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Activation of Human *ether-a-go-go*-Related Gene Potassium Channels by the Diphenylurea 1,3-Bis-(2-hydroxy-5-trifluoromethyl-phenyl)-urea (NS1643)

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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 antiarrhythmic principle, because it possibly would attenuate afterdepolarizations, ischemic leak currents, and reentry phenomena. Repolarization of the cardiac myocytes is crucially dependent on the late rapid delayed rectifier current (I_{Kr}) conducted by *ether-a-go-go*-related gene (ERG) potassium channels. We have developed the diphenylurea compound 1,3-bis-(2-hydroxy-5-trifluoromethyl-phenyl)-urea (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 human embryonic kidney 293 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 the specific HERG channel inhibitor 4′-[[1-[2-(6-methyl-2-pyridyl)ethyl]-4-piperidinyl]carbonyl]-methanesulfonanilide (E-4031) at 100 nM. Application of NS1643 also resulted in a prolonged postrepolarization refractory time. Finally, cardiomyocytes exposed to NS1643 resisted reactivation by small depolarizing currents mimicking early afterdepolarizations. In conclusion, HERG channel activation by small molecules such as NS1643 increases the repolarization reserve and presents an interesting new antiarrhythmic approach.

The action potential initiates and controls the contraction of cardiac cells. The shape and duration of the action potential are 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 ultrarapid 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 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 toward organic and inorganic blockers (Sanguinetti and Jurkiewicz, 1990). It is now widely accepted that the I_{Kr}

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ABBREVIATIONS: APD, action potential duration; HERG, human *ether-a-go-go*-related gene; NS1643, 1,3-bis-(2-hydroxy-5-trifluoromethyl-phenyl)-urea; HEK, human embryonic kidney; I-V, current-voltage; APD_{90}, action potential duration at 90% repolarization; EAD, early afterdepolarization; E-4031, 4′-[[1-[2-(6-methyl-2-pyridyl)ethyl]-4-piperidinyl]carbonyl]methanesulfonanilide; RPR260243, (3*R*,4*R*)-4-[3-(6-methoxyquinolin-4-yl)-3-oxo-propyl]-1-[3-(2,3,5-trifluoro-phenyl)-prop-2-ynyl]-piperidine-3-carboxylic acid.

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). However, this remains controversial (McDonald et al., 1997; Abbott et al., 1999; 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.

Most arrhythmias originate from disturbances in the function of ion channels that generate the normal action potentials. Class III antiarrhythmic 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. Antiarrhythmic drugs that compromise HERG channel function without effect on Ca^{2+} channels such as dofetilide or *d*-sotalol introduce a risk of proarrhythmic events because 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 prolongs APD, which is reflected on the ECG as a longer QT interval. Ventricular action potential prolongation can develop into torsade de pointes arrhythmia and ventricular fibrillation (Monahan et al., 1990; Sanguinetti and Jurkiewicz, 1990; Metzger and Friedman, 1993; Pohjola-Sintonen et al., 1993; Woosley et al., 1993).

Opening of HERG channels could thus present a novel antiarryhythmic principle, and several genetic studies support this notion. It has been demonstrated that increasing I_{Kr} by overexpression 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 toward an increase of the HERG current as a beneficial antiarrhythmic approach.

In a patent application submitted in 2003, we reported the first examples of small molecule HERG channel activators (Olesen et al., 2005). 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 antiarrhythmic 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 antiarrhythmic approach.

Materials and Methods

Chemical Synthesis

NS1643 was synthesized at NeuroSearch A/S (Ballerup, Denmark). The symmetrical bis-phenol NS1643 was synthesized in two steps from 2-methoxy-5-trifluoromethylaniline as shown in Fig. 1. Reaction of two equivalents of the aniline with phosgene provided the urea (Fig. 1), which was treated with boron bromide 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) or T7 mMessage machine kit (Ambion, Austin, TX) according to the manufacturers' instructions. mRNA was phenol/chloroform extracted, ethanol precipitated, and dissolved in Tris-EDTA buffer to approximate concentrations of 1 $\mu g/\mu l$. For proof of purity and integrity, mRNA was inspected by gel electrophoresis, and concentrations were determined photometrically. mRNA was stored at −80°C until injection.

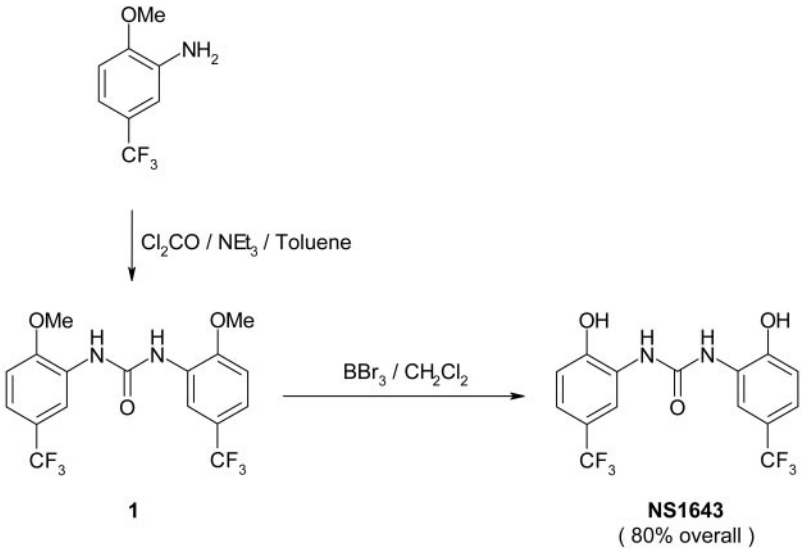

Fig. 1. Synthesis of NS1643.

