

Cardiac Ion Channel Effects of Tolterodine

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Running Title: Cardiac effects of tolterodine

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Text Pages: 24

Tables: 0

Figures: 6

References: 25

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Abbreviations: HERG: *human ether-a-go-go*-related gene; SEM: Standard error of the mean

Recommended Section: Cardiovascular

Abstract: Tolterodine is a muscarinic antagonist widely used in the treatment of urinary incontinence. Although tolterodine has not been reported to alter cardiac repolarization, it is chemically related to other muscarinic antagonist known to prolong cardiac repolarization. For this reason we studied the effects of tolterodine on cardiac ion channels and action potential recordings. Using patch clamp electrophysiology we found that tolterodine was a potent antagonist of the *human ether-a-go-go-related gene* (HERG) K^+ channel displaying an IC_{50} value of 17 nM. This potency was similar to that observed for the antiarrhythmic drug dofetilide ($IC_{50}=11$ nM). Tolterodine block of HERG displayed a positive voltage-dependence suggesting an interaction with an activated state. Tolterodine had little effect on the human cardiac Na^+ channel at concentrations of up to 1 μ M. Inhibition of L-type Ca^{++} currents by tolterodine was frequency-dependent with IC_{50} values measuring 143 and 1084 nM at 1Hz and 0.1Hz, respectively. Both tolterodine and dofetilide prolonged action potential duration in single guinea pig myocytes over the concentration range of 3-100 nM. However, prolongation was significantly larger for dofetilide compared to tolterodine. Tolterodine appears to be an unusual drug in that it blocks HERG with high affinity, but produces little QT prolongation clinically. Low plasma levels following therapeutic doses combined with mixed ion channel effects, most notably Ca^{++} channel blockade, may serve to attenuate the QT prolonging effects of this potent HERG channel antagonist.

Voltage-dependent ion channels control the electrical activity of the heart. During the heartbeat the influx of Na^+ and Ca^{++} , through their respective channels, serves to depolarize the myocardium while K^+ efflux through K^+ channels repolarizes the heart. Working in concert these channels give rise to the shape and the duration of the action potential on the cellular level, and to the electrocardiogram (ECG) waveform measured clinically. Any alteration in the activity of these channels can lead to changes in the ECG waveform and potentially to the development of cardiac arrhythmia. One such proarrhythmic condition is drug-induced (or acquired) long QT syndrome. In this case administration of a drug slows cardiac repolarization resulting in a prolongation of the QT interval on the electrocardiogram. This QT prolongation may be associated with the development of the ventricular arrhythmia known as torsades de pointes (Ben-David and Zipes, 1993). It is now believed that most cases of acquired QT prolongation are due to specific inhibition of the human cardiac K^+ channel known as HERG (*human ether-a-go-go-related gene*; Pearlstein et al., 2003). The HERG channel carries the rapid component of the delayed rectifier K^+ current in the human heart (I_{Kr}) (Sanguinetti et al., 1995). HERG channel inhibition is considered to be the mechanism that underlies the QT prolongation and ventricular arrhythmias associated with the administration of drugs such as astemizole (Zhou et al., 1999), terfenadine (Roy et al., 1996) and cisapride (Rampe et al., 1997; Mohammad et al., 1997).

Patch clamp electrophysiology studies using cloned HERG K^+ channels have become an important means for predicting the potential of a drug to cause QT prolongation in humans. Indeed, drugs that display high affinity block of HERG channel currents almost invariably demonstrate some degree of QT prolongation in clinical

practice (De Ponti et al., 2000; Redfern et al., 2003; Pearlstein et al., 2003). One striking exception to this trend is the drug verapamil. Although verapamil blocks HERG with an IC_{50} value of 143 nM, acquired long QT syndrome and torsades de pointe arrhythmia are not associated with its use (Zhang et al., 1999). This lack of proarrhythmia is presumably due to a concurrent inhibition of cardiac Ca^{++} channels by verapamil, an activity that counteracts the QT prolonging effects of HERG channel inhibition (Zhang et al., 1999). Such mixed ion channel effects may therefore mask clinical QT prolongation and confound the interpretation of HERG channel IC_{50} data.

Tolterodine (brand name Detrol) is a muscarinic antagonist used in the treatment of overactive bladder. QT prolongation or torsades de pointe arrhythmia are not associated with tolterodine treatment despite widespread clinical use (Hills et al., 1998; Larsson et al., 1999; Millard et al., 1999; Layton et al., 2001). Other drugs in this chemical and pharmacological class (e.g. terodiline) are known to prolong action potential duration, block cardiac K^+ channels and produce QT prolongation and arrhythmia in clinical use (Thomas et al., 1995; Jones et al., 1998). However, no data is currently available regarding the effects of tolterodine on cardiac ion channels. For this reason we examined the effects of tolterodine on a number of cardiac ion channels including HERG, Na^+ and Ca^{++} channels. We also examined the effects of tolterodine on action potentials measured in single cardiac myocytes and compared its activity to that of the potent antiarrhythmic drug dofetilide.

Materials and Methods

Cell Preparation. Chinese hamster ovary (CHO) cells (American Type Culture Collection, Manassas, VA) were stably transformed with the cDNA encoding the HERG cardiac K⁺ channel as previously described (Rampe et al., 1997). Cells were maintained in tissue culture incubators at 37°C in a humidified 95% air/5% CO₂ atmosphere. Stable transfectants were selected by coexpression of the HERG cDNA and neomycin resistance gene incorporated into the expression plasmid. Cells were cultured in Ham's F-12 medium containing 10% fetal calf serum and 500 µg/ml G418. The cDNA encoding SCN5A, the human cardiac Na⁺ channel, was stably transfected into HEK-293 cells (American Type Culture Collection) as previously described (Kuryshv et al., 2000). Cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and 500 µg/ml G418.

Single ventricular myocytes were isolated from guinea pigs using a method modified from that described by Salata et al (1995). Briefly, male guinea pigs (Hartley) were anesthetized with 5% of isoflurane (Baxter Healthcare corp., Deerfield, IL) in a mixture of nitrous oxide and oxygen (1:1). Then a thoracotomy was performed and the heart was removed and immediately transferred to oxygenated (100% O₂) cold saline. The heart was perfused retrogradely at 10 ml/min through the aorta with an oxygenated Ca⁺⁺-free saline at 37°C in three stages: first with standard Ca⁺⁺-free saline for 5 min, second with the same solution containing 280 U/ml Type II collagenase (Worthington Biochemical Corp., Lakewood, NJ) plus 0.75 U/ml Type XIV protease (Sigma Chemical Co., St. Louis, MO) for 8 min, and finally with saline containing 0.2 mM CaCl₂ for additional 7 min. The left ventricle cut into small pieces was gently shaken at room

temperature for about 5 min to disperse single myocytes. The isolated myocytes were then maintained at 10°C for electrophysiological recording, usually within 8 hours after isolation.

