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Biophysical characterization of the new HERG1 channel opener NS3623

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Abbreviations: APD, action potential duration; HERG, human ether a-go-go related gene.

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Abstract

Within the field of new anti-arrhythmic compounds the interesting idea of activating HERG1 potassium channels has recently been introduced. Potentially, drugs that increase HERG1 channel activity will augment the repolarising current of the cardiac myocytes and stabilize the diastolic interval. This might make the myocardium more resistant to events that causes arrhythmias. We here present the compound NS3623 that has the ability to activate HERG1 channels expressed in *Xenopus laevis* oocytes with an EC50 value of 79.4 μM. Exposure of HERG1 channels to NS3623 affects the voltage dependent release from inactivation, resulting in a half inactivation voltage that is right-ward shifted by 17.7 mV. Moreover the compound is affecting the time constant of inactivation leading to a slower on-set of inactivation of the macroscopic HERG1 currents. We also characterized the ability of NS3623 to increase the activity of different mutated HERG1 channels. The mutants S620T and S631A are severely compromised in their ability to inactivate. Application of NS3623 to any of these two mutants did not result in increased HERG1 current. In contrast, application of NS3623 to the mutant F656M increased HERG1 current to a larger extent than what was observed with wild-type HERG1 channels. Since the amino acid F656 is essential for high affinity inhibition of HERG1 channels it is concluded that NS3623 has a dual mode of action being both an activator and an inhibitor of HERG1 channels. Finally, we demonstrate that NS3623 has the ability to shorten action potential durations in guinea pig papillary muscle.

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Introduction

Potassium channels are essential for repolarising the membrane potential of excitable cells and for maintaining the resting membrane potential between action potentials. In the heart, the delayed inward rectifying repolarizing potassium current I_K is composed of two currents denoted I_{Kr} and I_{Ks}. Activation of the human ether-a-go-go related gene (HERG1) potassium channels underlies the I_{Kr} (Sanguinetti et al., 1995;Trudeau et al., 1995), while the molecular component behind I_{Ks} is KCNQ1 potassium channels in association with KCNE1 β-subunits (Barhanin et al., 1996;Sanguinetti et al., 1996). The HERG1 channel has an architecture like many other voltage gated potassium channels with homologous subunits containing six transmembrane domains that form a tetrameric channel. In addition, the HERG1 channel is recognized by its characteristic inward rectification properties. The inward rectification is caused by the unique ability of the HERG1 channel to slowly activate at depolarized membrane potentials followed by much faster C-type inactivation where the channel keeps a non-conducting configuration (Spector et al., 1996). When the membrane repolarizes, the channel is quickly released from inactivation and conducts a slowly deactivating current. The pore region of the HERG1 channel has aromatic residues at positions 652 and 656 which point toward the central cavity, thereby render the channel rather promiscuous with respect to lipophilic drugs that can bind in the pore region and thereby block channel conduction (Mitcheson et al., 2005). Although blocking I_{Kr} has been a strategy for prevention of ventricular and more recently also atrial fibrillation it is now known that this approach in some patients is likely to be pro-arrhythmic. Inhibiting HERG1 channel function prolongs cardiac action potential and under certain conditions this may lead to lethal ventricular arrhythmias mediated by transient Torsade de Pointes (reviewed by Fitzgerald and Ackerman, 2005). Recently, a series of new molecules that activate the HERG1 channel has been discovered (Hansen et al.,

2006;Kang et al., 2005;Zhou et al., 1998). The compounds all showed effective in increasing the repolarizing reserve in cardiac myocytes and thereby significantly shorten the action potential duration. Moreover, in one of the studies dofetilide was applied to increase ventricular dispersion in rabbit hearts. This is known to be pro-arrhythmic, and the introduced heterogeneity in action potential duration across the cardiac wall could be circumvented by application of HERG1 channel activators (Zhou et al., 1998). However, a more thorough examination of the effects of the HERG1 openers *in vivo* and in intact cardiac tissue has not been described. In a previous report we examined the *in vitro* effects of the compound NS1643 (Hansen et al., 2006;Zhou et al., 1998). This compound was difficult to formulate in order to perform studies on intact tissue and for this reason we identified another compound, NS3623 that also increased HERG1 channel activity and in addition can be dissolved in vehicles appropriate for *in vivo* applications. This paper presents a thorough *in vitro* characterization of NS3623 by experiments conducted after heterologous HERG1 expression in *Xenopus laevis* oocytes and in mammalian HEK-293 cells. In addition, the ability of NS3623 to affect HERG1 channels under native conditions was investigated using cardiac papillary muscle from guinea pig.

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Materials and Methods

Molecular biology

cDNA encoding either HERG1 (KCNH2, Kv11.1), KCNQ1 (Kv7.1), Kv1.5 (KCNA5) or Kv4.3 (KCND3) 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). The HERG1 channel mutants HERG1-F656M, HERG1-S620T and HERG1-S631A were introduced in pSP64. All HERG1 mutants were a kind gift from Michael C. Sanguinetti

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, 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.

Expression in Xenopus laevis oocytes

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. 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.

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Expression and recording in mammalian cells

Heterologous expression of HERG1 in mammalian HEK293 cells and whole-cell patch clamp recordings were performed as previously described (Hansen et al. 2006)

Isolation of native cardiomyocytes

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

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 Mg-ATP, 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 the following 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 depolarising test 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).

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Papillary muscle preparation

Animal handling was performed in accordance with the Helsinki convention. Male Dunkin Hartley Crl: HA guinea pigs (Charles River, Sulzfeld, Germany) of 280-350 g were sacrificed under light CO anaestesia. Hearts were excised and thin papillary muscles were removed from the right ventricles. The muscles were mounted in an organ bath continuously perfused with carbogenated Tyrode solution: (mM) 126.7 NaCl, 5.4 KCl, 1.8 $CaCl_2$, 1.05 $MgCl_2$, 22 $NaHCO_3$, 0.42 $NaPO_4$, 5 glucose, pH 7.4 at 37 ° C. One end of the muscle was pinned to the floor of the chamber and the free end was connected to a force transducer (AE 801, SensoNor, Dasing, Germany) with silk thread. The muscle was stimulated via Ag/AgCl elelectrodes at a regular frequency of 1 Hz. After at least 90 minutes of equilibration intracellular action potentials were recorded with conventional glass pipettes filled with 2 M KCl (tip resistance 10-20 MΩ). Drugs were added to the superfusion solution. Action potentials from stable impalements were recorded 30-45 min. after drug addition before the drug concentration was increased. Action potentials were recorded and the following parameters were analysed off-line: resting membrane potential, action potential amplitude, action potential duration at 20%, 50% and 90% of repolarization (APD_{20}, APD_{50}, APD_{90}), maximum upstroke velocity (dV/dt_{max}). All data acquisition and analysis were carried out with the ISO 2 system (MFK, Niedernhausen, Germany)

Analysis of data

Data analysis and drawings were performed using IGOR software (WaveMetrics, Lake Oswego, OR) 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. EC_{50} values were calculated from equilibrium concentration response experiments. Data were fitted with a sigmoidal dose-response

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equation: Y=Bottom + (Top-Bottom)/(1+$10^{((LogEC50-X)*HillSlope)}$), where X is the logarithm of concentration and Y is the response. Similar, activation and inactivation data were fitted with Boltzmann equations. The applied equations were for activation data: $I = I_{max}/(1+\exp[(V_{50} - V_t)/k])$ and for inactivation data: $I = I_{max}/(1+\exp[(V_t - V_{50})/k])$, 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. To avoid transient capacitance, current values for release from inactivation (figure 5) were calculated as the average current amplitude recorded from 10 to 20 ms after the second depolarization step to + 20 mV. Time constants for deactivation was calculated by fitting with the double exponential equation $I_{tail} = K_0 + K_{fast} * \exp[-(t/\tau_{fast})] + K_{slow} * \exp[-(t/\tau_{slow})]$, where t is the time in seconds and the fast and slow deactivation constants are given by τ_{fast} and τ_{slow}, respectively. Finally, the time constant for inactivation was calculated by fitting to the single exponential equation $I_{tail} = K_0 + K * \exp[-(t/\tau)]$, where t is the time in s and τ is the time constant for inactivation. Capacitative current was subtracted before fitting.

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*).

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Results

NS3623 was originally identified as a chloride channel blocker, and has shown effective in preventing dehydration of erythrocytes (Bennekou et al. 2001). However, this compound also showed an interesting profile when the effect was examined on cloned HERG1 channels and we therefore chose to examine these effects more thoroughly. Figure 1 presents the structure of NS3623.

