

Original article

Variability in the measurement of hERG potassium channel inhibition: Effects of temperature and stimulus pattern

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Abstract

Introduction: In vitro evaluation of drug effects on hERG K⁺ channels is a valuable tool for identifying potential proarrhythmic side effects in drug safety testing. Patch-clamp recording of hERG K⁺ current in mammalian cells can accurately evaluate drug effects, but the methodology has not been standardized, and results vary widely. Our objective was to evaluate two potential sources of variability: the temperature at which recordings are performed and the voltage pulse protocol used to activate hERG K⁺ channels expressed in HEK293 cells. **Methods:** A panel of 15 drugs that spanned a broad range of potency for hERG inhibition and pharmacological class was evaluated at both room and near-physiological temperatures using several patch-clamp voltage protocols. Concentration–response analysis was performed with three stimulus protocols: 0.5- and 2-s step pulses, or a step-ramp pattern. **Results:** Block by 2 of the 15 drugs tested, D,L-sotalol (antiarrhythmic) and erythromycin (antibiotic), was markedly temperature sensitive. hERG inhibition measured using a 2-s step-pulse protocol underestimated erythromycin potency compared with results obtained with a step-ramp protocol. Using conservative acceptance criteria and the step-ramp protocol, the IC₅₀ values for hERG block differed by less than twofold for 15 drugs. **Discussion:** Data obtained at near-physiological temperatures using a step-ramp pattern are highly repeatable and provide a conservative safety evaluation of hERG inhibition.

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1. Introduction

The prolongation of the electrocardiographic QT interval (long QT syndrome, LQTS) has been associated with increased risk of a serious ventricular arrhythmia, torsade de pointes. Several inherited forms of LQTS have been traced to cardiac ion channel mutations, including loss of function mutations affecting hERG (human ether-a-go-go-related gene) potassium channels. Moreover, hERG K⁺ channel inhibition by both cardiac and noncardiac drugs has been identified as the most common cause of acquired, drug-induced LQTS (Brown & Rampe, 2000; Lacerda,

Kramer, Shen, Thomas, & Brown, 2001; Weirich & Antoni, 1998; Yap & Camm, 1999). hERG K⁺ channels are responsible for a rapid component (I_{Kr}) of the repolarizing currents that terminate the cardiac action potential. Drugs that inhibit hERG have the potential to prolong the cardiac action potential and the QT interval and cause torsade de pointes.

In vitro evaluation of the effects of drugs on hERG K⁺ channels expressed heterologously in mammalian cells has been recommended as part of the preclinical safety package by the International Conference on Harmonization (ICH S7B Expert Working Group, 2002). Patch-clamp electrophysiological recording of K⁺ currents in hERG-transfected mammalian cells is considered the most accurate method for evaluating the drug inhibition of functional, voltage-

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gated K⁺ channels. However, the methodology for such assays has not been standardized, and wide variability in results has been reported (Witchel, Milnes, Mitcheson, & Hancox, 2002a). Some of the discrepancies in the literature relate to the use of different test systems (e.g., native I_{Kr} channel expression in cardiac myocytes, heterologous hERG expression in *Xenopus* oocyte, and heterologous hERG expression in mammalian cells). Our objective was to test two potential sources of variability within the same test system: the temperature at which the recordings are performed (Crumb, 2000) and the voltage pulse protocol used to activate hERG K⁺ channels (Rampe, Roy, Dennis, & Brown, 1997) stably expressed in human embryonic kidney cells (HEK293).

A diverse panel of drugs that spanned a broad range of potency for hERG inhibition and pharmacological class was tested at both room and near-physiological temperatures using several voltage protocols. We found that an accurate evaluation of these drugs required the adjustment of the pulse protocol and assessment at near-physiological temperature.

2. Methods

2.1. Cell culture

Human embryonic kidney (HEK293) cells were stably transfected with hERG cDNA. Stable transfectants were selected by coexpression with the G418-resistance gene incorporated into the expression plasmid. The selection pressure was maintained by including G418 in the culture medium (Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 supplemented with 10% fetal bovine serum, 100 U/ml penicillin G sodium, 100 µg/ml streptomycin sulfate, and 500 µg/ml G418). Cell culture stock plates (100-mm plastic culture dishes) were prepared each week. Cells for electrophysiological recording were cultured in 35-mm plastic dishes.