Patch Clamp Electrophysiology. All ionic currents were recorded using the whole-cell configuration of the patch-clamp technique (Hamill et al., 1981). Electrodes (2-4 MΩ resistance) were made from TW150F glass capillary tubes (World Precision Instruments, Sarasota, FL). For HERG channel recordings, electrodes were filled with the following solution: 120 mM potassium aspartate, 20 mM KCl, 4 mM Na₂ATP, 5mM HEPES, 1 mM MgCl₂, pH 7.2 with KOH. For Na⁺ channel recordings the electrode solution contained: 130 mM cesium aspartate, 5 mM MgCl₂, 2 mM Na₂ATP, 0.1 mM GTP, 10 mM HEPES, pH 7.2 with CsOH. For Ca⁺⁺ channel recordings the electrode solution contained: 130 mM cesium methanesulfonate, 20 mM tetraethylammonium chloride, 1 mM MgCl₂, 10 mM EGTA, 10 mM HEPES, 4 mM Tris-ATP, 0.3 mM Tris-GTP, 14 mM phosphocreatine, 50 U/mL creatine phosphokinase, pH 7.2 with CsOH. The external solution for HERG channel recordings contained the following: 130 mM NaCl, 5 mM KCl, 2.8 mM sodium acetate, 1.0 mM MgCl₂, 10 mM HEPES, 10 mM glucose, 1.0 mM CaCl₂, pH 7.4 with NaOH. For Na⁺ channel recordings the external solution contained 40 mM NaCl, 97 mM N-methyl-D-glucamine aspartate, 5.4 mM KCl, 1.8 mM CaCl₂, 1 mM MgCl₂, 5 mM HEPES, 10 mM glucose, pH 7.4 with N-methyl-D-glucamine aspartate. The external solution used for recording Ca⁺⁺ currents contained the following: 137 mM NaCl, 5.4 mM CsCl, 1.8 mM CaCl₂, 1 mM MgCl₂, 10 mM HEPES, 10 mM glucose, pH 7.4 with NaOH. All ionic currents were recorded at room temperature using an Axopatch 200B amplifier (Axon Instruments, Foster City, CA).

Currents were analyzed using the pCLAMP suite of software (Axon Instruments). IC_{50} values were obtained by nonlinear least-squares fit of the data (GraphPad Software, San Diego, CA).

Action Potential Recording. Myocytes were placed in a temperature-controlled ($37\pm 1^{\circ}C$) chamber and perfused with modified Tyrode's solution (in mM: NaCl 132, KCl 4, $MgCl_2$ 1.2, $CaCl_2$ 1.8, glucose 10 and HEPES 10, pH 7.4). Action potentials (AP) were recorded using a standard glass microelectrode filled with 3M KCl (resistance at 20-45 $M\Omega$). Action potentials were amplified using AxoClamp 2B amplifier (Axon Instruments) and data were stored and analyzed using the pCLAMP suite of software (Axon Instruments). Myocytes were allowed to equilibrate at a stimulation rate of 1 Hz for 30 min after which time action potential traces were recorded. The myocytes were then perfused and allowed to equilibrate for 5 minutes with ascending concentrations of drugs to generate dose-response relationships. Resting membrane potential (RMP), action potential amplitude (APA), action potential duration at 90% (APD_{90}) and at 50% (APD_{50}) of repolarization were determined for each concentration of drug.

Chemicals. (R) tolterodine and dofetilide were synthesized at Aventis Pharmaceuticals (Bridgewater, NJ). All other chemicals were obtained from Sigma (St. Louis, MO).

Results

Figure 1 shows the effects of tolterodine on HERG K⁺ channel currents. In these experiments, a 2-s depolarization to +20 mV was followed by repolarization of the cell to -40 mV to produce large, slowly deactivating tail currents characteristic of HERG (Sanguinetti et al., 1995). Figure 1A demonstrates that these tail currents were potently blocked by tolterodine. Block was evident over the concentration range of 3-100 nM and the IC₅₀ value measured 17 nM (15 nM - 19 nM, 95% C.L.; Fig. 1B). Tolterodine had no obvious effect on the waveform of the HERG channel currents under these conditions. The effects of tolterodine on HERG currents were mainly reversible upon washout. Following exposure to tolterodine (3-100 nM), currents recovered to within 62 ± 5% of pre-drug levels (n=3) upon washing the cells with drug-free solution for approximately 30 minutes. The affinity of tolterodine for blocking HERG was not significantly different from that observed for the potent antiarrhythmic drug dofetilide. The IC₅₀ value for dofetilide inhibition of peak HERG tail currents measured 11 nM (8 nM - 17 nM, 95% C.L.; Fig. 1B).

Figure 2 shows the effects of tolterodine on HERG channel currents measured over a wide range of test potentials. In these experiments, cells were held at -80 mV and currents were elicited by 2-s depolarizing pulses to potential ranging from -40 to +30 mV in 10 mV increments. The membrane potential was then returned to -100 mV and peak inward tail currents were recorded. Current traces in the absence and presence of 10 nM tolterodine are shown in Figs. 2A and B, respectively. The resultant current-voltage relationship averaged from 7 cells is presented in Fig. 2C. Tolterodine inhibited tail current amplitude in a voltage-dependent manner. When inhibition of HERG current is

plotted as a function of test potential, a statistically significant ($P < 0.05$, ANOVA) correlation between voltage and drug effect was observed with inhibition ranging from $5 \pm 2\%$ at -10 mV to $33 \pm 3\%$ at $+30$ mV (Fig. 2D).

The effects of tolterodine on the human cardiac Na^+ channel are illustrated in Fig. 3. Sodium channel currents were elicited by depolarizing pulses to -20 mV from a holding potential of -110 mV. Thirty of these depolarizing steps were delivered at a rate of 1 Hz. Tolterodine was then added to the cells and allowed to equilibrate for 2-3 minutes without pulsing. The pulse train was then repeated several times at approximately 1-minute intervals to allow the drug to equilibrate with the cells. Typical Na^+ channel currents in the absence and presence of $1 \mu\text{M}$ tolterodine are shown in Fig. 3A. The dose-response relationship for tolterodine block of the Na^+ channel is given in Fig. 3B. Tolterodine had little effect on Na^+ channel currents under these conditions with a maximal block of $6 \pm 2\%$ observed at $1 \mu\text{M}$ (Fig. 3B).