The following experiments were designed to obtain detailed information about the effect of NS3623 on cloned HERG1 channels expressed in *Xenopus laevis* oocytes. Figure 2 shows the effect of NS3623 on HERG1 expressing oocytes challenged with the outlined step protocol, where the HERG1 channels were activated by 1 s voltage increments ranging from –80 mV to +40 mV. These changes in membrane potential evoke a slow voltage dependent activation of the HERG1 channel followed by strong inactivation, a feature that is reflected in a bell-shaped current-voltage (*I-V*) relationship (Figure 2A and 2C). The potentials were increased with 10 mV steps and the tail current was elicited by clamping at –60 mV for 4 s. Summarized *I-V* relationship for tail currents are shown in figure 2D. When a stable control current was obtained, 30 μM NS3623 was added to the bath. In the presence of the compound both the steady-state and the tail HERG1 currents were increased to a level significantly different from initial control measurements (Figure 2B, 2C and 2D). The Boltzmann fits to the activation currents resulted in almost identical V_{50} where half-maximal activation was –20.0 ± 2 mV in control experiments and –20.0 ± 3 mV in the presence of NS3623. These experiments were repeated with similar results after co-expression of HERG1 and the β-subunit KCNE2 (data not shown). Finally, data were reproduced with HERG1 channels heterologously expressed in mammalian HEK293 cells (data not shown). In order to determine the concentration response of NS3623 on heterologously expressed HERG1 channels, oocytes were repeatedly activated by clamping 1 s at +20 mV followed by

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3 s at –60mV. Between steps cells were kept at –80 mV for 3 s. Oocytes were exposed to a series of seven concentrations of NS3623 ranging from 300 nM to 300 μM. In between application of drugs, the oocytes were washed with Kulori solution until a baseline comparable to the initial control values was obtained. The concentration response relationship with normalized current as a function of drug concentration is depicted in figure 3. These currents were sigmoidally fit and the increase in HERG1 channel activity as a function of concentration was found to have an EC_{50} value of 79.4 ± 14.9 μM.

At the single channel level an increase in current from heterologously expressed channels may stem from an increase in the amount of channels expressed or/and an increase in the probability that the ion channel spends a longer time in the open configuration or/and an increase in single channel conductance. An increase in single channel open probability could result in a number of changes in different kinetic parameters that each could contribute to augment the total HERG1 current. The increase in overall HERG1 channel conductance may for example be due to a slowing of the deactivation time constants. In order to test this hypothesis oocytes expressing HERG1 channels were subjected to a voltage clamping protocol where channels first were fully activated and inactivated by a clamp of +40 mV for 1 s, followed by steps from –130 to +40 mV lasting for 4 s in steps of 10 mV to reveal the deactivation kinetic. Oocytes were clamped for 3 s at –80 mV in between the steps and the resulting tail currents were measured. Representative current traces for control current are depicted in figure 4A and summarized *I-V* relationship in figure 4C. In the presence of 30 μM NS3623 the peak tail current increased relative to the control experiments as can be seen from representative current traces in figure 4B. In addition, the voltage that elicited the largest current was changed from –50 mV to –60 mV as can be seen from the *I-V* relationship presented in figure 4C (control) and 4D (NS3623). Deactivating tail currents were fitted with a two exponential function revealing the time constants for both fast and slow deactivation (Figure 4E and 4F). 30 μM of NS3623 did not induce a significant

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change in either the slow or fast time constants for deactivation. We then investigated the ability of NS3623 to change the response to voltage dependent release from inactivation on HERG1 channels (figure 5). Oocytes were exposed to a tri-pulse protocol where the channels were completely activated an inactivated by holding the membrane potential at +20 mV for 1s, followed by very short (10 ms) repolarizing steps from –150 to +20 mV and finally clamping again at +20 mV for 1 s. Application of this protocol makes it possible to reveal how release of inactivation is affected by the preceding voltage. This information is obtained by plotting the peak current resulting from the last step to +20 mV as a function of the previous short-step potential. The normalized data were fitted with a Boltzman function. In the control oocytes, V_{50} was –75.9 mV ± 0.6 mV and for oocytes exposed to 30 µM NS3623 V_{50} was calculated to –58.2 ± 0.4 mV (n=6) (figure 5C). This rightward shift of 17.7 mV in V_{50} may explain the increase in current through HERG1 channels exposed to NS3623.

To further investigate how NS3623 could affect the inactivation properties of HERG1 channels, a protocol designed to examine the time-course of the inactivation was applied. This protocol was initially described by Smith et al. (1996) and Spector et al. (1996) and is outlined in figure 6. Channels were first activated and then inactivated by a 1s pulse to +40 mV. Subsequently, the channels were subjected to a 10 ms pulse at –120 mV to allow full recovery from inactivation. The 10 ms time span at –120 mV is at the same time sufficiently short to prevent the channels from initiating deactivation. The third part of this pulse was a stepwise increment in the potential from –40 to +50 mV lasting for 1 s, to elicit a re-onset of channel inactivation. The currents representing this re-onset of inactivation were then fitted with a mono-exponential function. When comparing the resulting time constants from NS3623 treated oocytes with control experiments, it was revealed that time constants were significantly, and immensely increased at all measured potentials in the presence of 30 µM NS3623 (figure 6C).

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Having addressed the ability of NS3623 to affect activation, deactivation and inactivation of HERG1 channels, the mode of action of NS3623 was further investigated by application of different HERG1 channel mutants. To test whether NS3623 could activate HERG1 channels not capable of inactivation, the effect of the compound was investigated on channels that do not (HERG1 S620T) or only to a small degree (HERG1 S631A) inactivate (Zou et al. 1998, Casis et al. 2005). The step protocol described in figure 2 was used to elicit the mutant HERG1 current and 30 µM NS3623 was applied when stable control currents were obtained. The activity of the HERG1 S620T mutant in the absence (figure 7A) or presence (figure 7B) of 30 µM NS3623 was investigated. The steady-state *I-V* relationship in summarized in figure 7C revealed no significant difference in oocytes treated with the drug compared to the controls. Similar results were obtained with the S631A mutant that only has a very small degree of inactivation in the absence (figure 7D) or presence (figure 7E) of 30 µM NS3623. Summarized data are depicted in figure 7F. Although no significant difference was observed in the *I-V* relationship of the drug induced response compared to controls, there was tendency towards larger steady-state currents in the presence of NS3623. Having established that the ability of NS3623 to increase HERG1 current is severely reduced in non-inactivating channels, the mode of action of NS3623 was further explored using HERG1 channels mutated in the high affinity pore blocker residue F656. Mutating this residue from phenylalanine to methionine (F656M) greatly reduces the affinity of a number HERG1 channel inhibitors (Lees-Miller et al., 2000;Mitcheson et al., 2005). Interestingly, when the 30 µM NS3623 was examined on the mutated channel HERG1 F656M, a very large increase in the current response (approximately 5 times) was observed as can be seen form figure 8. This indicates that NS3623 also is acting as a partial blocker of the HERG1 channels. As a final biophysical characterization of NS3623 it was investigated if the compound could activate closed HERG1 channels. Continuous opening and closing of HERG1 channels were obtained by a repeated two step

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protocol with 200 ms at +20mV followed by 800 ms at -80mV. 30 µM NS3623 was applied and the time scale for complete compound effect determined (figure 9A). Compound application was also performed to channels where the repeated step protocol was interrupted by a continuous clamp at -80mV to keep the channels closed. After resuming the repeated step protocol an instant current increase to 144 ± 14 % was observed, indicating that NS3623 can bind to closed channels (n=4).

In order to test the ability of NS3623 to act as an anti-arrhythmic compound, HERG1 channel activity was monitored during a cardiac action potential stimulus. The action potential-like protocol is shown in figure 10. Application of the outlined protocol resulted in a gradual increase of the steady state current during the 'plateau phase' of the action potential, which reached a maximum during the end of this depolarized phase and a tail current was elicited upon repolarization. When the oocytes were exposed to 30 µM NS3623 both the steady state and the tail current increased but no change in the gradual onset of the steady state current was observed.

The selectivity of NS3623 for HERG1 over other important cardiac potassium ion channels was examined and representative traces are presented in figure 11. Measurements were performed from oocytes expressing either KCNQ1 (figure 11A), Kv1.5 (figure 11B) or Kv4.3 channels (figure 11C). KCNQ1 participates in cardiac I_{Ks} current, Kv1.5 in I_{Kur} current and Kv4.3 in I_{to} current. The protocols outlined in the figure legend were used to elicit the respective currents. KCNQ1, Kv1.5 and Kv4.3 channels were unaffected by the presence of 30 µM of NS3623. We then further examined the effect of NS3623 on native calcium and sodium currents measured from isolated guinea pig cardiomyocytes. The results obtained for 10 µM of the compound are shown in figure 12A and 12B (calcium current) and 12C and 12D (sodium current). Effect of NS 3623 on inward currents I_{Ca} in guinea-pig ventricular myocytes showed a substantial and variable run-down over time. Five minutes after establishing access, the cells were exposed to 10 µM NS 3623. $I_{Ca,L}$ density was 20.1 ± 3.4 pA/pF before and 19.1 ±

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3.7 pA/pF after drug exposure (n = 7). This decline was not different compared to spontaneous run-down in time-matched controls (data not shown). NS 3623 also did not affect $I_{Ca,T}$ (12A). However, a small block of 20 % of the L-type calcium currents was observed in the presence of 30 µM of the compound while 100 µM inhibited the current by 40 %. I_{Na} was stable over time and I_{Na} density two minutes after exposure to NS 3623 was not different from the control values (46.5 ± 7.3 pA/pF vs. 46.8 ± 7.5 pA/pF, n = 5). The small Na^+ current was unaffected by 30 and 100 µM NS3623 (data not shown). We then examined the effect of activating HERG1 channels by NS3623 in isolated papillary muscle (figure 13). Under control conditions APD_{20}, APD_{50} and APD_{90} was 80±3 ms, 133±4 ms and 154 ± 5 ms (n = 5) and remained stable in time-matched control experiments. Exposure to 10 µM NS3623 decreased APD_{20}, APD_{50} and APD_{90} to 67±3 ms, 112±6 ms and 131 ± 8 ms ($p < 0.05$). Moreover, NS3623 (10 µM) reduced force of contraction slightly when compared to the time-matched control (from 122 ± 45 µN to 53 ± 23 µN in NS 3623 vs. from 118 ± 12 µN to 78 ± 10 µN in TMC, n = 5 each group). Resting membrane potential, dV/dt_{max} and action potential amplitude were not affected by NS3623. Control values were: 87 ± 0.5 mV, 238 ± 13 V/s and 125 ± 1 mV, respectively (n = 5).

