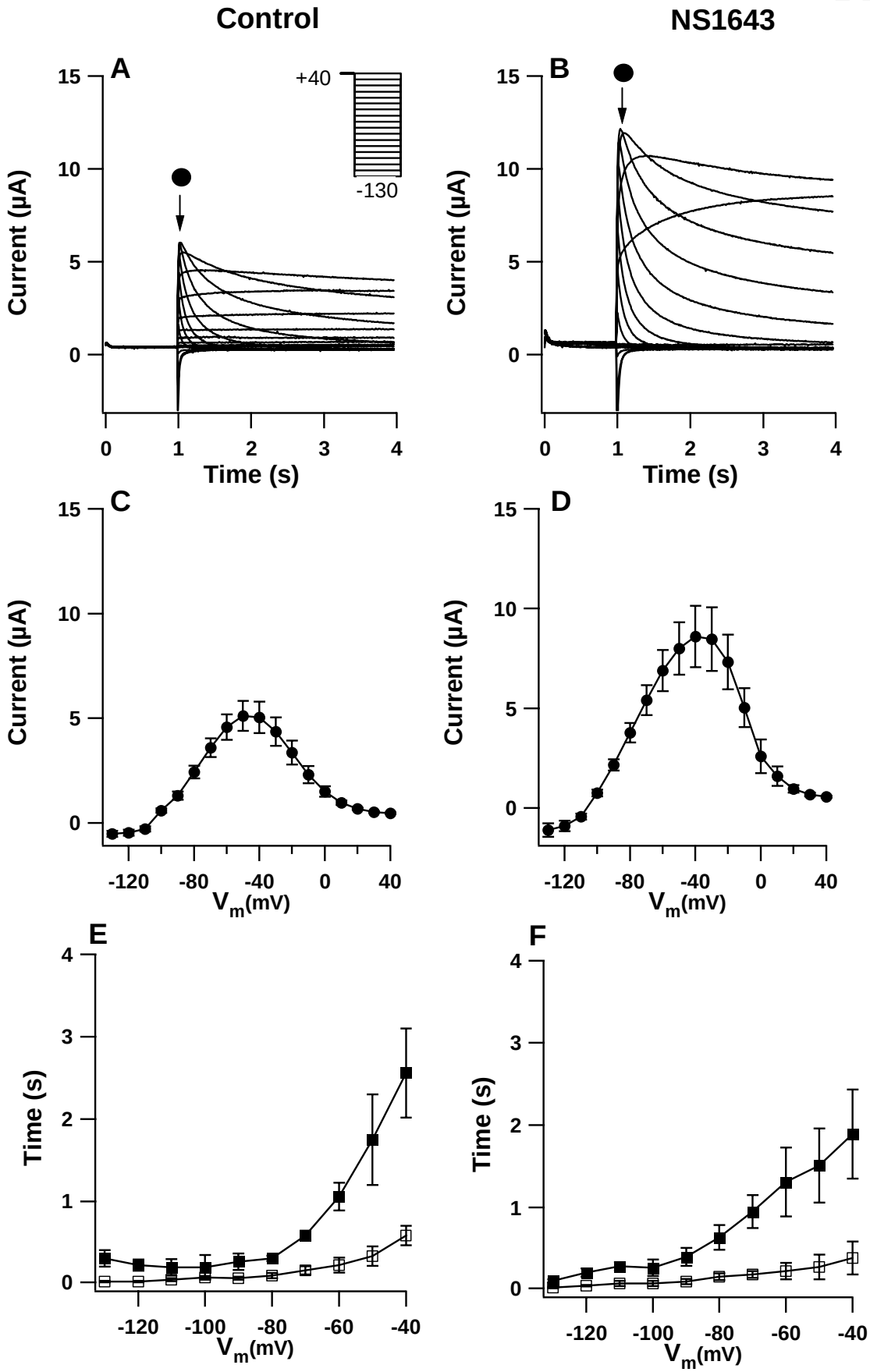

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Figure 4