Expression in *X. laevis* Oocytes and Mammalian HEK293 Cells

X. laevis surgery and oocyte treatment were done as described previously (Grunnet et al., 2001). Oocytes were collected under anesthesia (2 g/l tricain; catalog no. A-5040; Sigma-Aldrich, St. Louis, MO) according to guidelines approved by the Danish National Committee for Animal Studies. Before injection of 50 nl of mRNA (approximately 50 ng), oocytes were kept for 24 h at 19°C in Kulori solution consisting of 90 mM NaCl, 1 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, and 5 mM HEPES, pH 7.4 with NaOH. Injection of mRNA was accomplished using a Nanoject microinjector from Drummond Scientific (Broomall, PA). Oocytes were kept at 19°C for 2 to 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's medium with ultraglutamin 1 (Cambrex Bio Science Walkersville, Inc., Walkersville, MD) supplemented with 10% fetal calf serum at 37°C in 5% CO_2.

Isolation of Single Ventricular Cardiomyocytes

Ventricular cardiomyocytes were isolated using a method slightly modified from that of Mitra and Morad (1985). Perfusion velocity was 10 to 15 ml/min for all solutions, and all solutions were heated to 37°C and filtered before use. Guinea pigs were anesthetized with intraperitoneal injection of pentobarbital (50–75 mg/kg). In addition, 1 ml/kg heparin (1000 IU/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 setup. To release intracardiac 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) Tyrode's solution (with Ca^{2+}) consisting of 135 mM NaCl, 4 mM KCl, 1 mM $MgCl_2$, 0.33 mM NaH_2PO_4, 10 mM HEPES, 10 mM glucose, and 2 mM $CaCl_2$, pH 7.4 with NaOH. This was followed by 5-min perfusion with oxygenated (100% O_2) no-Ca^{2+} Tyrode's solution. Perfusion was continued for 5 min with an oxygenated potassium gluconate solution consisting of 120 mM potassium gluconate, 20 mM NaCl, 1 mM $MgCl_2$, 10 mM HEPES, and 10 mM glucose, pH 7.4 with KOH. Glucose and potassium gluconate (28.10 g/l) were added just before use. Finally, enzymatic digestion was performed by perfusion with oxygenated (100% O_2) potassium gluconate solution containing 0.5 mg/ml collagenase (type CLS-2; catalog no. LS004176, Medinova Scientific A/S, Glostrup, Denmark) and 50 μM $CaCl_2$. The first 25 ml of this solution was discarded. The remaining solution was continuously recycled until the heart seemed 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) potassium 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 potassium 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 (CA-1B; Dagan, Minneapolis, MN). Electrodes were pulled from borosilicate glass capillaries on a horizontal patch-electrode puller (DMZ universal puller; Zeitz Instruments, Munich, Germany) and had tip resistance between 0.3 and 2.5 MΩ when filled with 1 M KCl. During the 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 (90 mM NaCl, 1 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, and 5 mM HEPES, pH adjusted to 7.4 with NaOH). 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, Lambrecht/Pfalz, 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^+) consisting of 150 mM NaCl, 4 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, and 10 mM HEPES, pH 7.4 with NaOH. Pipettes were filled with solution consisting of 144 mM KCl, 10 mM EGTA, 10 mM HEPES, and 4 mM 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 were 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 signals were low-pass filtered at 3 kHz and acquired using PULSE software (HEKA).

Native Cardiomyocytes. Cells in suspension were transferred to coverslips and left for 15 min before recordings. Before performing patch-clamp experiments, cells were transferred to the perfusion chamber, and superfused for 5 min with potassium gluconate solution consisting of 120 mM potassium gluconate, 20 mM NaCl, 1 mM $MgCl_2$, 10 mM HEPES, and 10 mM glucose, pH 7.4 with KOH, before changing to Tyrode's solution consisting of 135 mM NaCl, 4 mM KCl, 1 mM $MgCl_2$, 0.33 mM NaH_2PO_4, 10 mM HEPES, 10 mM glucose, and 2 mM $CaCl_2$, 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 that deviated more than ±15 mV from −80 mV were discarded. At the outset, 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).

Native Cardiomyocytes for Recordings of Ca^{2+} and Na^+ Current. Currents were measured with the single electrode voltage-clamp method as described in detail previously (Christ et al., 2005).

The pipette solution had the following composition: 90 mM cesium methanesulfonate, 20 mM CsCl, 10 mM HEPES, 4 mM MgATP, 0.4 mM Tris-GTP, 10 mM EGTA, and 3 mM $CaCl_2$, with calculated free Ca^{2+} concentration of ~60 nM, pH 7.2 (EqCal; Biosoft). Ca^{2+} currents were measured with Na^+-free superfusion solution consisting of 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). L-type calcium current ($I_{Ca,L}$) 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 sodium current (I_{Na}), 5 mM NaCl 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 vicinity of the cells (Cell Micro Controls, Virginia Beach, VA; ALA Scientific Instruments, Long Island, NY).

Drugs and Solutions

Unless otherwise mentioned, all chemicals were of analytical grade and were obtained from Sigma-Aldrich. Nisoldipine was a gift of Baver AG (Wuppertal, Germany). Drugs were dissolved in dimethyl sulfoxide as concentrated stock solutions and diluted directly into the superfusion solution to yield the final concentration. Dimethyl sulfoxide concentration never exceeded 0.1% in final solutions. At this concentration, no influence on any measurements was observed (data not shown).

Analysis of Data

Data analysis and drawings were performed using IGOR software (Wavemetrics, Lake Oswego, OR) or Prism software (GraphPad Software Inc., San Diego, CA). All deviations of calculated mean averages are given as S.E.M. values. Significance was calculated as paired t test or by analysis of variance.