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Discussion

Very recently, we have shown that the diphenylurea NS1643 is an activator of HERG1 potassium channels (Hansen et al., 2006). It has so far been impossible to obtain a formulation of NS1643 for *in vivo* purposes. Since we strongly believe there is a need for a HERG1 activating compounds that can be used in experiments using isolated cardiac tissue and *in vivo* we searched for another compound that had the ability of activating the HERG1 channel. The present experiments were performed to examine the *in vitro* effects of the compound NS3623. In contrast to NS1643, this compound can be dissolved in vehicles appropriate for *in vivo* experiments. Initially, NS3623 has been tested *in vivo* and identified as a chloride channel blocker with the availability to inhibit the dehydration of red blood cells that occurs in sickle cell disease (Bennekou et al., 2001). It can not be completely ruled out that any observed effect of NS3623 in the present and future assays is to some extent mediated by a block of chloride channels. However, although it has been reported that $I_{Cl.vol}$ is present in the mammalian myocardium (Decher et al., 2001;Zou et al., 1998), a block of this current is not expected to influence the duration of the action potential in normal cardiac tissue.

Until very recently, no activators of the HERG1 channel have been known, and understanding how compounds can increase the current through this important cardiac channel is crucial, since it can be speculated that such a drug is beneficial in the treatment of certain kinds of arrhythmias. An overall increase of HERG1 channel current may stem from an increase in channel protein insertion in the cell membrane, and/or an increase in the open probability of the channel and/or a change in single channel conductance. In the present experiments, the increase of current was immediate as the drug was added to the perfusion solution, which makes it highly unlikely that NS3623 induces a higher expression of HERG1 channel in the oocyte. NS3623 was found to predominantly increase HERG1 current by

affecting channel inactivation. When investigating the voltage dependent release from inactivation we observed a right ward shift of 17.7 mV of the half inactivation voltage, *i.e.* channels will more easily be released from inactivation at any given membrane potential during repolarization and at the resting membrane potential in the cardiac diastole. It therefore follows that in the presence of NS3623 more channels are expected to be available at physiological relevant potentials compared with controls (figure 5C). We also found that the onset of inactivation was dramatically slowed at all measured potentials (figure 6B). The profound influence of NS3623 on the inactivation kinetics of the HERG1 channel may fully account for the observed increase in current. Since inactivation holds channels in a non-conducting conformation, rightward shifting the voltage at which half of the channels are inactive makes more channels available at most physiologically relevant membrane potentials. It can therefore be hypothesized that during the action potential exposure of the cardiomyocyte to NS3623 will increase the repolarizing reserve.

We also investigated the effect of NS3623 on channel activation, and although there was an overall increase in the steady-state and tail current in the drug treated oocytes we found no significant difference in the voltage dependence of activation (figure 2). When deactivation time course was investigated, we did not find any significant changes in the presence of NS3623 compared to control (figure 4). However, NS3623 induced a shift in the voltage that induced the largest tail current (figures 4C and 4D). Under control conditions the largest current was induced at –50 mV, whereas application of NS3623 resulted in a 10 mV shift towards a more negative potential. Such a shift may be speculated to increase the repolarizing reserve and the diastolic HERG1 current of cardiac myocytes exposed to drugs like NS3623, since the larger tail current will appear later in the repolarizing phase of the action potential.

In mutant channels where the inactivation ability was compromised (HERG1 S620T and HERG1 S631A) or were the blocking site was eliminated (HERG1 F656M), the mechanism of action of NS3623 was further revealed. When the compound was examined by application to the non-inacitvating HERG1 mutant S620T, no significant increase in current was observed (figure 7C). Neither did NS3623 significantly increase currents measured from the S631A mutated channel (figure 7C), a channel that has been reported to posses a slight inactivation (Zou et al., 1998). This is consistent with the finding that NS3623 most dominantly affects the rapid C-type inactivation that occurs during normal HERG1 channel activation. Addition of NS3623 to channels mutated in one of two amino acids necessary for high affinity HERG1 channel inhibition (F565M) resulted in a ~5 fold increase of the steady state current at 0 mV and a ~5 fold increase in the tail current likewise measured at 0 mV (Figure 8). When these data are compared to results obtained after NS3623 application to wild type HERG1 channels (figure 2) it is obvious that NS3623 is a far more potent HERG1 channel activator when part of the high affinity HERG1 channel inhibitor site is mutated. It can therefore be concluded that NS3623 must have a dual mode of action with a combined inhibitory and activating function. It should be emphasized that the overall mode of action of NS3623 is to increase wild type HERG1 current, demonstrating that the activating function of NS3623 is superior to the inhibitory activity of the compound. Finally, it was demonstrated that NS3623 can bind to and activate closed HERG1 channels. This indicates that the binding site for NS3623 might be at the extracellular part of the channel.

Recently, attention has been drawn to the short QT syndrome, where an apparent gain-of function mutation of the HERG1 channels gives rise to lethal cardiac arrhythmias. Gussak et al. (2000) first described that short QT intervals on the electrocardiogram increase the risk of sudden cardiac death to the same extent as the long QT, and it can be speculated whether drugs that increase the

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HERG1 current and thereby decrease the action potential duration will be pro-arrhythmic. It has been shown that the short LQT1 syndrome is caused by a gain-of-function mutation in the HERG1 channel by a N588K (aspargine to lysine) mutation that is located in the S5-pore linker region and gives rise to a HERG1 current with a large steady state current but a very small tail current (Brugada et al., 2004;Cordeiro et al., 2005;McPate et al., 2005;Zou et al., 1998)). The small tail current will result in less repolarisation reserve in the myocardium. It can therefore be argued that even though HERG1 N588K is reported as a gain-of-function in the steady-state current it can equally well be revealed as a loss of function when it comes to participation in cardiac action potential repolarization. Moreover, this mutation affects the recovery of inactivation extensively resulting in an almost 100 mV rightward shift in the voltage dependence HERG1 channel availability. We observed a rightward shift of 17 mV, which means that NS3623 induces a shift in the potential where half of the channels will be inactivated towards more depolarized potentials, resulting in a larger steady state current. However, HERG1 channels exposed to NS3623 will still be expected to inactivate during the plateau phase of the action potential considering the moderate shift in the estimated halfpoint of mid-inactivation. Further, McPate et al. (2005) and Cordeiro et al. (2005) reported that the N588K mutation resulted in a HERG1 current that monotonously followed the action potential wave-form when such a protocol was applied, in contrast to the wild type HERG1 current where the steady state reaches a peak current during the plateau phase. When we applied an action potential waveform to elicit HERG1 current we observed that the shape of the current did not change when the oocytes were exposed to NS3623, although the current did increase in magnitude as seen on figure 10. It is therefore highly likely that HERG1 channel activation by NS3623 is incomparable to the HERG1 N588K gain-of-function mutation.

In the isolated papillary muscle from guinea pigs we observed a shortening of the action potential (figure 13). In the presence of 10 μM NS3623, APD_{90}, APD_{50} and APD_{20} decreased to the

same extent indicating a uniform abbreviation of repolarization. In the studies of selectivity for NS3623 for HERG1 channels over other relevant cardiac channels we did not observe any significant changes in the native sodium and calcium currents (figure 12) finding that is consistent with the maintenance of 'normal' action potential appearance. Neither did NS3623 significantly affect KCNQ1, Kv1.5 or Kv4.3, indicating that the abbreviation of the action potential duration is mainly through activation of the HERG1 channel.

We conclude that NS3623 can be seen as a selective HERG1 channel opener when applied in cardiac tissue. Further, NS3623 activation of HERG1 channels is fundamentally different from currents recorded from gain-of-function HERG1 N588K mutations. Having the good solubility profile of NS3623 in mind we therefore believe that a proper tool compound has been obtained to address questions regarding HERG1 opening in intact tissue and after *in vivo* applications. These approaches will be addressed in future studies.

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Aknowlegdements

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Figure legends

Figure 1

The structure of N-(4-bromo-2-(1H-tetrazol-5-yl)-phenyl)-N'-(3'-trifluoromethylphenyl)urea (NS3623).

Figure 2

Activation of the HERG1 channel by 30 µM NS3623. Activating currents elicited by depolarizing voltage in steps of 10 mV in *Xenopus laevis* oocytes injected with HERG1 cRNA in control measurements (A) and after exposure to 30 µM NS3623 (B). C is displaying the characteristic bell-shaped I/V curve of the steady state curve that is obtained when the oocytes are challenged with the indicated protocol, and the corresponding I/V relationship obtained when oocytes were exposed to 30 µM NS3623 (grey). D depicts the tail currents recorded at -60 mV obtained in control situation (black) and after exposure to NS3623 (grey) (n = 7).