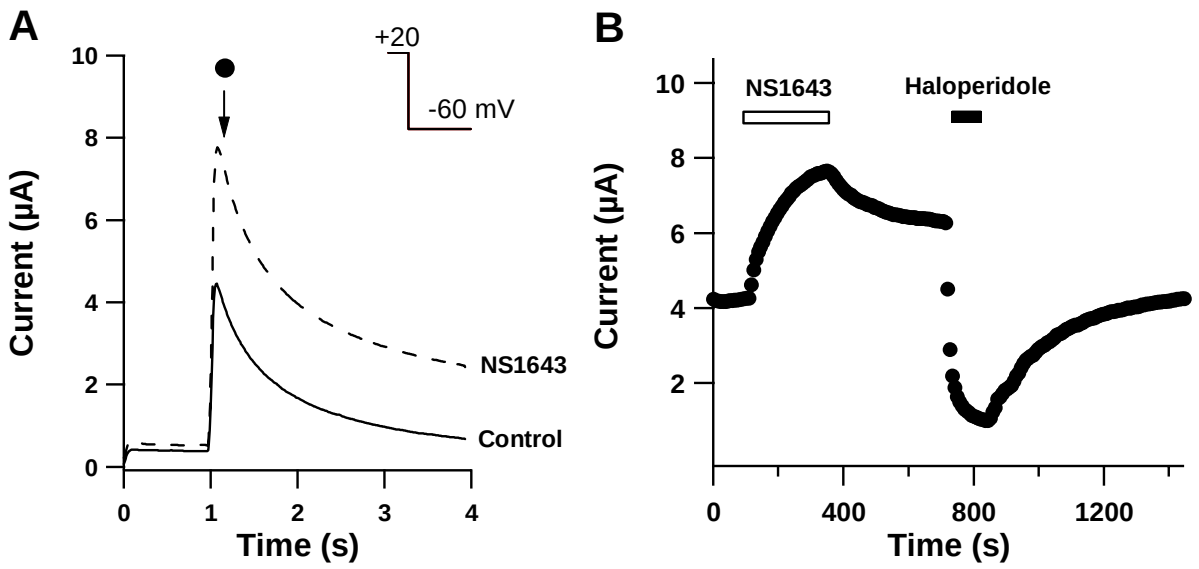

Figure 5

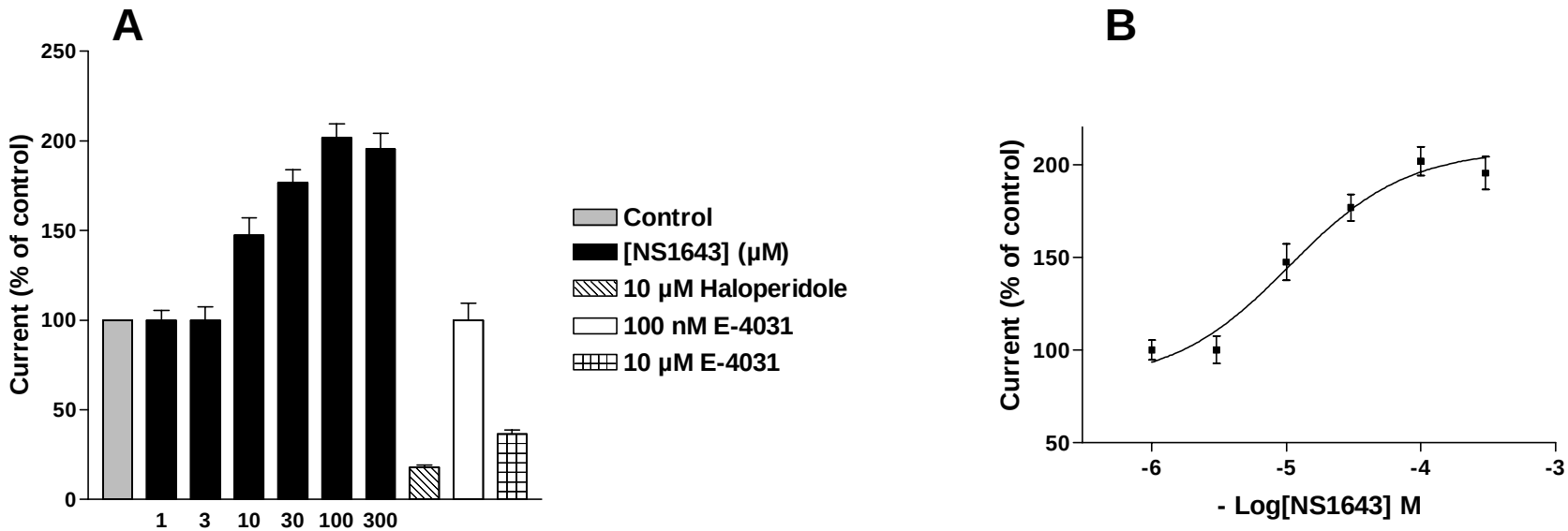