2.2. Electrophysiological procedures

Cells were in plastic culture dishes and were transferred to the stage of an inverted phase-contrast microscope and continuously superfused with HEPES-buffered physiological saline (HB-PS) solution. Patch pipettes were made from borosilicate glass capillary tubing using a P-97 micropipette puller (Sutter Instruments, Novato, CA) and filled with pipette solution consisting of (in mM) potassium aspartate, 130; MgCl₂, 5; EGTA, 5; ATP, 4; HEPES, 10; pH adjusted to 7.2 with KOH. The pipette solution was prepared in batches, aliquoted, stored frozen, and a fresh aliquot thawed each day.

Experiments were performed at either ambient room (20–24 °C) or near-physiological temperature (35 ± 2 °C). In the latter case, the bathing solution temperature was maintained

using a combination of an in-line solution preheater, chamber heater, feedback temperature controller, and thermistor probe in the bath. A commercial patch-clamp amplifier was used for the whole cell recordings. Before digitization, current records were low-pass filtered at one fifth of the sampling frequency.

The onset and steady-state block of hERG K⁺ current at room temperature were evaluated using a pulse pattern with fixed amplitudes (conditioning prepulse: +20 mV for 2 s; test pulse: –50 mV for 2 s) repeated at 10-s intervals, from a holding potential of –80 mV. Peak tail current was measured during the 2-s step to –50 mV. Solution exchange was complete within 30 s, and the total exposure time of an individual cell to each drug concentration was limited to approximately 10 min or until reaching steady-state effect. Steady state was defined by the limiting constant rate of change with time (linear time dependence).

The following criteria were used to determine data acceptability:

- (1) Initial seal resistance greater than 1 GΩ;
- (2) Access resistance ≤ 5 MΩ and a series resistance voltage error (product of test pulse current amplitude and access resistance) < 5 mV;
- (3) Stable leakage current < 100 pA at a holding potential of –80 mV, or < 10% of the amplitude of the peak test pulse current;
- (4) Normal test pulse current waveform (e.g., hERG peak tail) current amplitude greater than prepulse current amplitude;
- (5) Apparent rundown of test pulse current amplitude < 2% per minute.

Measurements at near-physiological temperature were obtained using one of three stimulus patterns:

- (1) Step pulse (2 s) as described above for room temperature experiments.
- (2) Step pulse (0.5 s) consisting of a conditioning prepulse (0.5-s duration, +20 mV amplitude) followed by a test pulse (0.5-s duration, –50 mV amplitude) repeated at 10-s intervals, from a holding potential of –80 mV.
- (3) Step ramp consisting a conditioning step (+20 mV amplitude, 1-s duration) followed by a repolarizing test ramp (+20 to –80 mV at –0.5 V/s) repeated at 5-s intervals from a holding potential of –80 mV.

2.3. Data analysis and statistics

Data acquisition and preliminary analysis were performed using the suite of pCLAMP (Version 8.2) programs (Axon Instruments, Foster City, CA). The steady state before and after drug application was used to calculate the percentage of current inhibited at each concentration (4–5

concentrations/drug). Concentration–response data were fit to an equation of the following form:

$$\%Block = \left\{ 1 - 1 / \left[1 + \left([Test] / IC_{50} \right)^N \right] \right\} \times 100$$

Where [Test] is the drug concentration, IC_{50} is the drug concentration at half-maximal inhibition, %Block is the mean percentage of the hERG K^+ current inhibited at each drug concentration (3–12 cells/concentration), and N is a slope coefficient. Nonlinear least squares fits were solved with the solver add-in for Excel 2000 (Microsoft, Redmond, WA).

Confidence intervals for concentration–response parameter estimates were obtained with JMP 5.0.1 (SAS Institute, Cary, NC) statistical software using the nonlinear regression platform. The algorithm found the upper and lower 95% profile confidence intervals by iteratively searching for parameter estimates above and below the best-fit value until an F test of the sample variances rejected the null hypothesis at the specified probability limit ($P < 0.05$).

The statistical significance of the differences between mean values was determined by Student's t test. $P < 0.05$ was considered statistically significant.