Figure 3

Concentration response of NS3623 on cloned HERG1 channels. HERG1 channels 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 -80mV for 3 s. After establishment of stable control current the oocytes were exposed to increasing doses of NS3623 ranging from 300 nM to 300 µM. Oocytes were washed with Kulori in between concentrations. Currents were measured at steady state at + 20 mV, normalized to the control current and plotted as a function of the NS3623 concentration. The dose effect relationship was sigmoidally fit and the EC_{50} value was calculated to 79.4 ± 14.9 µM (n = 5).

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Figure 4

NS3623 does not affect deactivation of the HERG1 channel. The voltage dependence and time constants of channel deactivation was investigated using the outlined protocol, where current was activated with a 1 s pulse at +40 mV, followed by a 10 mV stepped test potential ranging from -130 to +40 mV in order to determine the voltage dependence of deactivation in control oocytes (A) and after exposure to 30 µM NS3623 (B). The peak tail current as a function of the applied voltage used to release the channels from inactivation is depicted in C. In the presence of 30 µM NS3623 the tail current increased at most potentials (D). Time constants for deactivation were calculated from two exponential fits to the recorded tail currents. Neither the fast (E) nor the slow (F) component changed significantly when the channels were exposed to NS3623 (n = 6).

Figure 5

NS3623 influences the half-point of voltage dependent inactivation. Channels were completely activated and inactivated by holding the membrane potential at +20 mV for 1s followed by very short (10 ms) repolarizing steps from -150 to 20 mV and finally clamping at 20 mV. Representative data for control oocytes (black) and oocytes exposed to 30 µM of NS3623 (grey) are demonstrated in A and B, respectively. The peak tail current was then recorded and plotted as a function of the previous short-step potential, which makes it possible to investigate the voltage dependence of release from inactivation (C). The normalized data for control oocytes (black) and oocytes exposed to 30 µM of NS3623 (grey) were fitted to a Boltzmann function. In control oocytes V_{50} was -75.9 mV +/- 0.6 mV and for oocytes exposed to 30 µM NS3623 V_{50} was calculated to -58.2 +/- 0.4 mV (n=6).

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Figure 6

The time constant for re-onset of inactivation is affected by NS3623. Current was first activated and then inactivated by a 1 s pulse to 40 mV. Subsequently, the channels were subjected to a 10 ms pulse to allow complete recovery from inactivation. The third part of the outlined pulse was a stepwise increment in the potential from -40 to +50 mV to elicit a re-onset of channel inactivation. The currents representing the re-onset of inactivation were then fitted to a mono-exponential function. When comparing the resulting time constants from NS3623 treated oocytes with control experiments, it was revealed that these constants were significantly and immensely increased at all measured potentials (n = 4).

Figure 7

No effect of NS3623 on non-inactivating mutants. To test whether NS3623 could increase activity of HERG1 channels not capable of inactivation, the effect of the compound was investigated on channels that do not (S620T) or only to a small extent (S631A) inactivate. The step protocol outlined in figure 2 was used to elicit the mutant HERG1 current and 30 μM NS3623 was applied when stable control currents were obtained. Control recordings of S620T are demonstrated in black in A and recordings in the presence of 30 μM NS3623 in grey in B. The summarized steady state I/V relationship depicted in figure C reveals no significant difference in oocytes treated with NS3623 compared to the controls. Similar results were obtained with the S631A mutant that only has a very small degree of inactivation (figure D, E and F) (n = 4).

Figure 8

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Increased potency of NS3623 on mutant with altered blocking site. F656M HERG1 channels expressed in oocytes were challenged with the protocol described in figure 2. A and B show representative traces of results obtained from control oocytes and from oocytes exposed to NS3623, respectively. Both steady state (C) and tail currents (D) were increased approximately 5 times in the presence of NS3623 (grey traces) (n = 5).

Figure 9

NS3623 can bind to closed HERG1 channels. HERG1 channels were continuously opened and closed by a repeated two step protocol with 200 ms at +20mV followed by 800 ms at -80mV. Black bars indicate application 30 μM NS3623. The time scale for full compound effect was determined (A). Compound application was also performed to channels where the repeated step protocol was interrupted by a continuous clamp at -80mV to keep the channels closed (B). After resuming the repeated step protocol an instant current increase to 144 ± 14 % was observed (n=4).

Figure 10

Maintenance of normal action potential shape of the HERG1 current upon exposure to NS3623. HERG1 currents were elicited with stimuli imitating cardiac ventricular action potentials. The black trace is representative of currents obtained in control situations with a gradual increase of the steady state current during the 'plateau' phase of the action potential that reaches maximum at the end of this depolarized phase and a smaller tail current that is elicited upon repolarization. When the oocytes were exposed to 30 μM NS3623 both the steady state and the tail current were increased but no change in the gradual onset of the steady state was observed (grey trace) (n = 5).

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Figure 11

NS3623 is not affecting other important cardiac potassium channels. The selectivity of NS3623 for HERG1 over other potassium channels was examined and representative traces are presented for KCNQ1 (A), Kv1.5 (B) and Kv4.3 (C) channels. The protocols outlined next to the traces were used to elicit the respective currents. None of the investigated channels were significantly affected by the presence of 30 μM NS3623 (n = 3).

Figure 12. *Effect of NS 3623 on I_{Ca} and I_{Na} in guinea-pig ventricular myocytes*

Current voltage relationship for Ca^{2+} current recorded before and 2 minutes after addition of 10 μM NS 3623 (A). 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. Time course of peak $I_{Ca,L}$ (B) Current voltage relationship for I_{Na} (C). Time course for peak I_{Na} (D). Insets in A and C**:** voltage protocols; in B and D**:** original traces obtained at times marked by "a" and "b", respectively. The horizontal bars indicate the time of drug exposure.

Figure 13 Papillary muscle

Effect of NS3623 on action potentials recorded in papillary muscle. Application of 10 μM NS3623 to isolated papillary muscle caused a significant shortening of action potential duration at 20, 50 and 90 % of repolarization (APD_{20}, APD_{50} and APD_{90}) relative to the stable control recordings. The resting membrane potential and the upstroke velocity was not affected during drug application.