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Figure 6

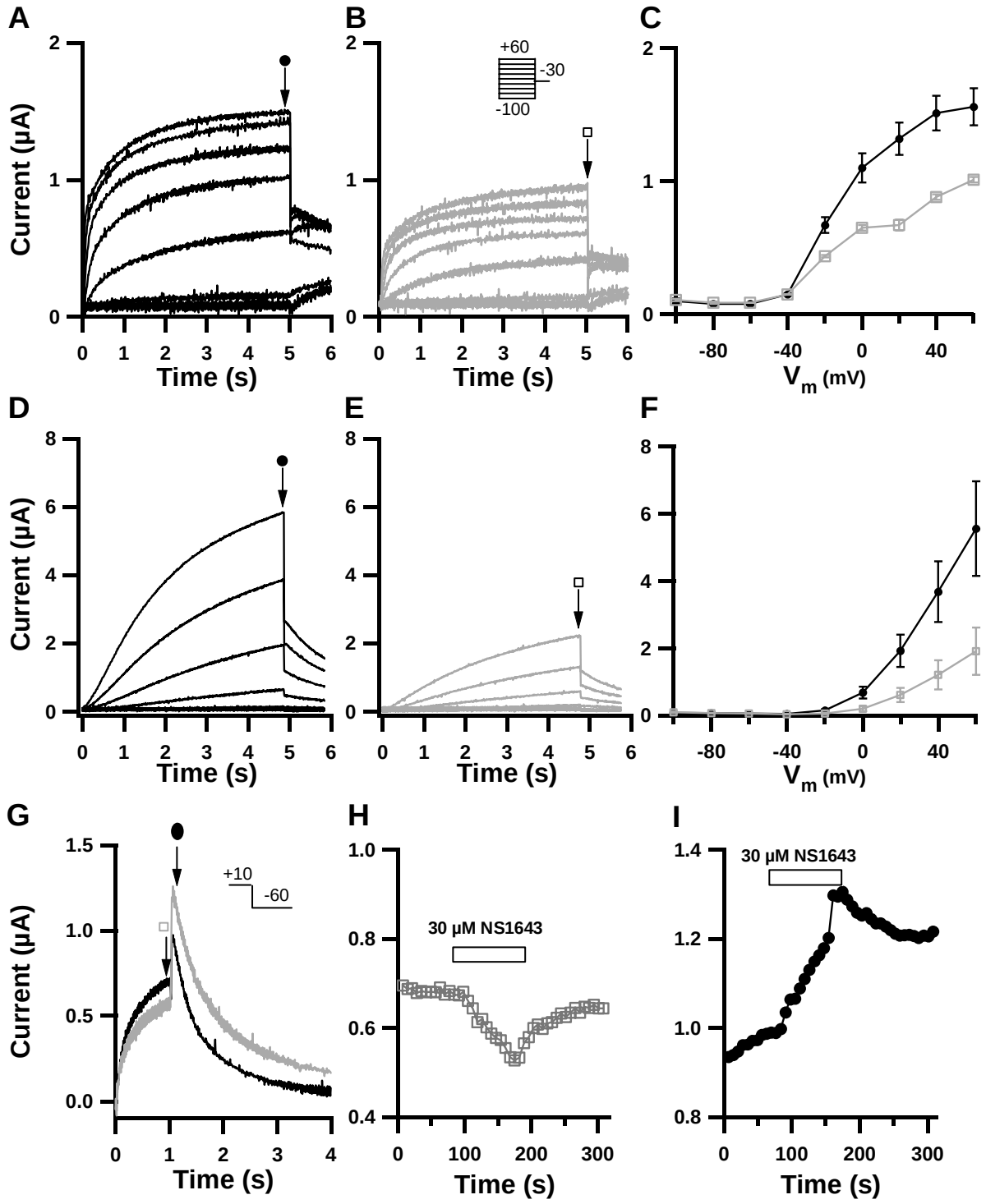