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: $I_{tail} = K_0 + K_{fast} \times \exp[-(t/\tau_{fast})] + K_{slow} \times \exp(t/\tau_{slow})$, where t is time in seconds and fast and slow deactivation constants are determined by τ_{fast} and τ_{slow}, respectively.

Results

A number of drugs and chemical compounds inhibit current flow through HERG 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 *X. 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 I-V curve recorded during the step protocol from −80 to +60 mV had a bell-shaped appearance with maximal amplitude at 0 mV (Fig. 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 Fig. 2E. When

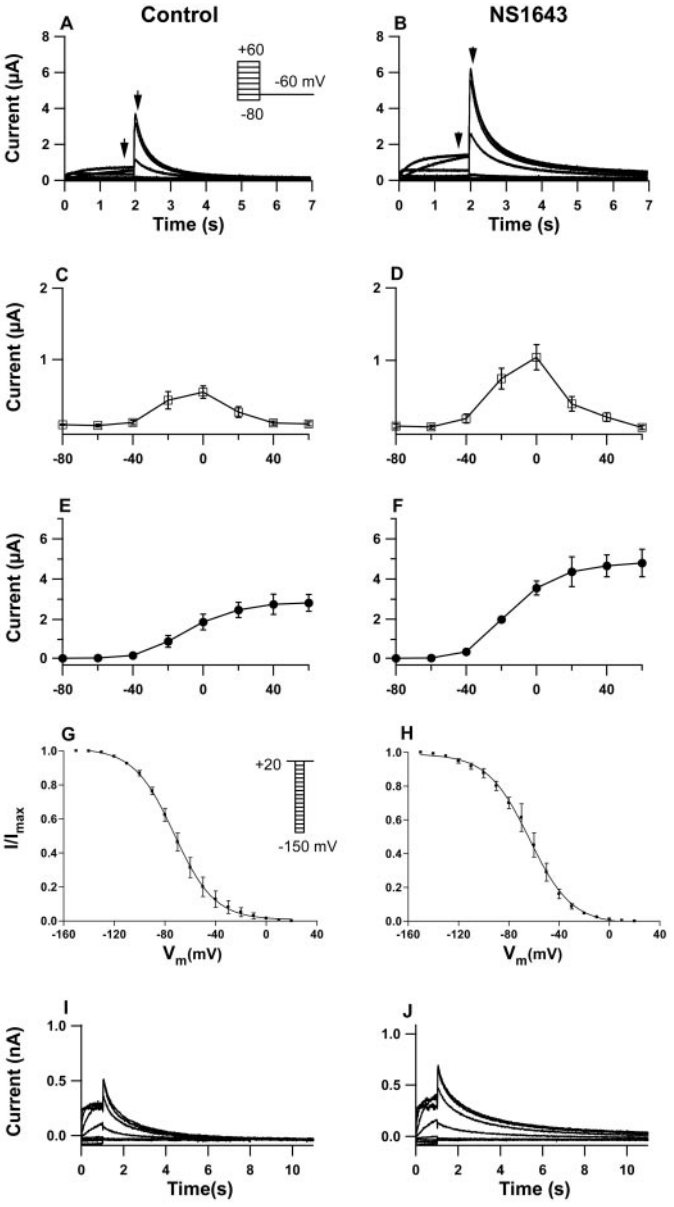

Fig. 2. The current amplitude of HERG channels expressed in *X. laevis* oocytes is increased in the presence of NS1643. HERG channel activation was obtained by application of a step protocol from −80 to +60 mV with 20-mV increments between steps. Tail current was recorded at −60 mV. Representative traces are shown in A and B. The steady-state current revealed strong inactivation as seen by the bell-shaped I-V curve (open squares; C), whereas 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 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 was 16.8 ± 3 mV. The difference in V_{50} is significant ($p = 0.02$). For all summarized experiments, $n = 6$. I and J show the effect of NS1643 on HEK293 cells stably expressing the HERG channel and the KCNE1 subunit. Voltage-clamp recordings were performed by applying the 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 ($n = 3$).

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 (Fig. 2, B, 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 membrane potential to +20 mV for 1 s. This was followed by brief (10-ms) hyperpolarized steps from -150 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 dependence 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 (Fig. 2, G and H). Data were normalized and fitted to a Boltzmann function. In control oocytes, V_{50} was -72.7 ± 2 mV and the slope factor was 15.9 ± 2 mV. For oocytes exposed to NS1643, V_{50} was -63.7 ± 3 mV and the slope factor was 16.8 ± 3 mV. Summarized data represent $n = 6$. Because 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 (Fig. 2, I and J). 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 because 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 $45.1 \pm 10.1\%$ ($n = 3$) (Fig. 2J). To confirm expression of HERG channels and to determine the magnitude of leak, current experiments were completed by adding the specific HERG channel blocker E-4031 at 100 nM, 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 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 Fig. 3. Fast activation and inactivation were 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 with controls. In addition, a rightward 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, whereas results obtained in the presence of NS1643 revealed a maximal tail current at -40 mV. Summarized data are plotted as I-V 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 Fig. 3, E and F. No significant difference in deactivation kinetics could be observed after application of NS1643, even though there was a tendency toward 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 re-