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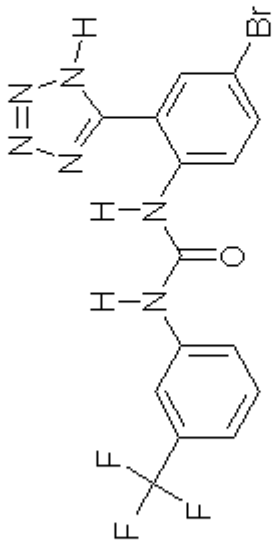
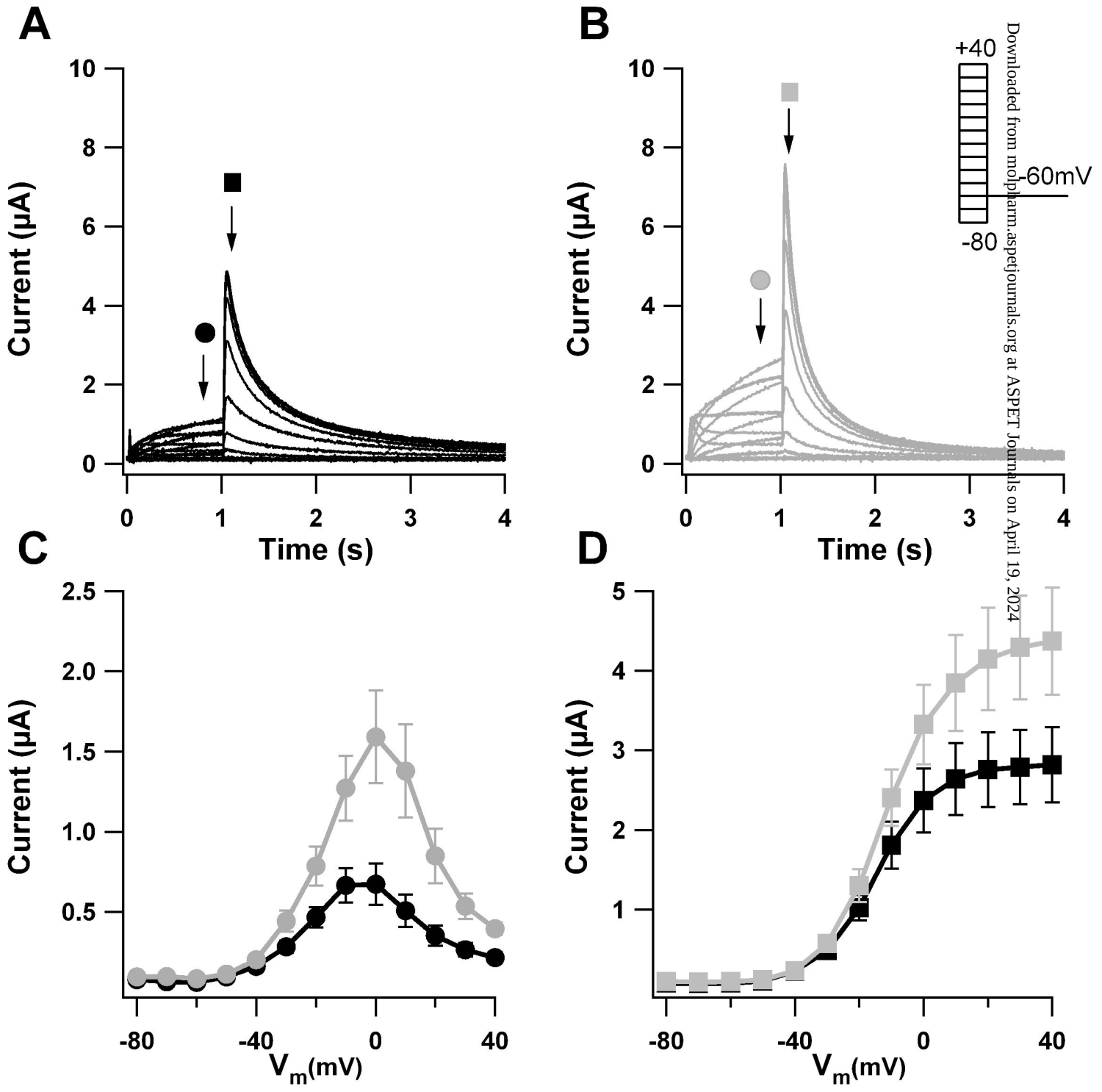
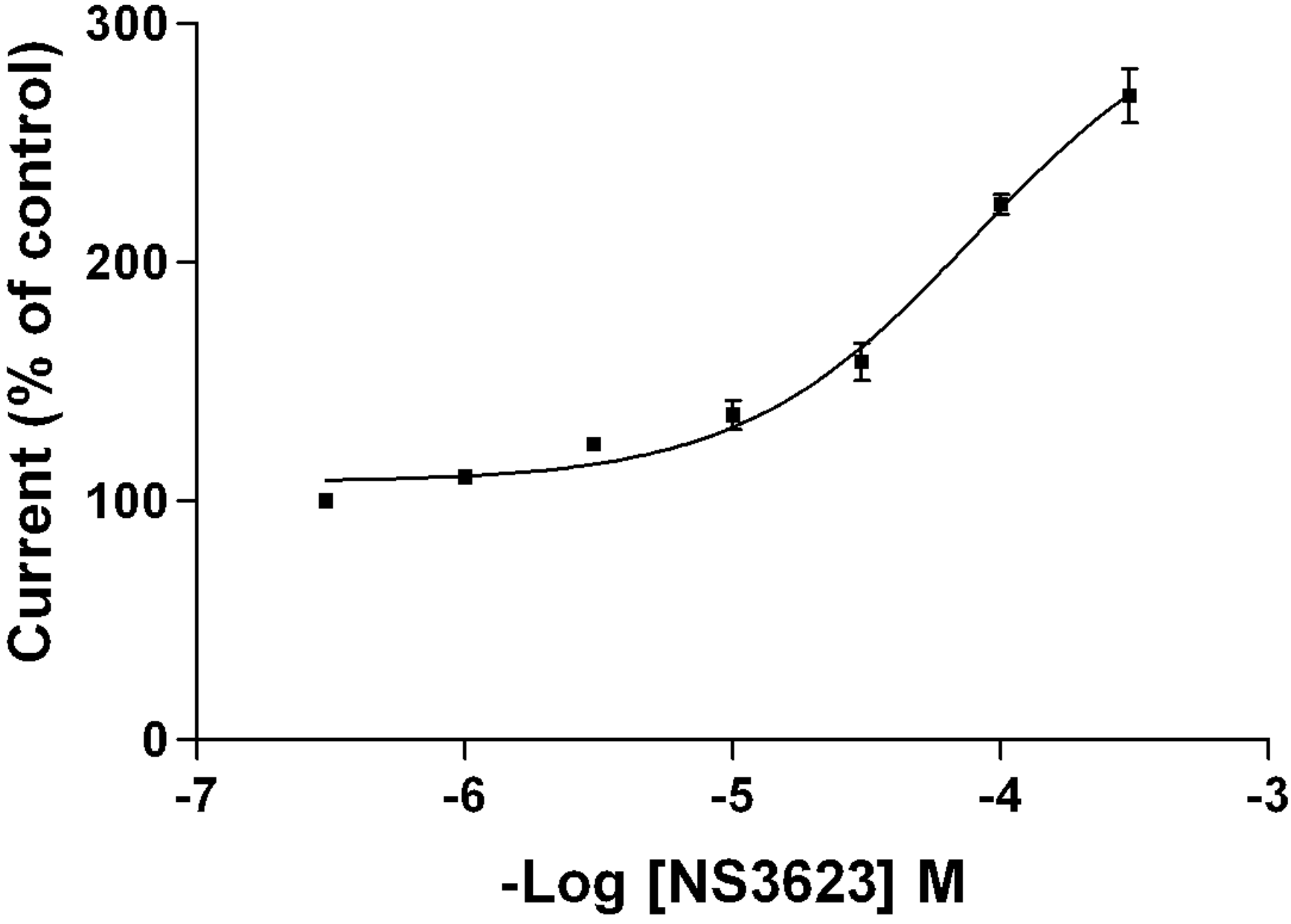
Figure 1



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200
100
0
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-6
-5
-4
-3
-Log [NS3623] M