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Figure 7

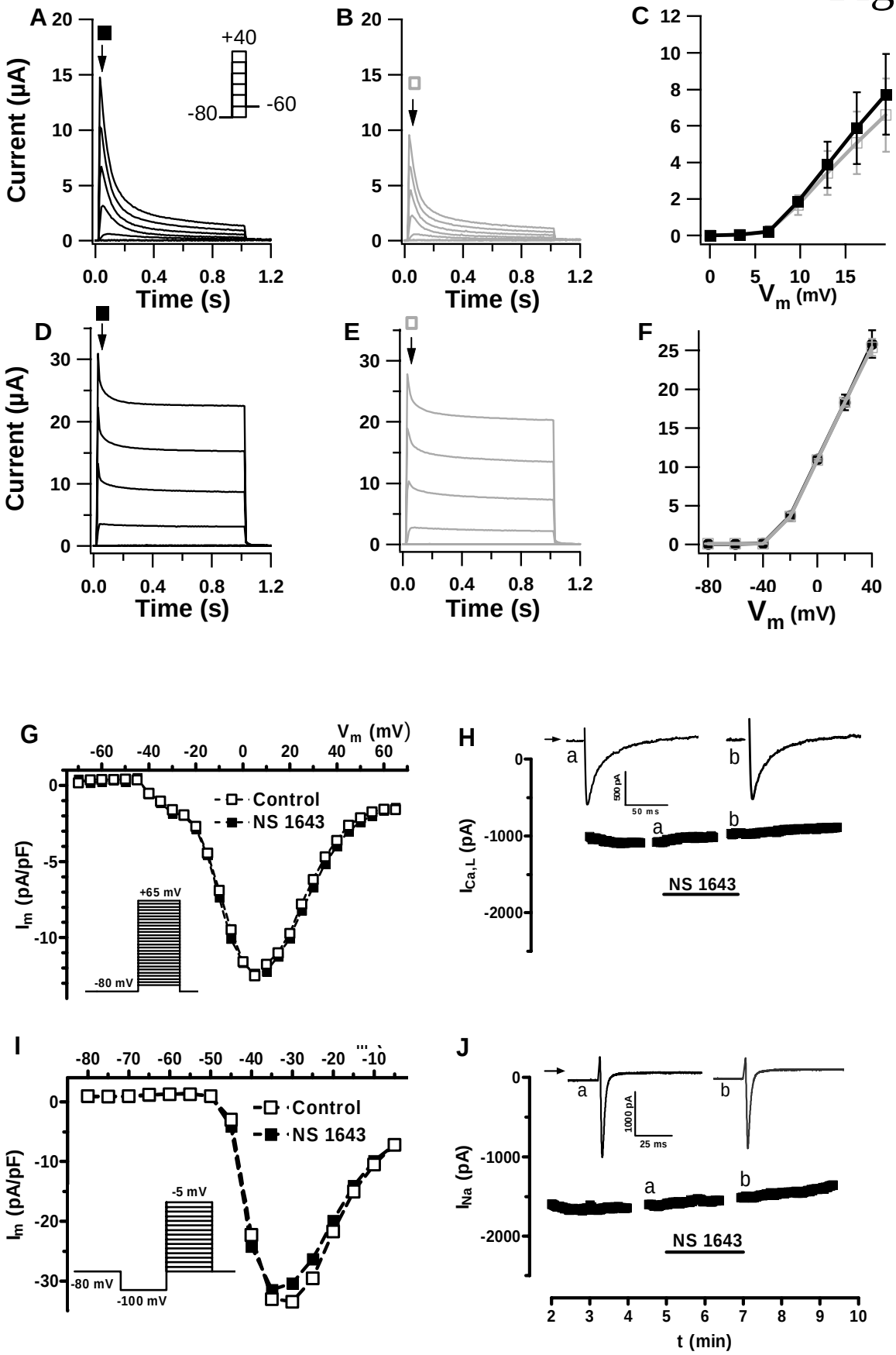