We used single ventricular myocytes isolated from guinea pigs to examine the effects of tolterodine on L-type Ca^{++} channel currents. Ca^{++} currents were elicited by 200 msec depolarizing pulses to 0 mV from a holding potential of -40 mV. As was the case for the Na^+ channel, thirty depolarizing steps were delivered at a rate of 1 Hz. Tolterodine was then added to the cells and allowed to equilibrate for 2-3 minutes without pulsing. The pulse train was then resumed and repeated several more times at approximately 1 minute intervals until the current traces from separate trains overlapped. Typical Ca^{++} channel current traces in the absence and presence of 30 and 300 nM tolterodine are shown in Fig. 4A. Under these conditions tolterodine inhibited Ca^{++} channel currents with an IC_{50} value of 143 nM (98–208 nM, 95% C.L., Fig. 4B). We also examined the

effects of tolterodine on Ca^{++} channels stimulated at a constant frequency of 0.1 Hz. At this frequency the drug was less potent displaying an IC_{50} value of 1084 nM (818-1437 nM, 95% C.L., Fig. 4B). To further examine this frequency dependence, we stimulated cells in absence of drug for 3 minutes at a rate of either 0.1 Hz or 1 Hz (Figs. 4C and D, respectively). After this, cells were allowed to equilibrate with 300 nM tolterodine for 3 minutes without pulsing. Following this equilibration period the pulse trains were repeated. Tolterodine was more effective at blocking Ca^{++} currents at 1 Hz compared to 0.1 Hz. When cells were stimulated at 0.1 Hz, Ca^{++} current decreased by $4 \pm 2\%$ in the absence of drug and by $24 \pm 6\%$ in the presence of 300 nM tolterodine (Fig. 3C). At a 1 Hz stimulation frequency, Ca^{++} current declined by $20 \pm 3\%$ under drug-free conditions and by $60 \pm 6\%$ in the presence of tolterodine (Fig. 3D). It appears that the drug block observed in Figs. 3C and 3D did not reach a complete steady state. Therefore the IC_{50} values reported in Fig. 3B may underestimate, to some degree, the true affinity of tolterodine for cardiac Ca^{++} channels, especially at the 1 Hz stimulation rate.

We next examined the effects of tolterodine on action potentials recorded from guinea pig ventricular myocytes and compared these effects to those observed for dofetilide. Figure 5A shows the effects of tolterodine (3 – 100 nM) on the action potential waveform. Figure 5B illustrates the effects of dofetilide (3-100 nM) on the action potential waveform. Both drugs increased action potential duration in a dose dependent manner but the effects of dofetilide were more pronounced. APD_{90} was increased by 4 ± 1 , 8 ± 1 , 16 ± 3 , and $28 \pm 6\%$ in the presence of 3, 10, 30 and 100 nM tolterodine, respectively. These same concentrations of dofetilide prolonged APD_{90} by 8 ± 1 , 30 ± 6 , 53 ± 13 and $65 \pm 17\%$, respectively (Fig. 6A). A similar patter was observed for APD_{50}

where 100 nM tolterodine produce a maximal prolongation of $25 \pm 6\%$ while dofetilide increased APD_{50} by $53 \pm 14\%$ at this same concentration (Fig. 6B). Neither drug displayed any significant effects on the resting membrane potential or the action potential amplitude at the concentrations tested.

Discussion

This report is the first to detail the effects of tolterodine on cardiac ion channels. We found that tolterodine was a potent inhibitor of the HERG cardiac potassium channel displaying an IC_{50} value of 17 nM. Some inhibition of HERG was evident at concentrations as low as 3 nM. The effects of tolterodine on HERG displayed a positive voltage-dependence suggesting that tolterodine interacts with an activated state of the HERG channel. Furthermore, we have found that the affinity of tolterodine for blocking HERG is similar to that of the potent Class III antiarrhythmic drug dofetilide. In addition to its effects on HERG, tolterodine was also an effective antagonist of cardiac Ca^{++} channels. When Ca^{++} channels were stimulated at 1 Hz, tolterodine displayed an IC_{50} value of 143 nM. At a lower stimulation rate (0.1 Hz) the drug was less potent suggesting the block of Ca^{++} channels was frequency dependent. Tolterodine had little effect on voltage-dependent Na^{+} channels even at concentrations up to 1 μ M. We believe the data support the notion that tolterodine is a potent mixed ion channel antagonist in the heart with prominent effects on both HERG and the L-type Ca^{++} channel. In contrast, dofetilide is known to be a specific inhibitor of HERG/ I_{Kr} with no effects on Ca^{++} channels up to 10 μ M (Paul et al., 2001). A head to head comparison of these two drugs on action potential waveforms revealed that tolterodine produced far less APD prolongation relative to dofetilide. We feel that this may reflect the added Ca^{++} channel blocking properties of tolterodine and that this activity serves to attenuate the APD lengthening that can be observed from pure I_{Kr} inhibition.

Drugs that block HERG/ I_{Kr} with high affinity are often associated with QT prolongation on the electrocardiogram, and the development of the ventricular arrhythmia known as torsades de pointes. Indeed, HERG channel affinity is now a widely used to both predict and to explain drug-induced QT prolongation and attending ventricular arrhythmia (Redfern et al., 2003; Pearlstein et al., 2003). The affinity of tolterodine for HERG is similar not only to dofetilide, but also to cisapride (Mohammad et al., 1997; Rampe et al., 1997), terfenadine (Wang et al., 2003) and pimoziide (Kang et al., 2000). All of the aforementioned drugs are well known to produce significant QT interval prolongation and, in some cases, torsades de pointes arrhythmia. However, tolterodine, despite widespread clinical use, has not been reported to produce QT prolongation either in controlled clinical trials or during post-marketing surveillance (Millard et al., 1999; Larsson et al., 1999; Nilvebrant, 2001; Layton et al., 2001). Following therapeutic doses, C_{max} levels of tolterodine in normal subjects average about 12-16 nM of which 96.3% is bound to serum proteins (Brynne et al., 1998; Ollson and Szamosi, 2001) resulting in free plasma levels of <1 nM. It is therefore quite possible that these plasma levels of tolterodine are simply not high enough to produce QT prolonging effects. However, in these same studies, poor metabolizers of the drug (cytochrome P450 2D6 polymorphism) display average C_{max} values of 51-116 nM. Yet even at these high plasma levels, electrocardiographic studies have reportedly revealed no prolongation of the QT interval (Brynne et al., 1998). In this patient population, pharmacodynamic factors, specifically block of the cardiac L-type calcium channel, could play a role in limiting QT prolongation. In this respect we feel that tolterodine may be similar to verapamil. Verapamil blocks HERG with an IC_{50} value of 143 nM, but the drug does not produce