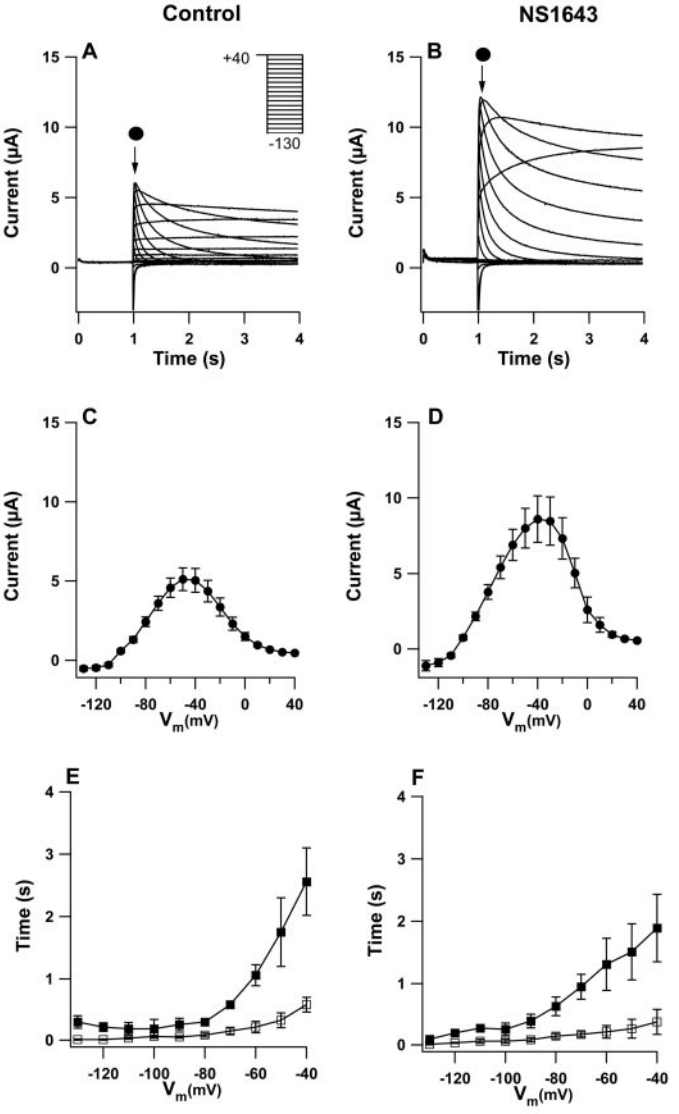

Fig. 3. HERG tail current and deactivation kinetics in the presence of NS1643. HERG channels expressed in *X. laevis* oocytes were activated and completely inactivated by holding at +40 mV for 1 s. Voltage dependence of release from inactivation, in the presence and absence of NS1643, was studied by a tail protocol stepped from -130 to +40 mV. Current recorded at the peak of the tail resulted in a bell-shaped I-V 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 kinetics could be observed after application of NS1643, even though there was a tendency toward decreasing τ_{fast} and τ_{slow} values in the presence of NS1643 ($n = 6$ for summarized data).

sponded to a single pulse of this protocol. Current recorded at +20 mV was slightly increased in the presence of NS1643 compared with 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 is depicted in Fig. 4B. Peak tail current as a function of time reveals how addition of 30 μM NS1643 increases HERG current approximately 100% within a time scale of roughly 200 s. Washing for 400 s only reduced the current level approximately 50%. To confirm specificity, the experiment was finalized by addition of HERG channel inhibitor haloperidol (10 μM). A rapid decrease in current amplitude that could be reverted by washing was observed. In noninjected 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 Fig. 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 HERG channels expressed in *X. laevis* oocytes, however is, severely compromised. E-4031 (100 nM) 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 previously (Sanguinetti et al., 1995). The EC_{50} value for NS1643 activation of HERG channels was calculated to 10.5 ± 1.5 μM.

Because 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 logical to investigate whether NS1643 could have an 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 Fig. 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 (Fig. 6B). On average, application of 30 μM NS1643 reduced KCNQ1 current to 64.7 ± 9% of control level (n = 5). Because 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 Fig. 6, D

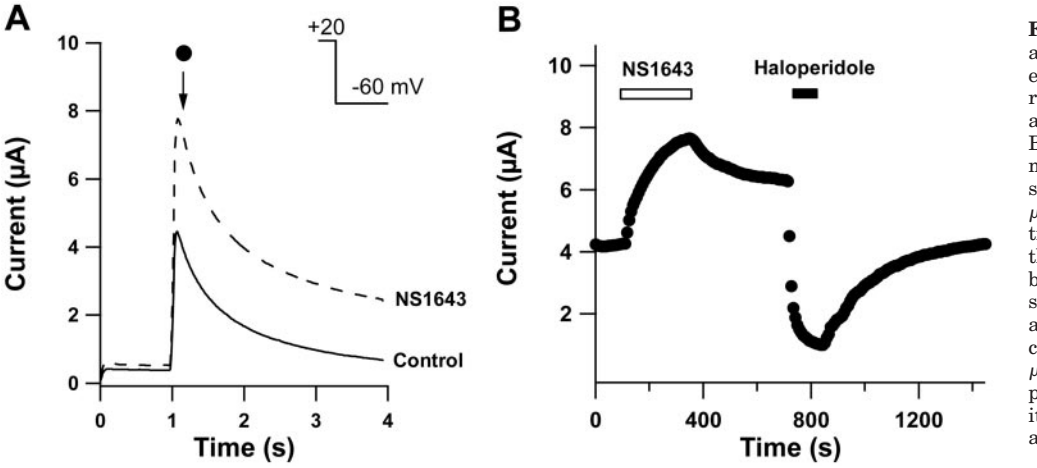

Fig. 4. Time course of HERG channel activation by NS1643. HERG channel expressed in *X. laevis* oocytes were repeatedly activated by clamping 1 s at +20 mV followed by 3 s at −60 mV. Between steps, cells were kept at −80 mV for 3 s. Current traces for a single step in the presence and absence of 30 μM 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 approximately 4 μA, application of 30 μM NS1643 gave a current increase to approximately 8 μA. The effect of NS1643 could be partly reversed by washing. Specificity of HERG current was confirmed by application of 10 μM haloperidol.