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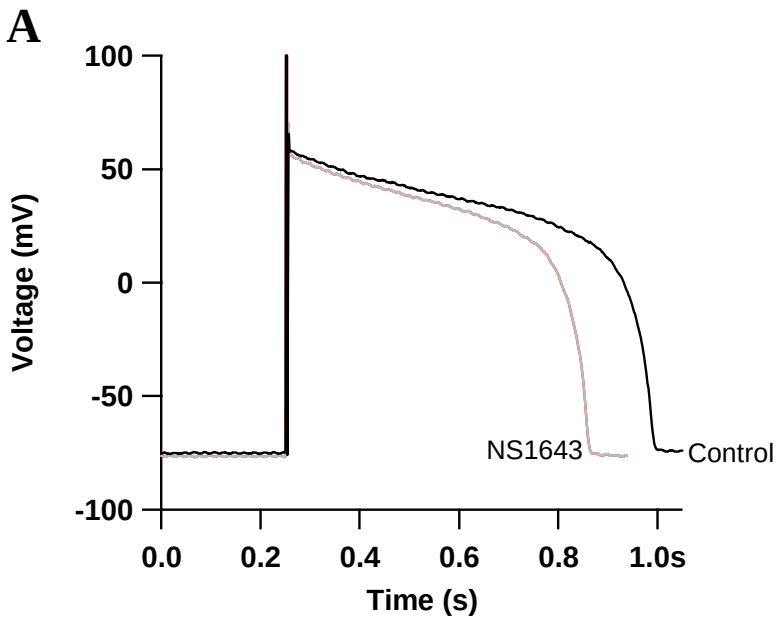
Figure 8