long QT syndrome despite the fact that plasma levels can reach the micromolar range (Zhang et al., 1999). This apparent discrepancy is explained by verapamil's block of cardiac L-type Ca^{++} channels that occurs over a similar concentration range, and acts to blunt the QT prolonging effects that would be expected from HERG channel inhibition alone (Zhang et al., 1999). This study is of great interest as it represents one of the only examples where potent block of the HERG channel *in vitro* did not translate into clinically significant QT prolongation (i.e. a false positive finding in the HERG assay). We believe that tolterodine joins verapamil in this atypical class of drugs that display high affinity for HERG, but no clearly evident clinical QT prolongation. Like verapamil, it is possible that block of the cardiac L-type Ca^{++} channel, at least in part, serves to counteract the QT prolonging effects of HERG channel blockade. Detailed clinical electrocardiographic studies, especially in poor metabolizers of the drug, would be valuable for exploring this possibility.

In summary, we have found tolterodine to be a potent blocker of both the L-type Ca^{++} channel and the HERG K^+ channel *in vitro*. These activities result in a prolongation of action potential duration, but not to the extent observed with the pure HERG/ I_{Kr} antagonist dofetilide. Tolterodine demonstrates that drugs with very high HERG affinity *in vitro*, do not necessarily produce obvious QT prolongation clinically. However, HERG channel testing is now a widely used safety screen in the drug development process and is considered as an important early predictor of clinical QT prolongation. It is therefore difficult to imagine that drugs like tolterodine, with low nanomolar affinity for HERG, will be developed for human use in the hope that some combination of biological factors will ultimately limit their QT prolonging potential. Just how many marketed drugs

currently share these attributes with tolterodine, and how many will be abandoned in the drug development process, remains unclear.

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Figure Legends

Figure 1. Effects of tolterodine on HERG. A, whole-cell HERG channel currents were elicited by 2-s depolarizing pulses to +20 mV from a holding potential of -80 mV at 40-s intervals. The cell was then returned to -40 mV to generate large outward tail currents. The effects of 3, 10, 30 and 100 nM tolterodine are shown. B, dose-response relationships comparing tolterodine and dofetilide block of peak tail currents at -40 mV. The IC_{50} values for tolterodine and dofetilide measured 17 nM and 11 nM respectively. Error bars denote S.E.M. (n=5-7). The chemical structures of tolterodine and dofetilide are shown as insets.

Figure 2. Effects of membrane potential on tolterodine block of HERG. Cells were held at -80 mV and depolarized for 2-s to potential ranging from -40 mV to +30 mV in 10 mV increments. The cells were then returned to -100 mV to generate inward tail currents. Traces in the absence and presence of 10 nM tolterodine are shown in A and B, respectively. C, Peak tail current amplitudes at -100 mV were normalized to those obtained after the +30 mV pulse in the absence of drug. The normalized tail currents are plotted as a function of test potential. Data in the absence of drug (filled squares) and after the addition of 10 nM tolterodine (open circles) are shown. Error bars indicate S.E.M. (n=7). D, inhibition of peak inward tail current amplitude is plotted as a function of test potential. Asterisks denote statistical significance compared to the inhibition observed after the +30 mV test pulse ($p < 0.05$, ANOVA). Error bars indicate S.E.M. (n=7).

Figure 3. Effects of tolterodine on Na⁺ channel currents. Cells were held at -110 mV and trains of 30 depolarizing pulses to +20 mV were delivered at a frequency of 1 Hz as described in Results. A, current traces after pulses 1, 10, 20 and 30 are shown in the absence and presence of 1 μM tolterodine. B, Dose-response relationship for tolterodine block of Na⁺ channels. Peak inward current at the last pulse (pulse 30) of each train was used to determine the inhibitory effects of tolterodine on Na⁺ currents. Little inhibition was noted under these conditions even with concentrations as high as 1 μM. Error bars denote S.E.M. (n=5).

Figure 4. Effects of tolterodine on Ca⁺⁺ channel currents. A, Ca⁺⁺ currents were elicited by trains of 30 depolarizing pulses to 0 mV from a holding potential of -40 mV delivered at 1 Hz as described in Results. Typical current traces after pulses 1, 10, 20 and 30 of this train are shown in the absence and presence of 30 and 300 nM tolterodine. Peak inward current at the last pulse of each train (pulse 30) was used to generate the dose-response relationship illustrated in panel B. The IC₅₀ value for tolterodine block of peak Ca⁺⁺ currents under these conditions measured 143 nM. Panel B also shows the dose-response relationship for tolterodine block of Ca⁺⁺ currents generated at a frequency of 0.1 Hz. Panels C and D illustrate the effects of 300 nM tolterodine on Ca⁺⁺ currents stimulated at 0.1 and 1 Hz, respectively. In both cases currents were normalized to the first pulse in the train. For the data obtained at 1 Hz, every 10th pulse is shown.

Figure 5. Effects of tolterodine and dofetilide on action potentials recorded from single guinea pig myocytes. A. Action potential waveforms in the absence and presence of 3, 10, 30 and 100 nM tolterodine are illustrated. B. Action potential waveforms in the absence and presence of 3, 10, 30 and 100 nM dofetilide are shown. For all experiments cells were stimulated at 1 Hz frequency and drugs added in ascending concentrations as described in Materials and Methods. Note that the time scales differ between panels A and B.

Figure 6. Effects of tolterodine and dofetilide on action potential duration in single guinea pig myocytes. Dose-response relationships for the lengthening of APD₉₀ (panel A) and APD₅₀ (panel B) by tolterodine and dofetilide are shown. Asterisks indicate significant difference from tolterodine value ($p < 0.05$ unpaired t-test). Error bars denote S.E.M. (n=6).