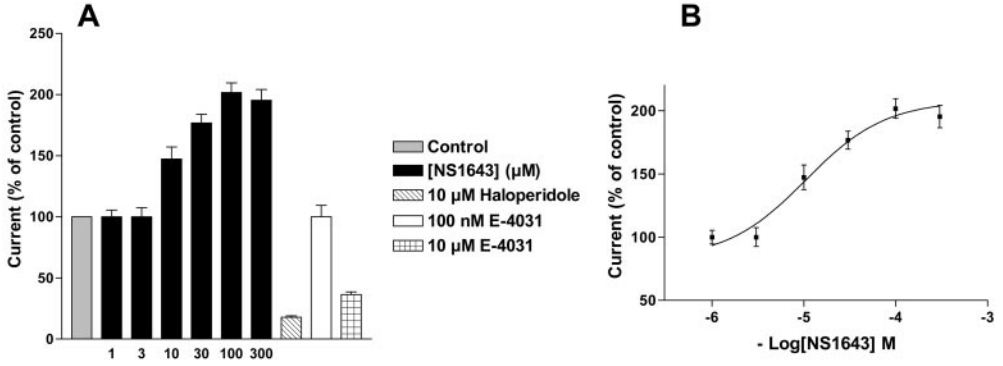

Fig. 5. Concentration-response relationship of NS1643. Oocytes expressing HERG channels were activated by a continuously step protocol from +10 to −60 mV, and peak tail current was recorded in the presence of different concentrations of NS1643. Only a single concentration of compound was applied to each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

to F, 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, coexpression studies were performed in *X. 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 because of the strong inactivation of HERG current at this potential, whereas the HERG current contribution will have a large impact at −60 mV as a consequence of the release from inactivation. When NS1643 was applied to coexpressing oocytes, a current reduction was observed at +10 mV, whereas the tail current recorded at −60 was increased (Fig. 6G). The corresponding on-line analysis recorded at +10 and −60 mV, respectively, is shown in Fig. 6, H and I. 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 toward 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 *X. laevis* oocytes. Channel activation was obtained by voltage step protocols from −80 to +40 mV. NS1643 (30 μM) inhibited Kv4.3 current to 84.6 ± 8% of control levels when current was recorded at +40 mV (Fig. 7, A–C; n = 4). In contrast, no effect was observed on Kv1.5 currents (Fig. 7, D–F; n = 3). The effect of NS1643 on $I_{Ca,L}$ and T-type calcium currents ($I_{Ca,T}$) as well as 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 rundown over time when exposed the drug; however, this decline was not different compared with the spontaneous rundown in time-matched controls (i.e., from 12.9 ± 1.5 to 10.8 ± 1.4 pA/pF; 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 $I_{Ca,T}$ (Fig. 7, G and H). Furthermore, we examined the effect of NS1643 on native sodium currents. I_{Na} was stable over time, and the current density 2 min after exposure to 10 μM NS1643 was not different from the respective control values (25.8 ± 3.9 versus 25.3 ± 4.1 pA/pF; n = 7) (Fig. 7, I and J).

Because NS1643 was observed to activate HERG in both *X. laevis* oocytes and mammalian expression systems, it was