Figure 4

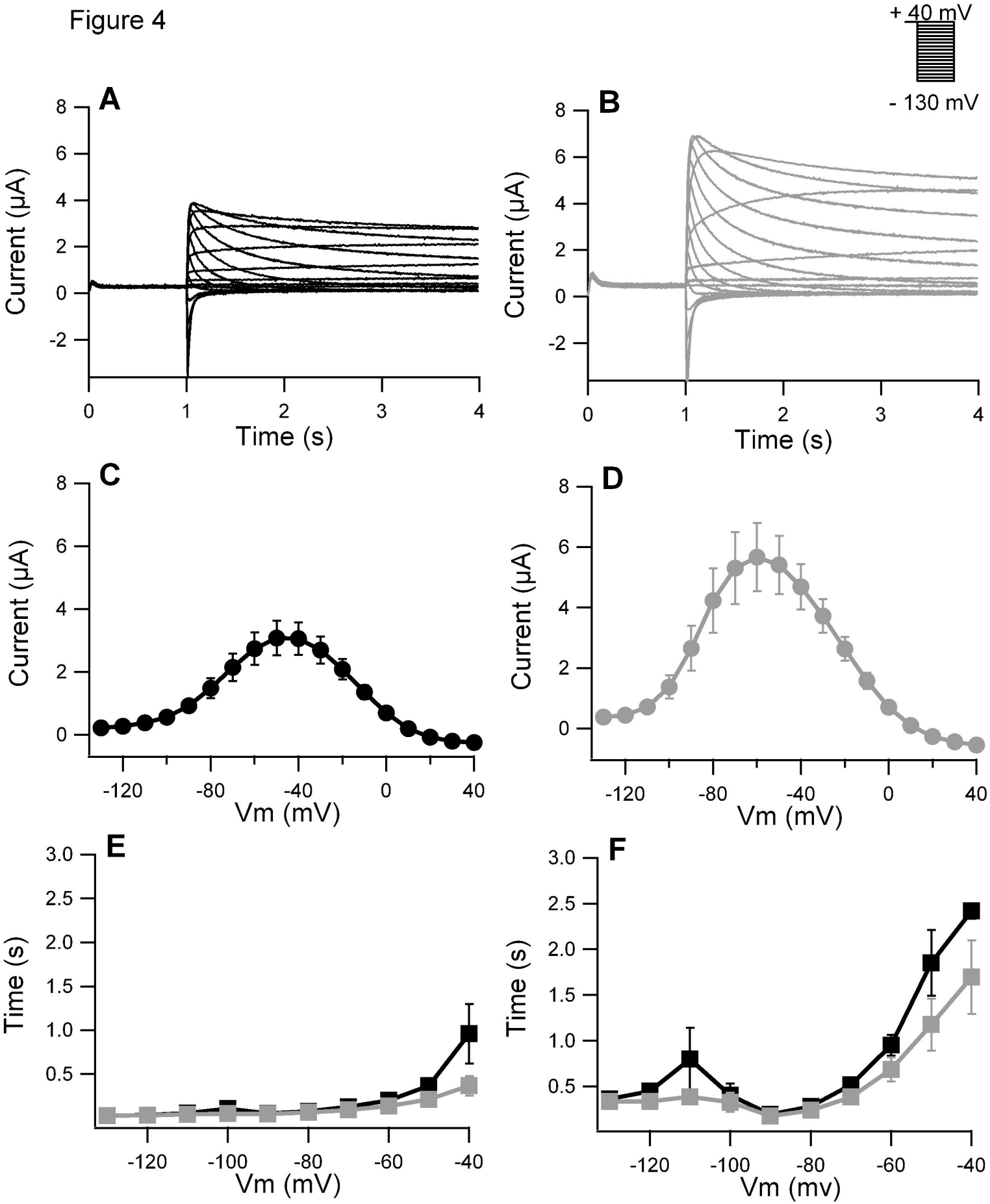


Figure 5

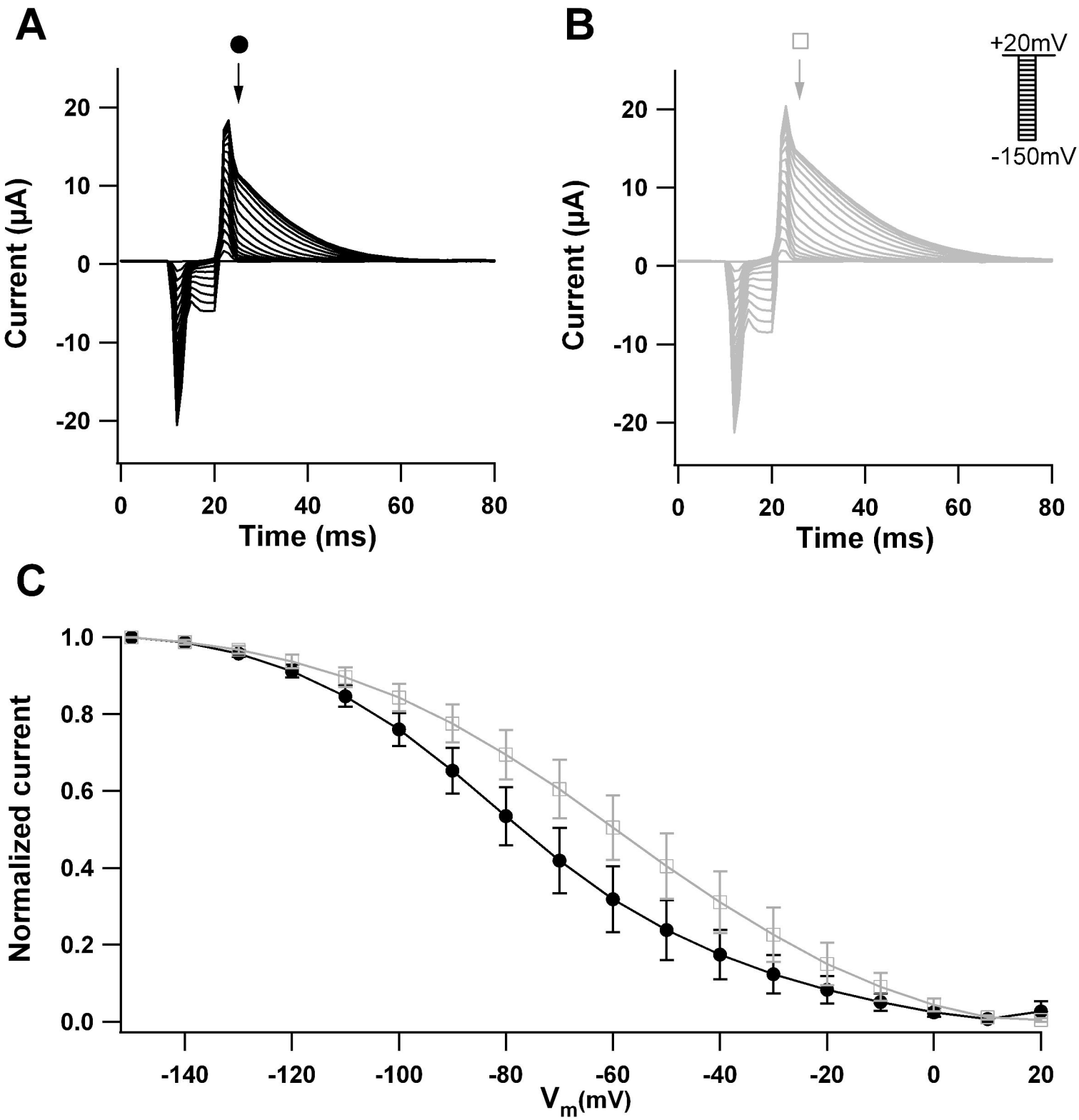
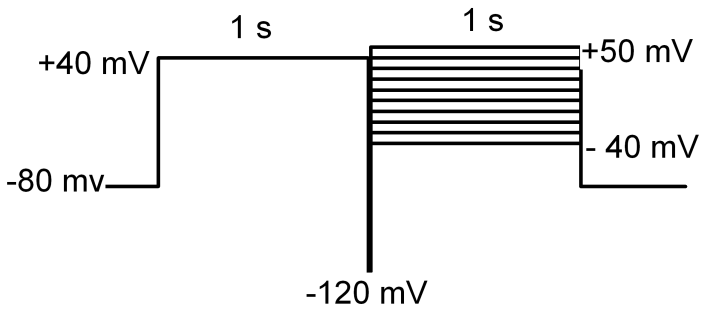
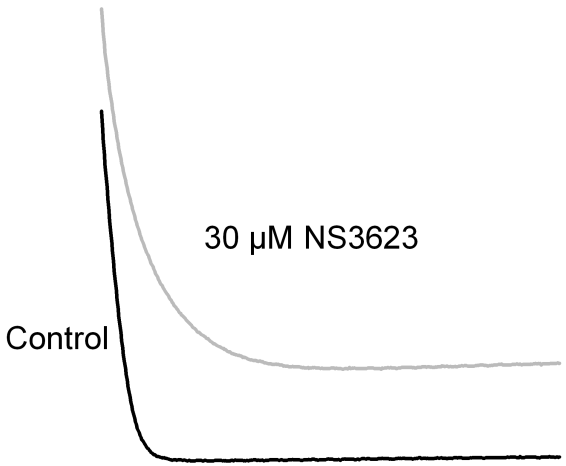
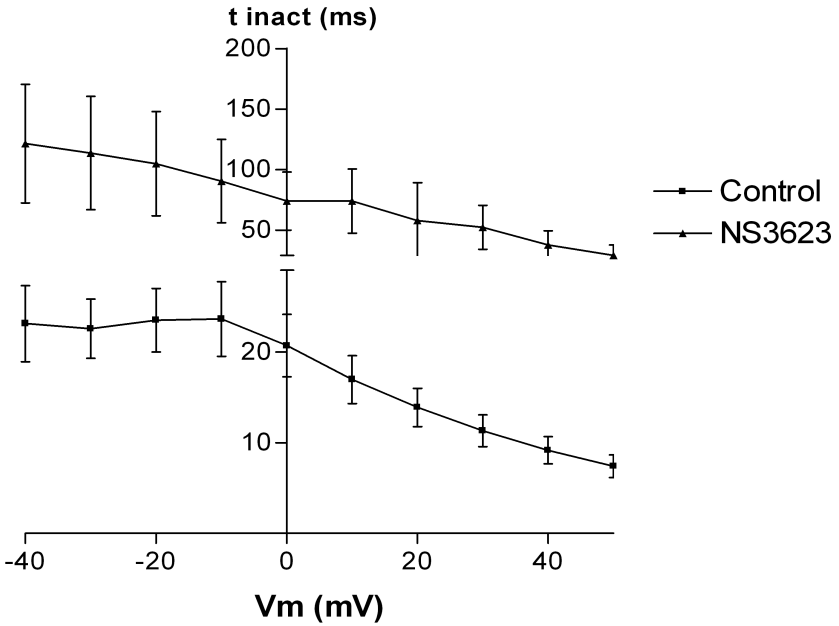


Figure 6







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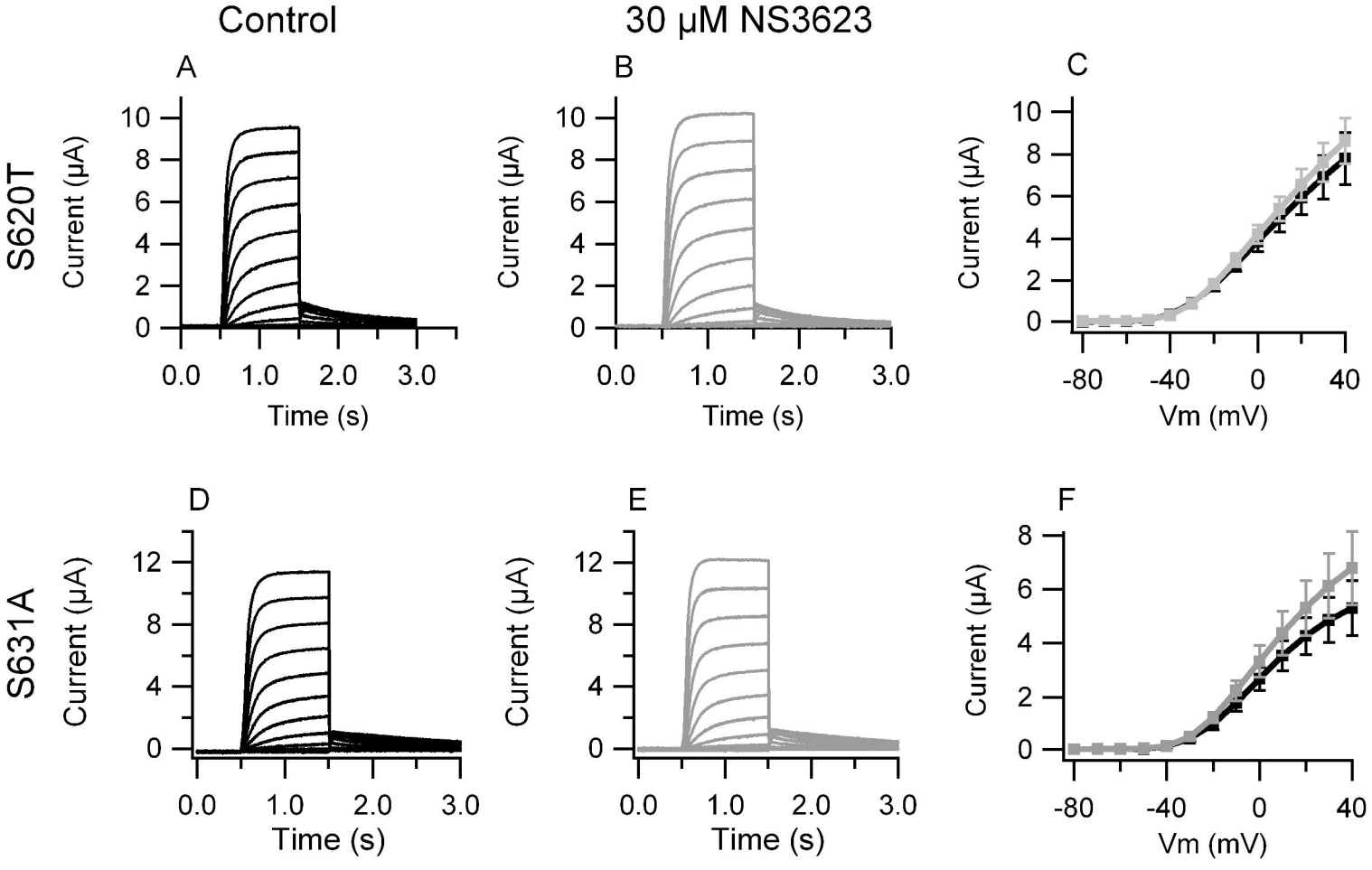