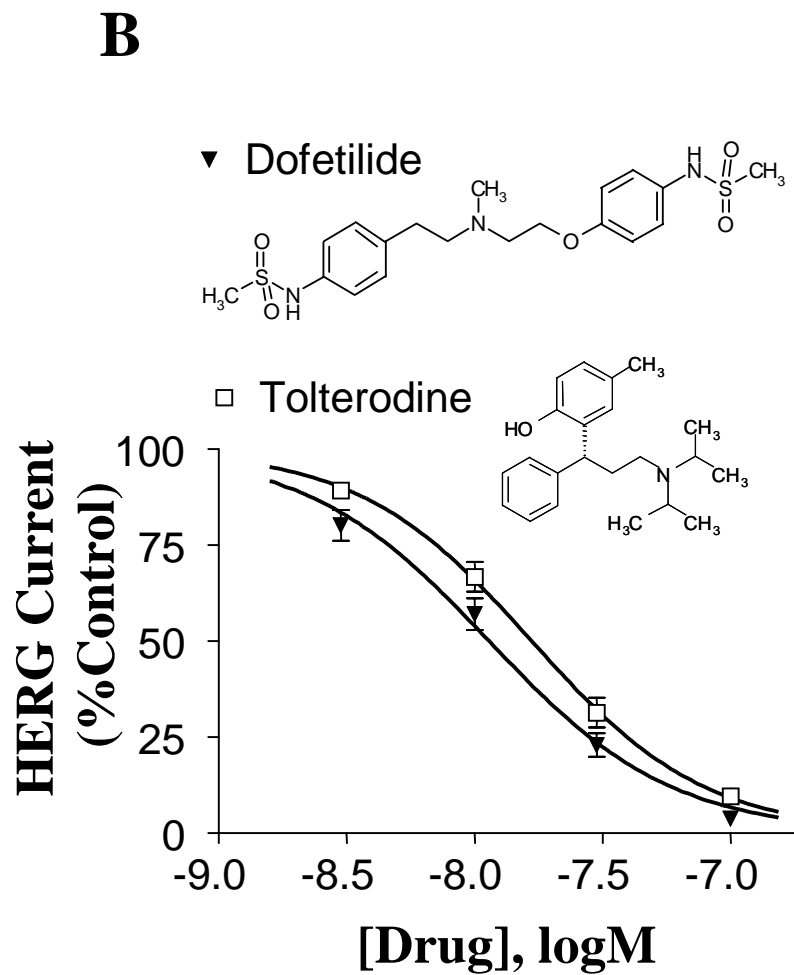
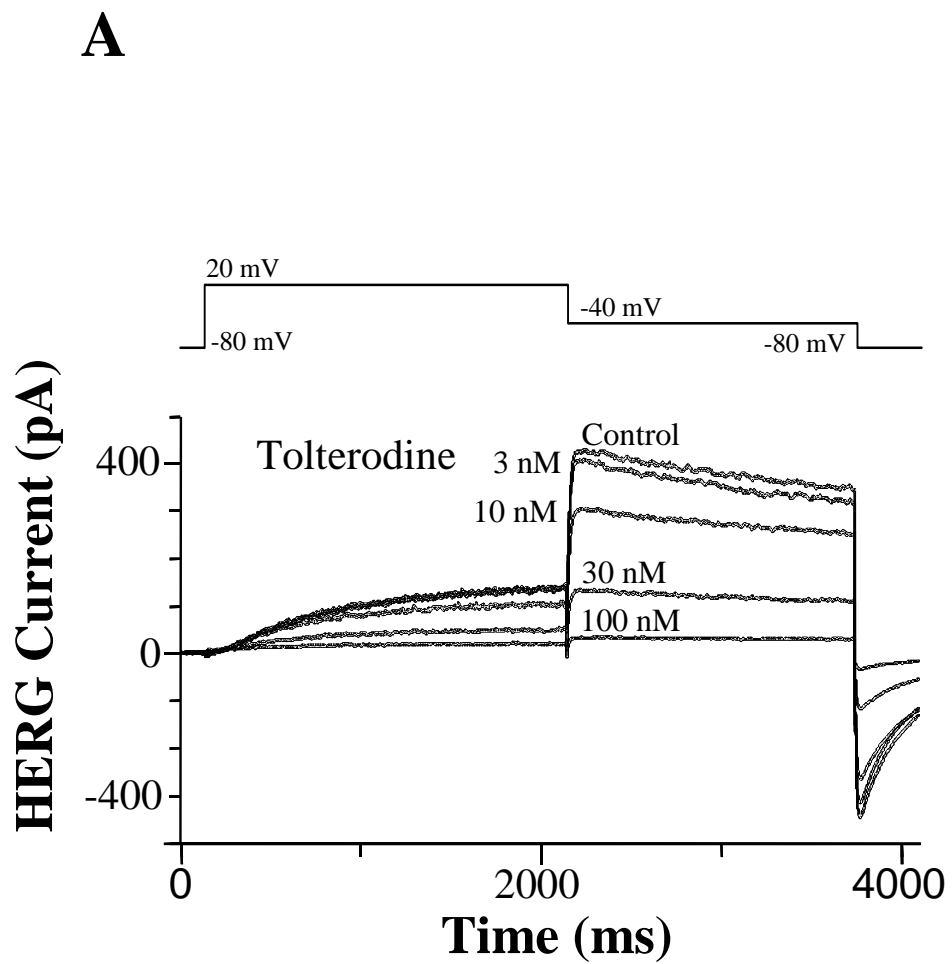