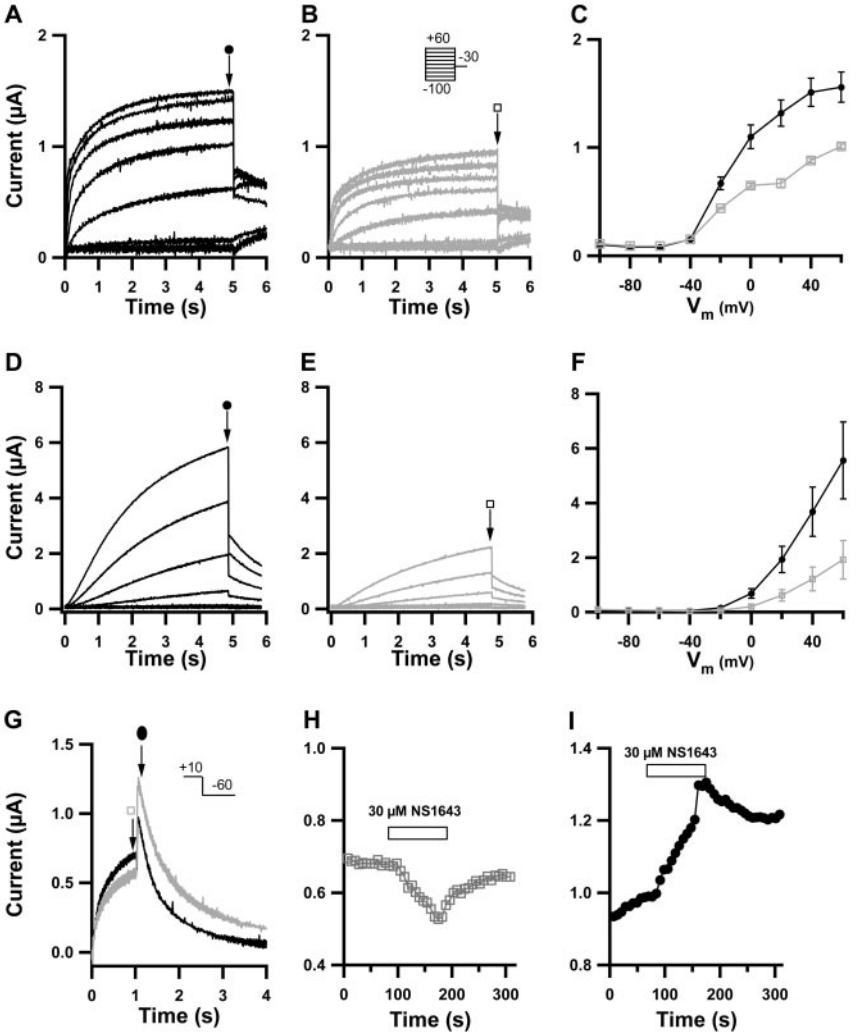

Fig. 6. NS1643 inhibits KCNQ1 current. Oocytes expressing KCNQ1 channels were activated by step protocols from −100 to +60 mV. Current activity was measured in the absence (A) or in the presence (B) of 30 μM NS1643. I-V curves are summarized in C. Using the same voltage protocol the effect of NS1643 on oocytes expressing both KCNQ1 and KCNE1 was addressed (D and E). I-V curves are summarized in F. Dual effect of NS1643 on HERG and KCNQ1 current was investigated by coexpressing both channel types in oocytes. Current was activated by a repeated step protocol from +10 to −60 mV (G). Current trace in the absence of NS1643 is labeled in black, whereas 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), whereas current recorded at −60 mV is depicted in I (closed circles).

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 to 5 nA (1.2 times rheobase value) to induce action potentials. Upon application of 10 μM NS1643, action potential duration at 90% repolarization (APD_{90}) was shortened significantly to 66 $\pm$ 8% (n = 3) (Fig. 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 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).

Because of 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 above, 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 described previously (Nuss et al., 1999). As can be seen from Fig. 9, application of 10 μM NS1643 dramatically and significantly ($P < 0.01$) altered the refractoriness of the guinea pig cardiomyocyte from 19 $\pm$ 9 to 156 $\pm$ 36 ms (n = 3). Subsequent application of 100 nM E-4031 resulted in a prolonged action potential and a decrease in the postrepolarization refractory period, although the second stimulus was unable to elicit full-size action potentials (Fig. 9C).

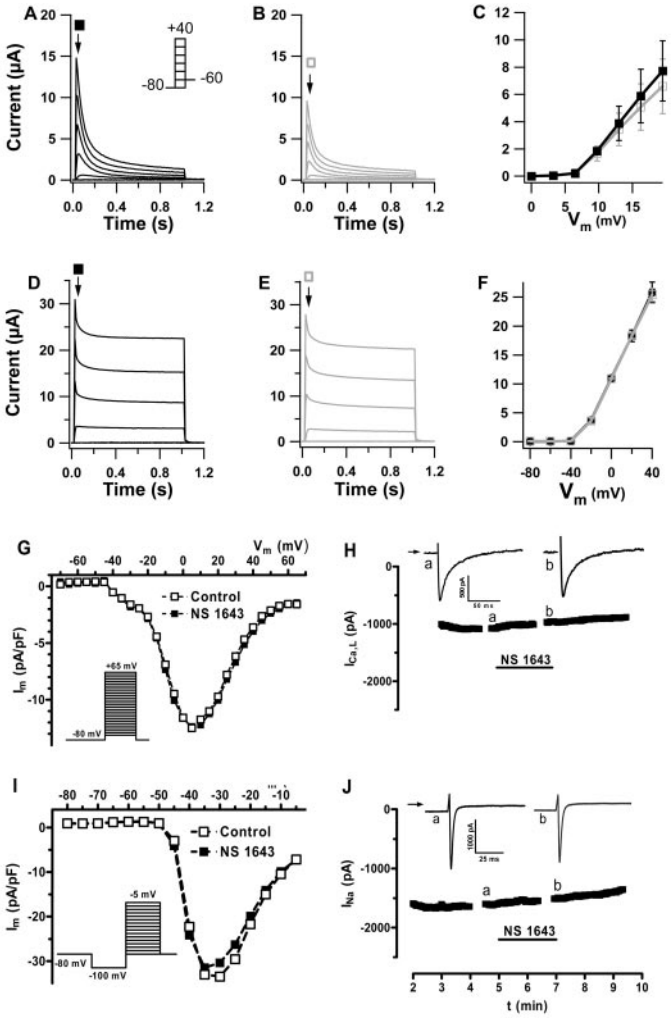

Fig. 7. Specificity of NS1643. The activity of NS1643 toward Kv1.5 (representing I_{Kur}) and Kv4.3 (representing I_{to}) potassium channels was investigated after expression in oocytes. Currents were evoked by step protocols from -80 to $+40$ mV. Oocytes were clamped at -80 mV between steps and tail currents recorded at -60 mV. As demonstrated in A to C, NS1643 applied at 30 μM had a minor inhibitory effect on Kv4.3 current. The current amplitude was reduced to 84.6 $\pm$ 8% of control levels. No effect on Kv1.5 current was observed after application of 30 μM NS1643 (D–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 min after addition of 10 μM NS1643 (G). The small shoulder in the I-V curve between -45 and -20 mV represents T-type Ca^{2+} current; the remaining current (peak at $+5$ mV) 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 recorded before and after application of 10 μM NS1643 is demonstrated in I and the time course for peak I_{Na} in J. H and J show original traces obtained at times marked by a and b, respectively. The horizontal bars indicate the time of drug exposure.