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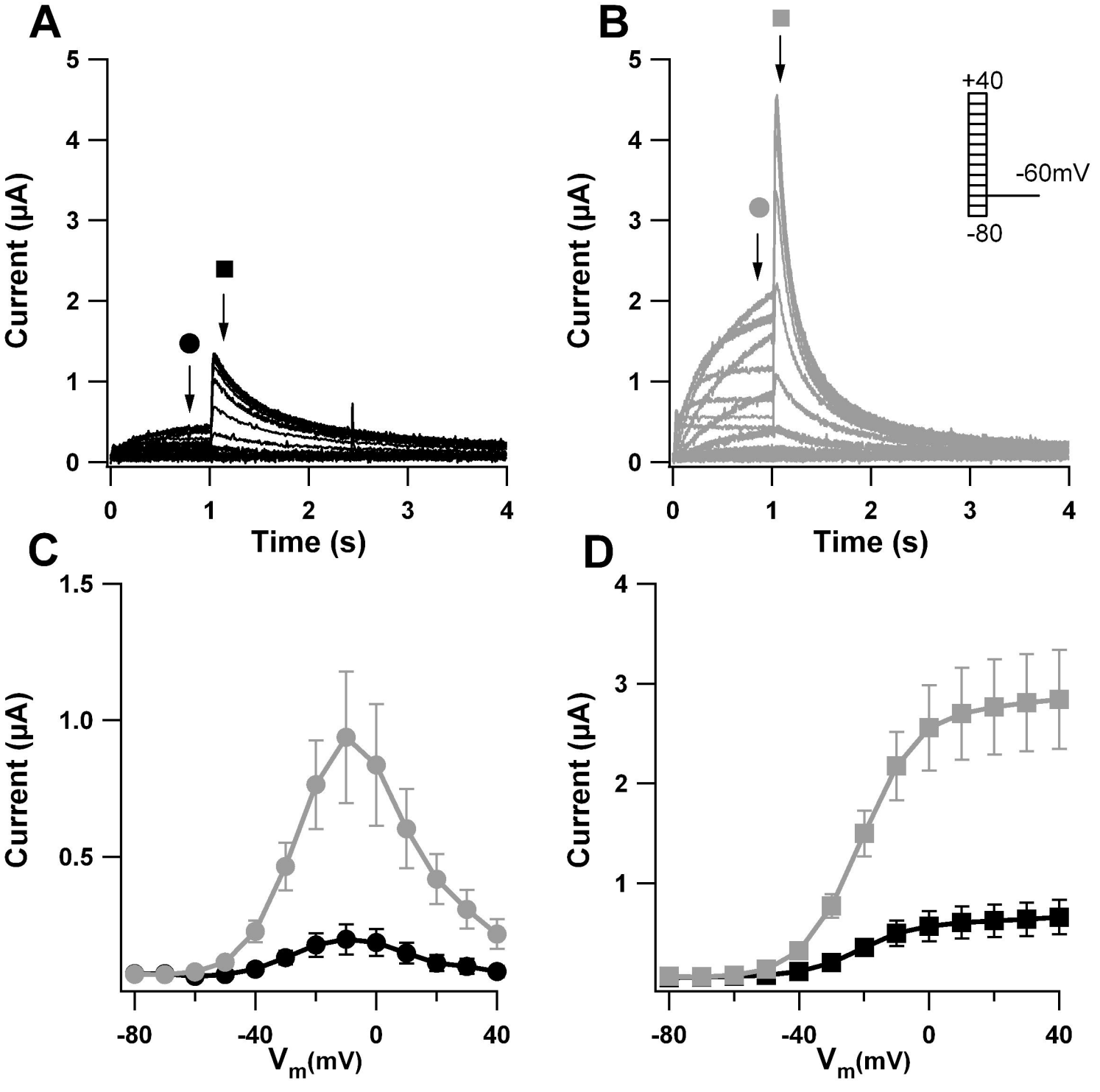


Figure 9

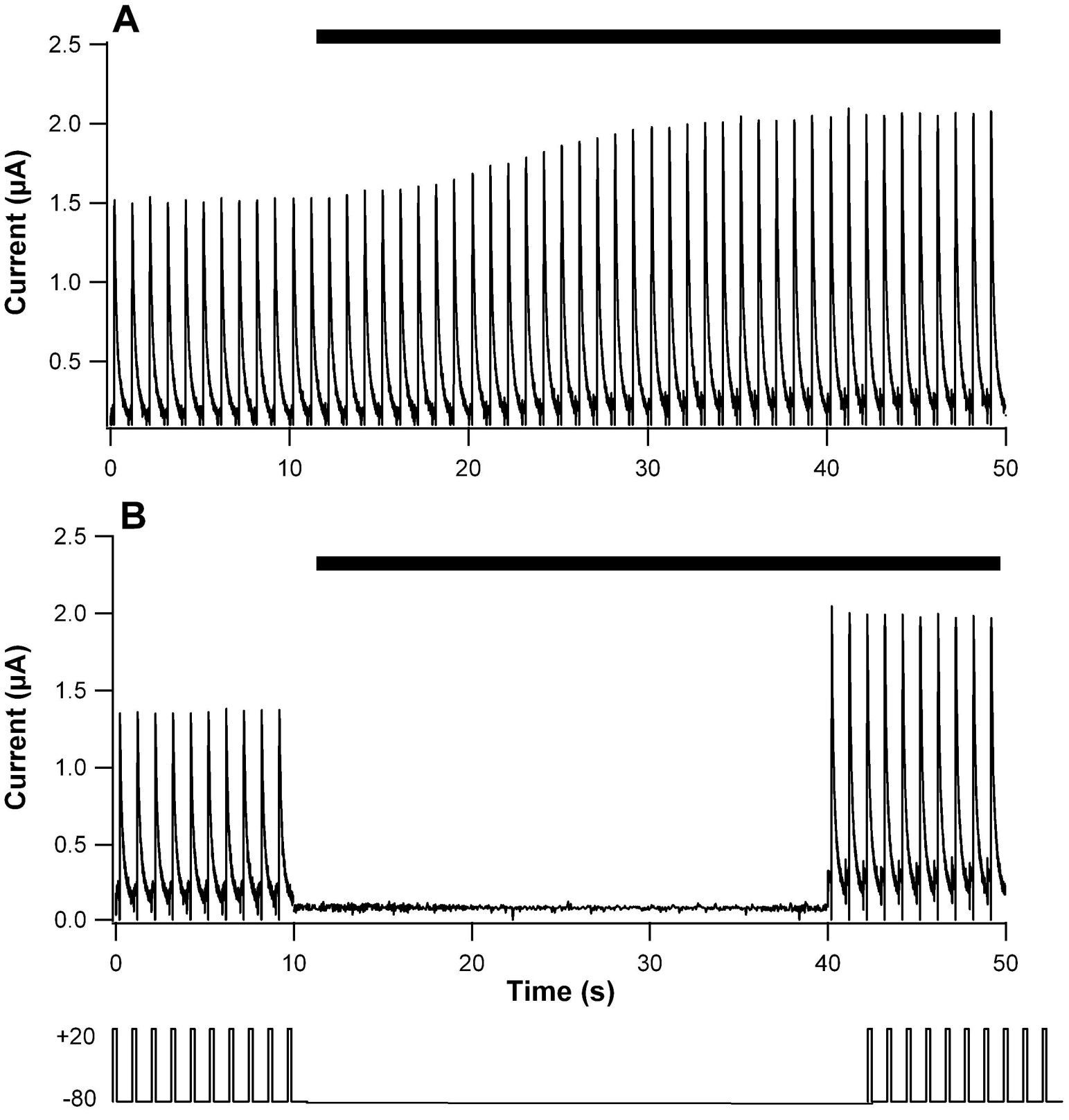
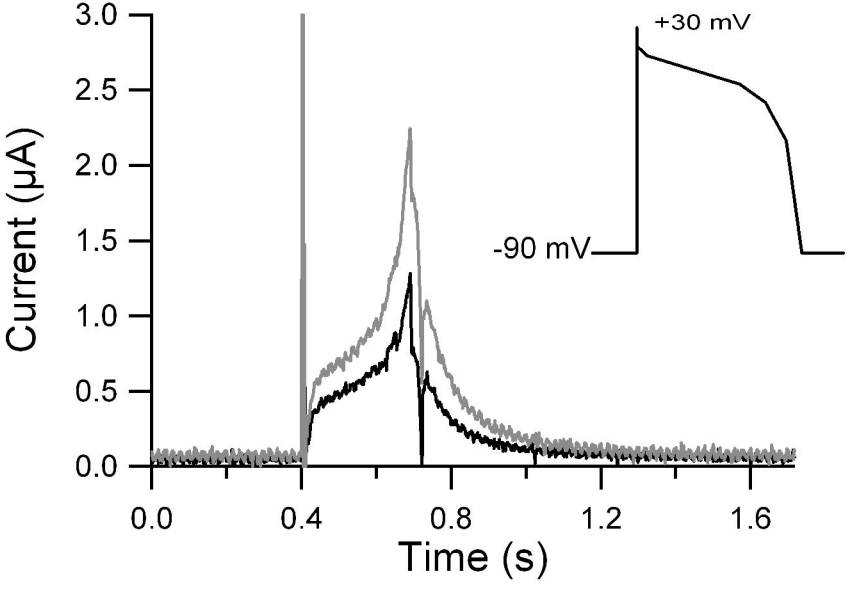