Figure 1

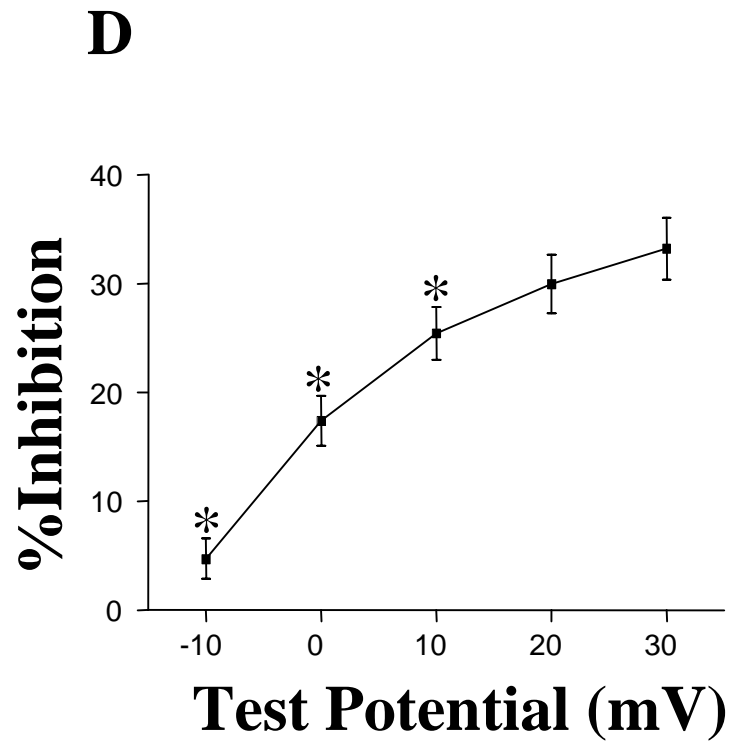
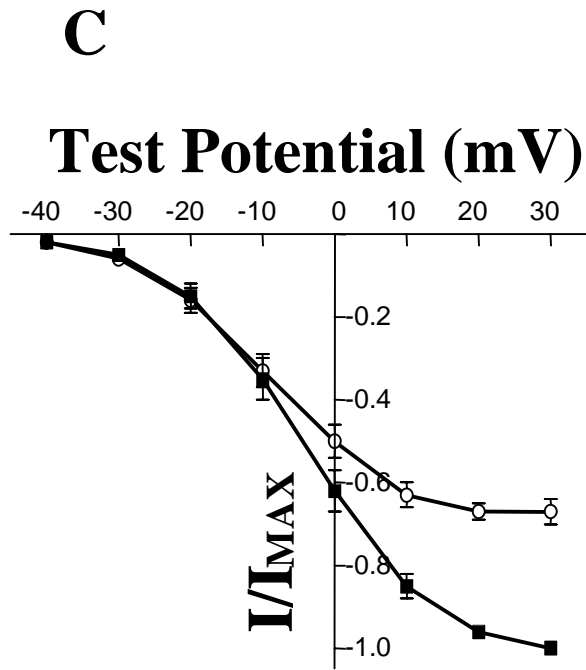
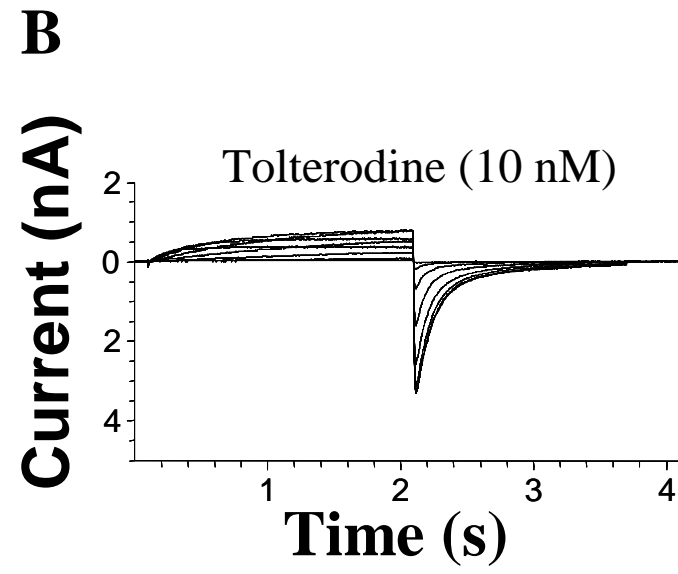
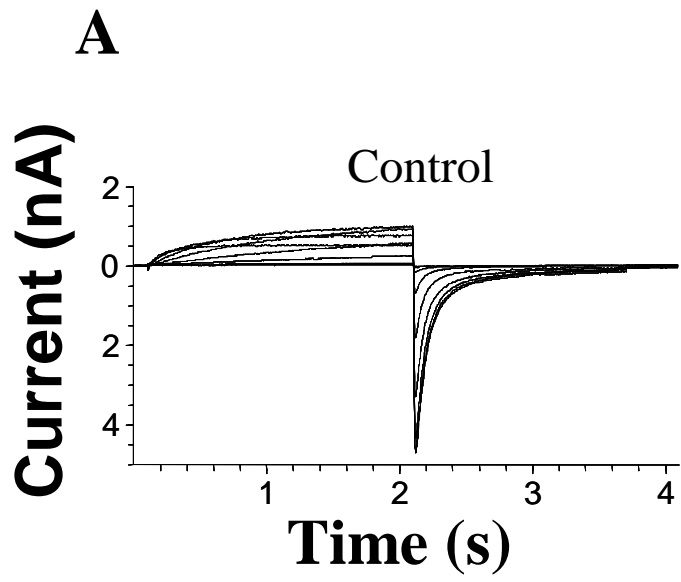