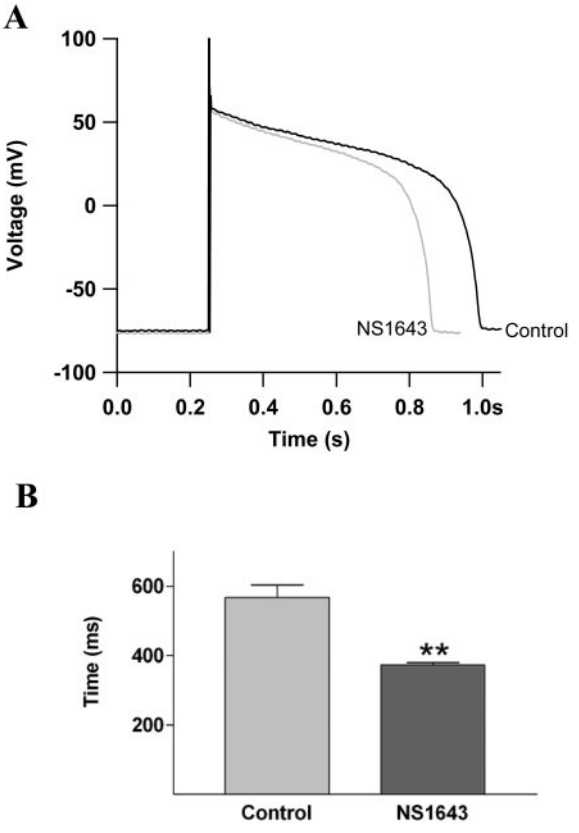

Fig. 8. APD measured from acutely isolated ventricular guinea pig cardiomyocytes. Cells were held in the current-clamp mode and stimulated to induce action potentials with injection of 120% threshold current at 0.5 Hz. When stable action potentials were obtained, APD was measured at APD_{90} (A). Addition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

Early afterdepolarizations (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 one of the cellular mechanisms underlying initiation of torsade de pointes and related polymorphic tachycardias (Nattel and Quantz, 1988; Szabo et al., 1995; Patterson et al., 1997; Burashnikov and Antzelevitch, 1998). L-type Ca^{2+} channels are activated at membrane potentials around −30 mV, and because 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 were elicited, and the cell was stimulated at APD_{90} with a sequence of 10 to 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 (Fig. 10, A and C). As seen on Fig. 10, B and E, 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} (Fig. 10, D and E; $n = 3$), 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).

Discussion

In the present study, the bis-phenol NS1643 was shown to activate cloned 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.

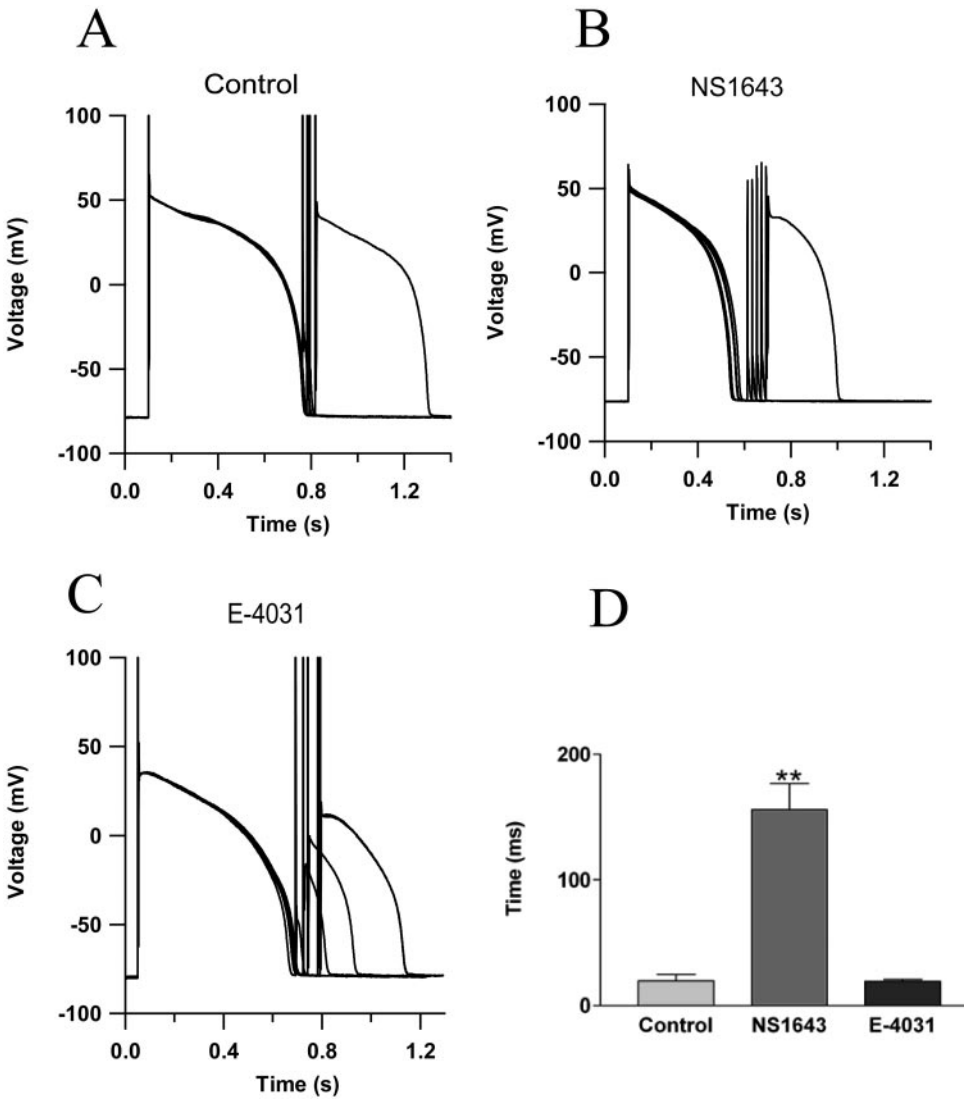

Fig. 9. Influence of NS1643 on the refractoriness of guinea pig cardiomyocytes. Action potentials were elicited as described in Fig. 8, APD was determined, and a 115% threshold current was injected as close to the repolarizing action potential as possible, to determine the postrepolarization refractory 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 postrepolarization 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 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).