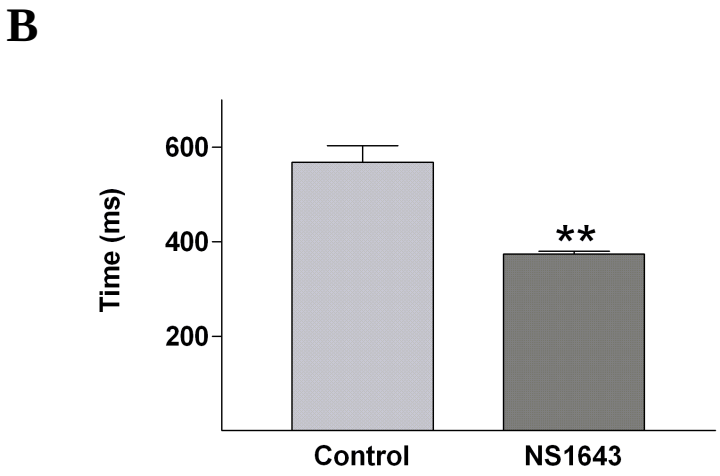


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Figure 9

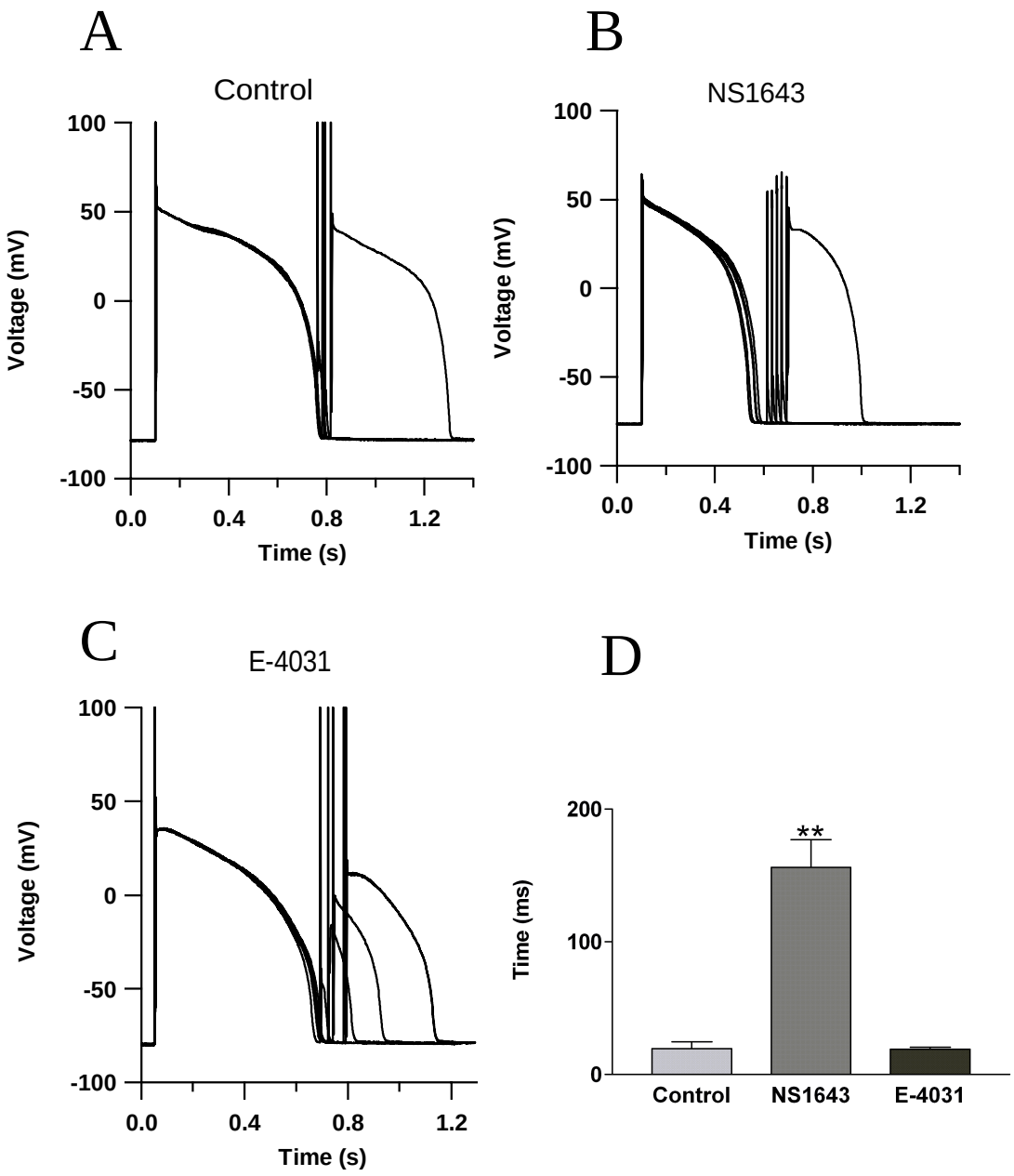

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Figure 10