Figure 2

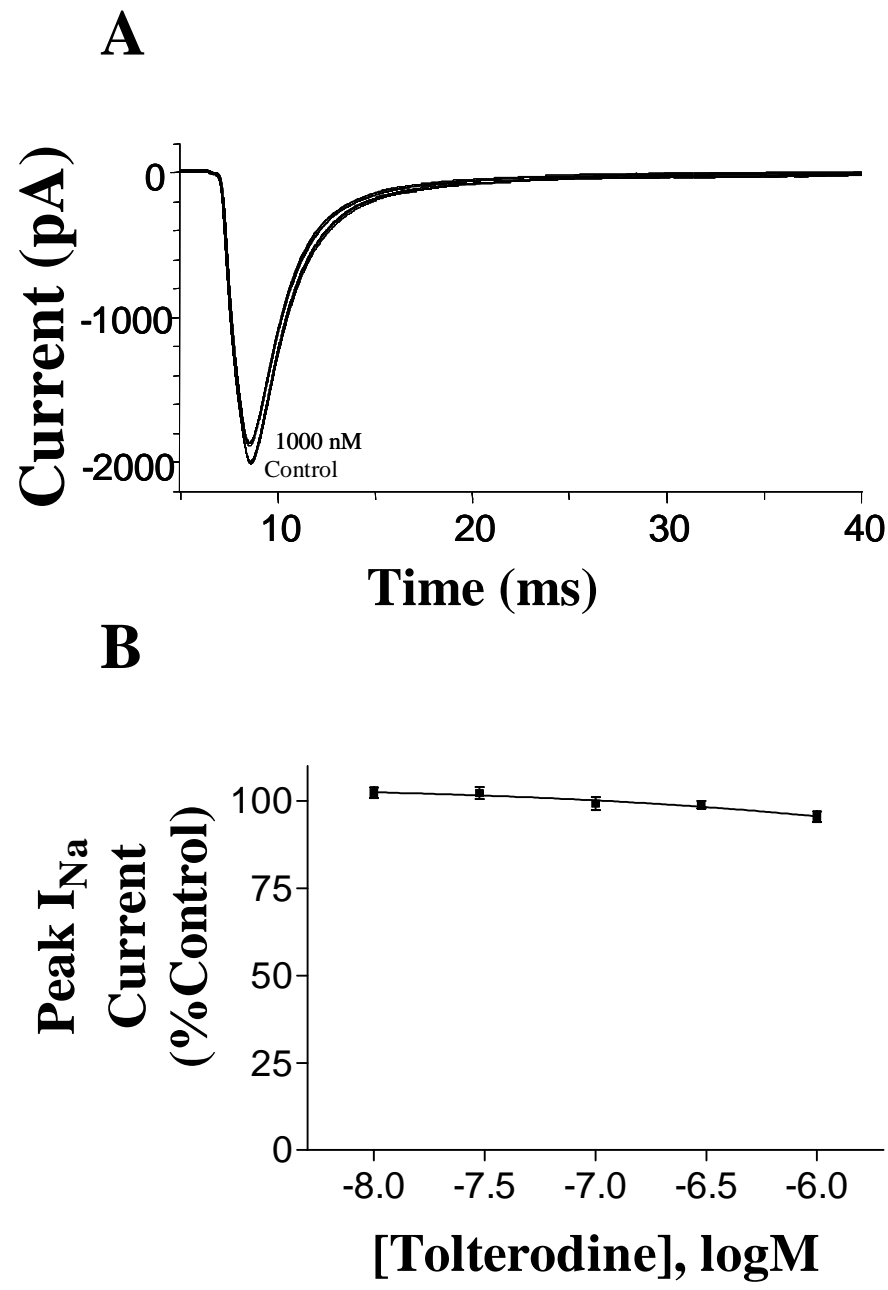


Figure 3

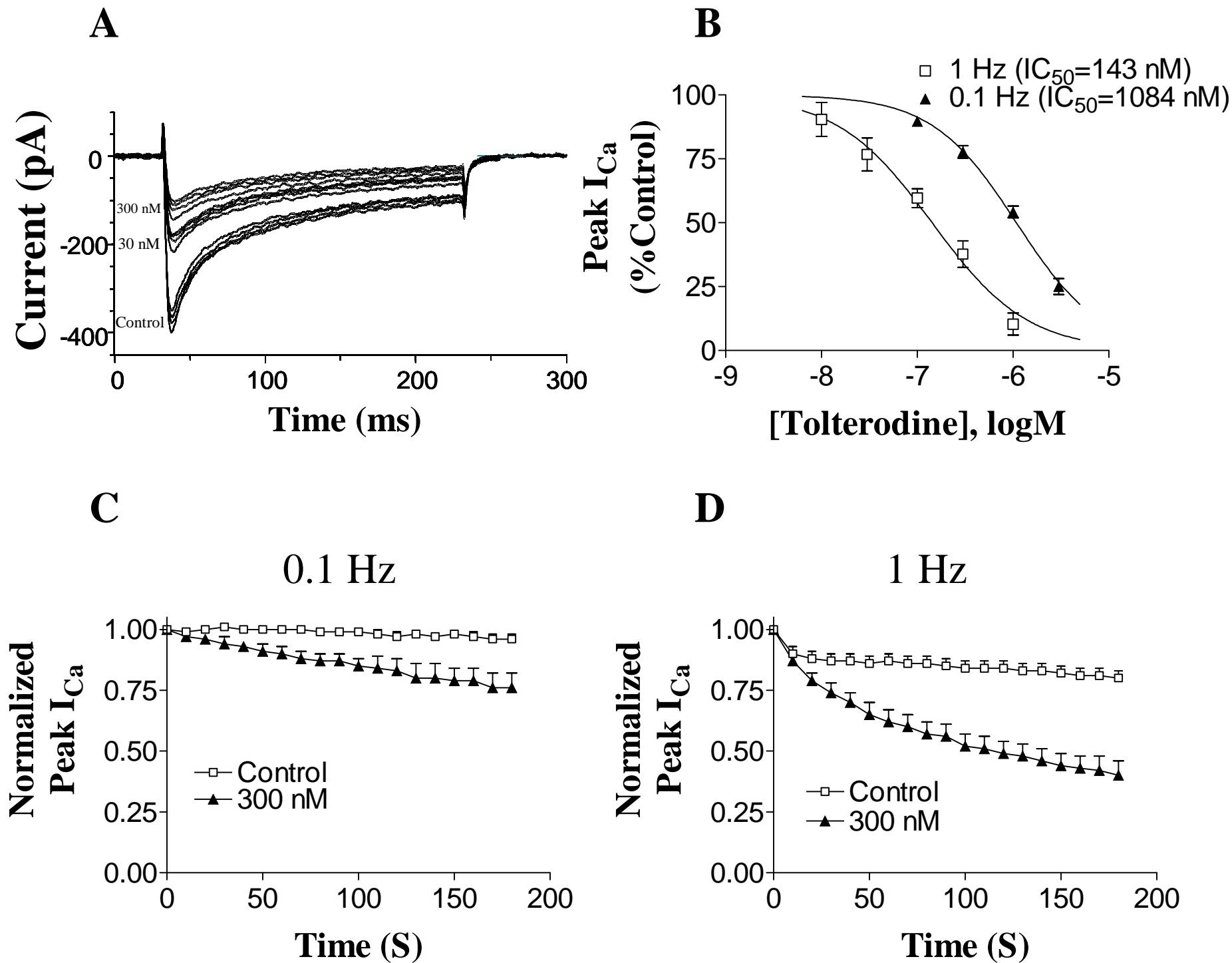