Figure 10



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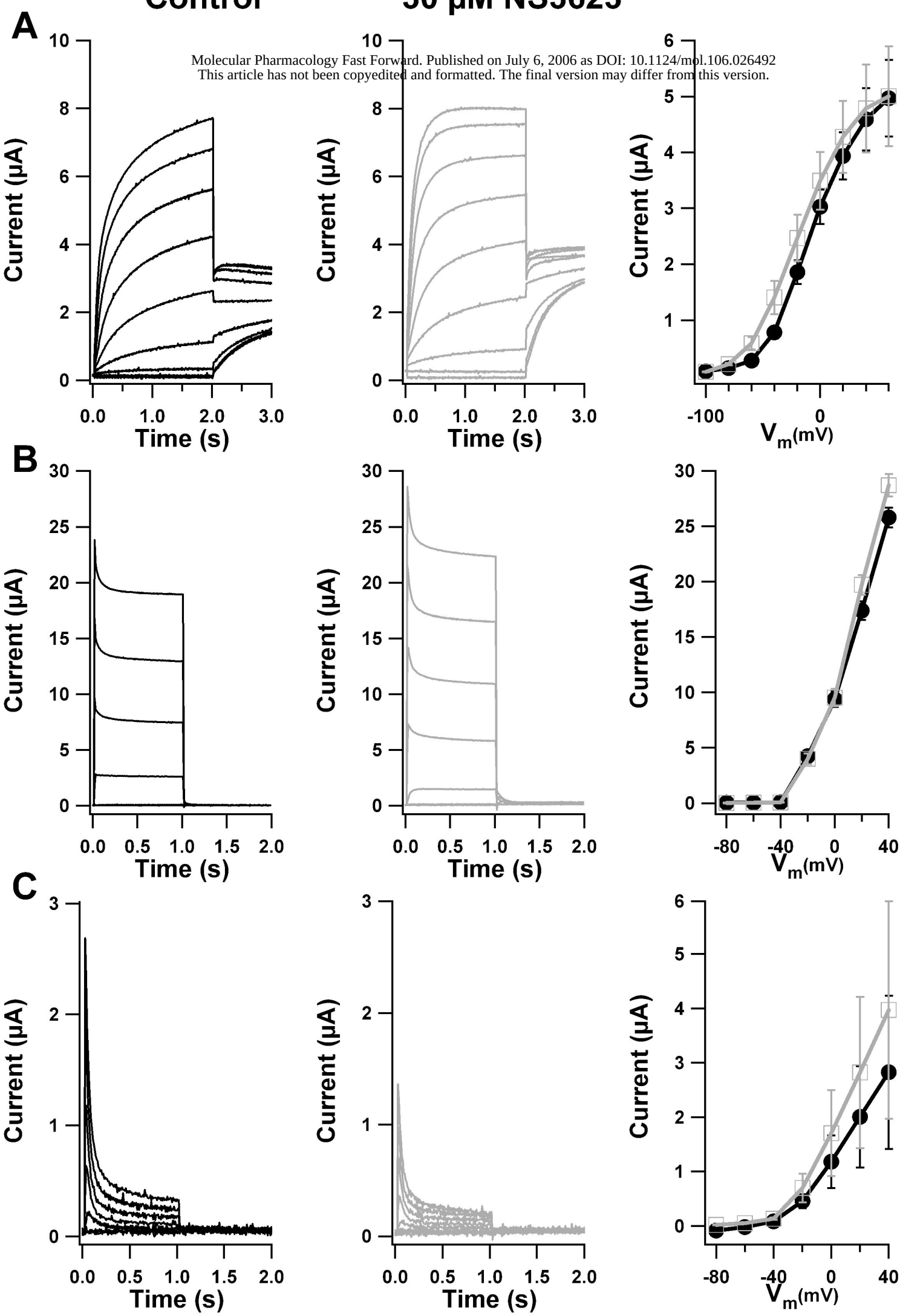

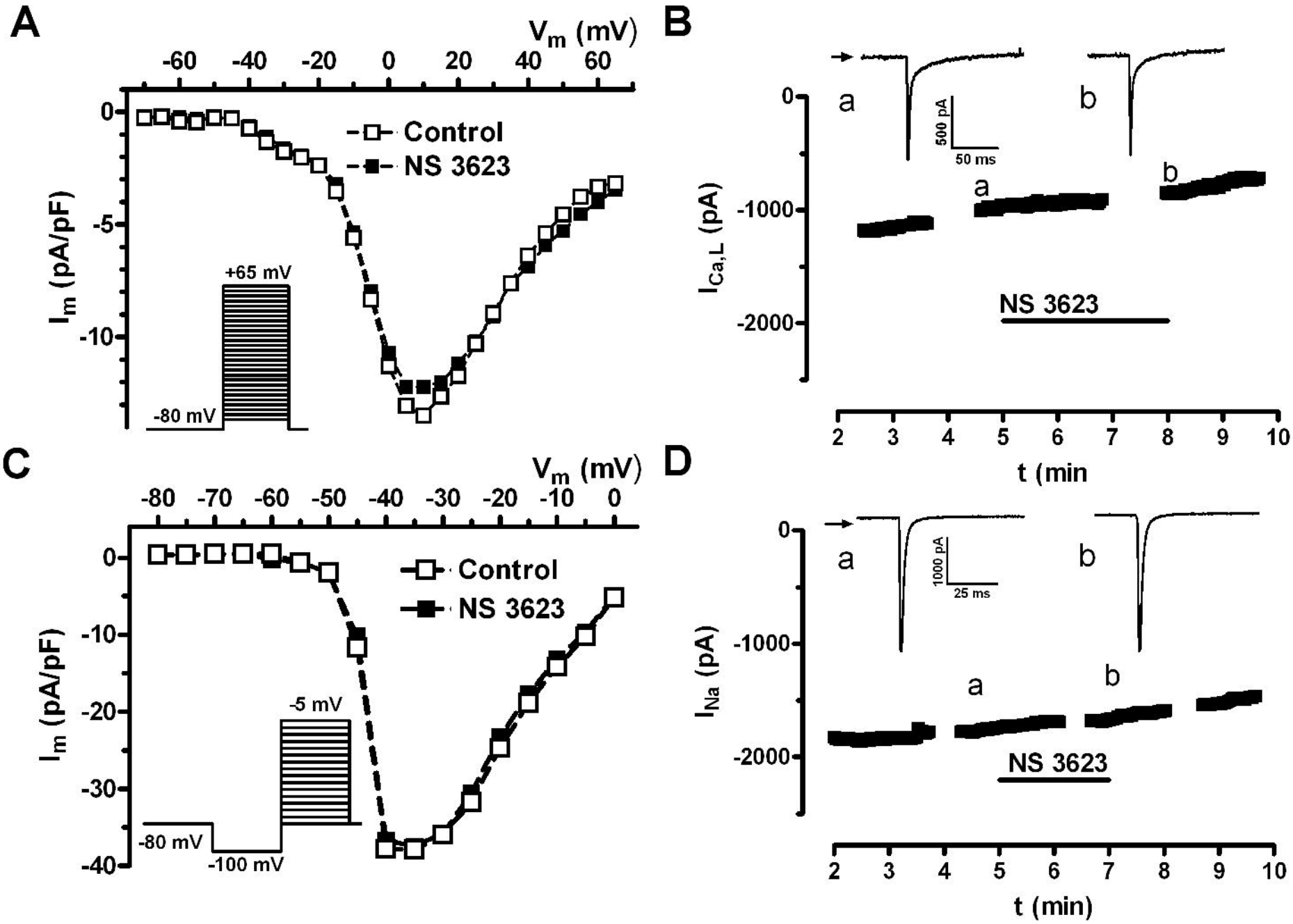
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0
-5
-10
I_m (pA/pF)
Control
NS 3623
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-80 mV
B
a
b
500 pA
50 ms
0
-1000
-2000
$I_{Ca,L}$ (pA)
NS 3623
2 3 4 5 6 7 8 9 10
t (min
C
V_m (mV)
-80 -70 -60 -50 -40 -30 -20 -10 0
0
-10
-20
-30
-40
I_m (pA/pF)
Control
NS 3623
-5 mV
-80 mV
-100 mV
D
a
b
1000 pA
25 ms
0
-1000
-2000
I_{Na} (pA)
NS 3623
2 3 4 5 6 7 8 9 10
t (min)

Figure 13