During the course of the cardiac action potential, the HERG channels open at membrane depolarizations positive to −40 mV. The channel open state, however, is transient, and the inactivation time constant is very fast (Zhou et al., 1998; Kiehn et al., 1999). 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 rightward shift of V_{50} for inactivation of 9 mV in the presence of NS1643 (Fig. 2, G and H). 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. First, increase of the steady-state current may not be prominent enough to affect the plateau phase. Another possibility is 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 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.

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, whereas the compound affected neither 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, in contrast, slowed down by 10 μM RPR260243. In the present work, we showed that 10 μM NS1643 increased both the

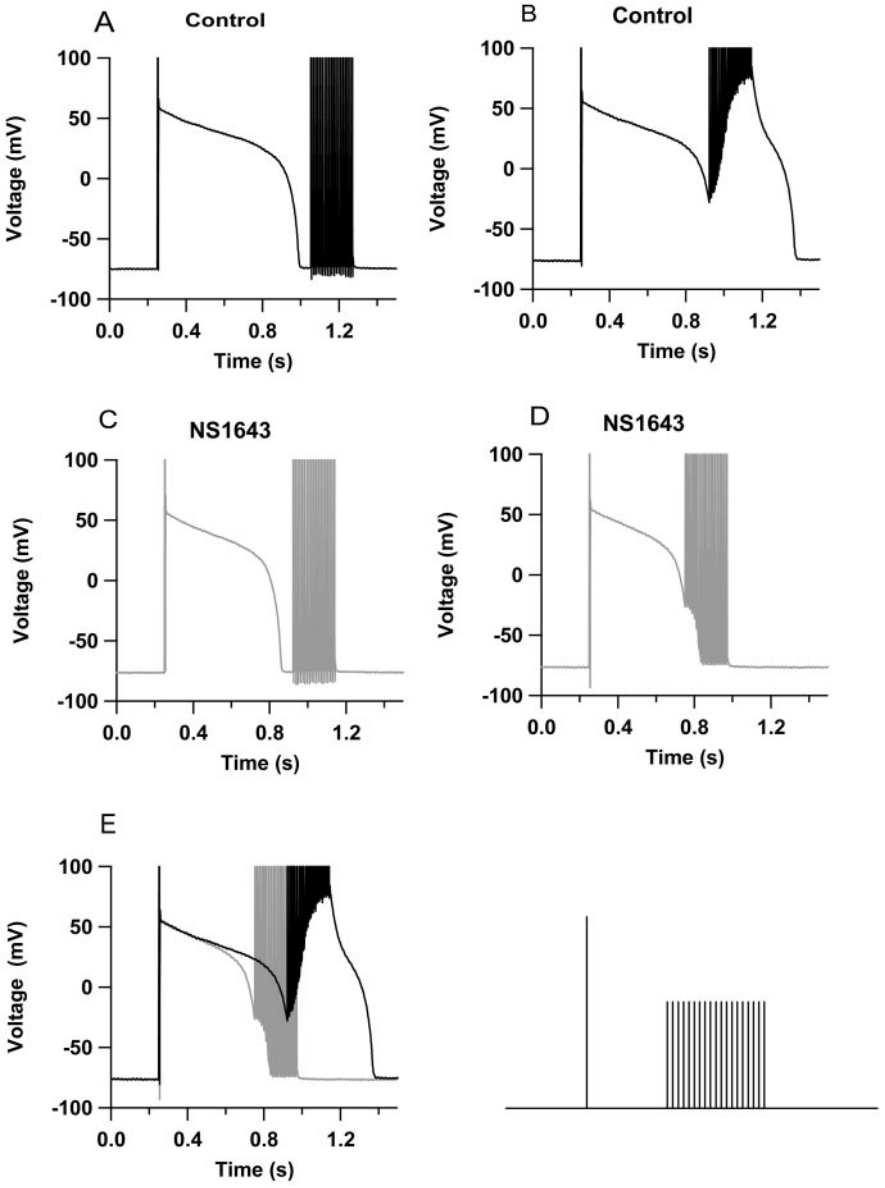

Fig. 10. Response of cardiomyocytes to simulated EADs. After an elicited action potential, a train of 10 to 20 50% subthreshold stimuli were applied either at control situations after full repolarization (A and C), or, to induce EADs, at APD_{90} (B and D). In the absence of NS1643, subthreshold stimuli applied at APD_{90} were 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, left. Current injection protocol is shown in E, right.

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 extent. An alternative explanation could simply be that the two compounds have different affinity for the HERG channel.

Antiarrhythmic 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, however, are proarrhythmic in other patients, which is probably caused by their reduction in the repolarization capacity and increased tendency to early aferdepolarization. The present HERG channel opener has the opposite effect in increasing the repolarizing reserve and reducing hyperexcitability. NS1643 also increases the postrepolarization refractory period. Whether the compound will increase or decrease the effective refractory period cannot be determined based on the cellular studies, because 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 because their mechanism is very different from that of the class III compounds, they are not expected to be associated with the same proarrhythmic potential. In fact, it has been shown that overexpression of HERG channels in mouse led to a smaller susceptibility to atrial and ventricular fibrillation (Royer et al., 2005), and it is very likely that the same may be true 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 phases of the action potential. EADs probably underlie the initiation of torsade de pointes 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 toward 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 overexpression in rabbit cardiomyocytes reduces the susceptibility to EADs in cultured cardiomyocytes. Nuss et al. (1999) 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 modeling, 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 abnormally long action potential, probably because of 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 antiarrhythmic drugs preventing EADs.

In conclusion, we have described the abilities of the bisphenol 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 increases both the steady-state and the peak tail current of the cloned HERG channels, gives a rightward shift in V_{50} for inactivation, and slows the deactivation of the tail current. In the native cardiomyocytes, the drug abbreviates action potential duration, increases the postrepolarization refractoriness, and suppresses hyperexcitability. The present data support the concept of use of HERG channel activators as a novel antiarrhythmic principle.

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