Figure 4

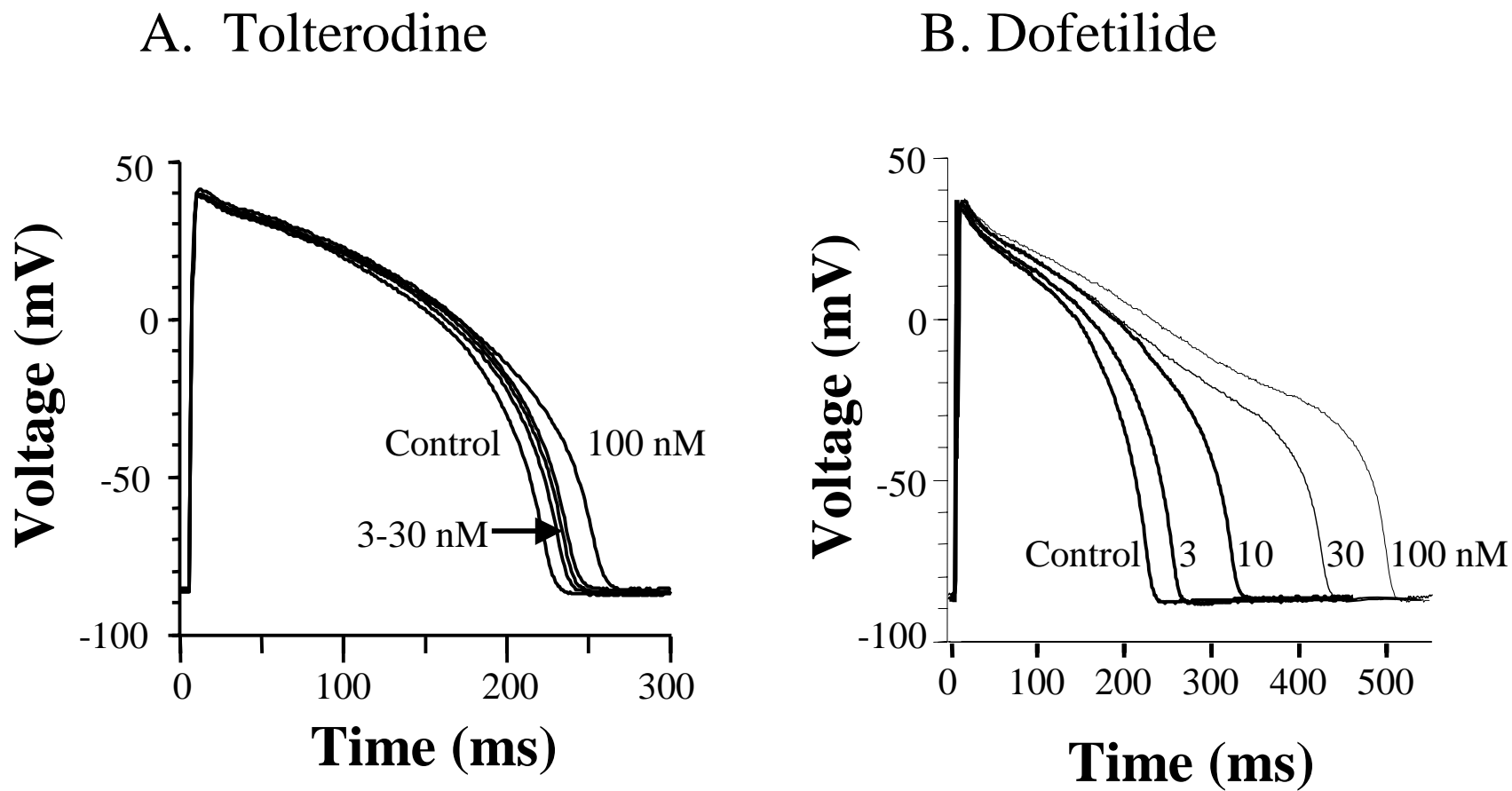


Figure 5

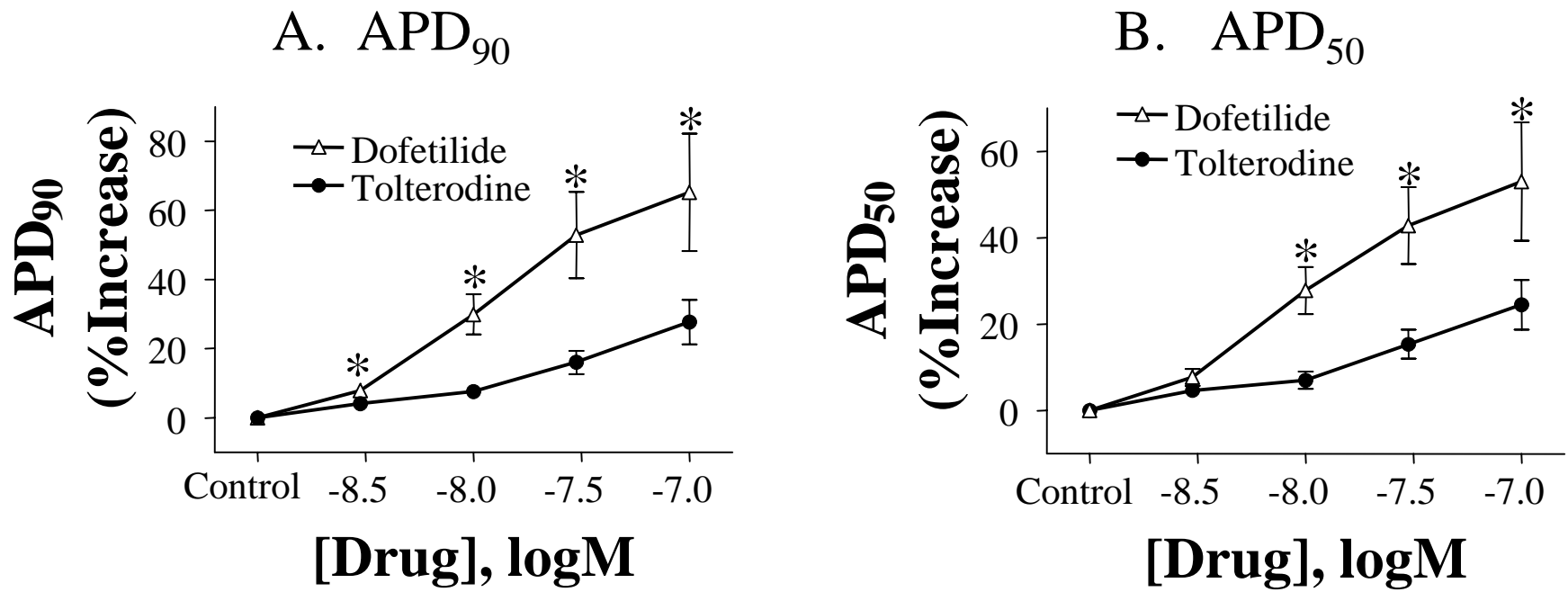


Figure 6