2.4. Drug and vehicle formulations

The stock solutions of the drugs were prepared using dimethyl sulfoxide (DMSO), aliquoted and stored frozen. Test concentrations were prepared fresh daily by diluting stock solutions into a buffered vehicle (HB-PS composition in mM): NaCl, 137; KCl, 4.0; $CaCl_2$, 1.8; $MgCl_2$, 1; HEPES, 10; glucose, 10; pH adjusted to 7.4 with NaOH (prepared weekly and refrigerated until use). Because previous results have shown that $\leq 0.3\%$ DMSO does not affect channel current, all test solutions contained 0.1% DMSO. All chemicals used in the preparation of the vehicle and micropipette-filling solutions were obtained from Sigma-Aldrich (St. Louis, MO) and were of ACS reagent grade purity or higher. All drugs were obtained from Sigma-Aldrich, except cisapride (Research Diagnostics, Flanders, NJ).

3. Results

3.1. hERG assay repeatability

In drug development programs, patch-clamp assays are often used to screen large numbers of compounds for hERG K^+ channel inhibition. However, because conventional patch-clamp methods have low throughput, a lack of repeatability over time may compromise the assay. Fig. 1 illustrates repeated measurements of the effects of two well-known hERG blockers that have been used as positive controls for patch-clamp assays of hERG K^+ channels stably expressed in HEK293 cells. The effects of 60 nM

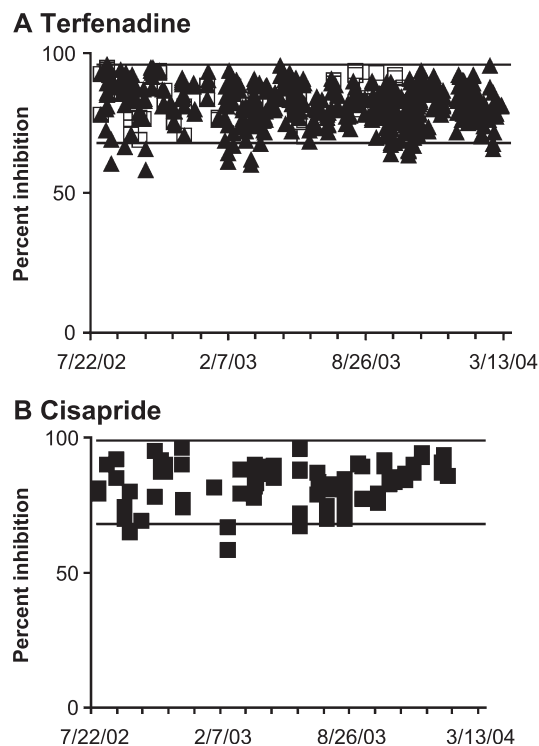


Fig. 1. hERG assay repeatability. The effects of two positive controls, (A) 60 nM terfenadine and (B) 90 nM cisapride, were tracked (8/2002–3/2004, the approximate time period during which this study was performed) in stably transfected hERG/HEK cells (cells passed weekly). Cells were passed weekly. Data were obtained at 22 °C (filled symbols, Panels A–B) using a step-pulse protocol and at 35 °C (Panel A) using either a step-pulse or a step-ramp protocol. Each symbol (\blacktriangle and \square : terfenadine; \blacksquare : cisapride) represents single cell measurements of the effect of the positive control normalized to baseline current amplitude in the same cell before the drug application. The horizontal lines indicate ± 2 S.D. from the mean.

terfenadine (Fig. 1A, \blacktriangle) and 90 nM cisapride (Fig. 1B, \blacksquare) from experiments conducted at room temperature were compiled from data obtained over periods of 20 and 18 months, respectively. Terfenadine and cisapride produced (mean inhibition \pm S.D.) $81.7 \pm 7.0\%$ ($n=459$ cells) and $83.4 \pm 7.7\%$ ($n=83$ cells) inhibition, respectively. For terfenadine, 97% of the single cell measurements fell within two standard deviations of the mean. Similarly, for cisapride, 96% of the data were within two standard deviations of the mean. The repeatability of the effect of terfenadine when measured at near-physiological temperature is also illustrated in Fig. 1A (\square); data compiled over a 15-month period gave a mean value $83.2 \pm 6.9\%$ ($n=78$ cells) inhibition. All of the data points fell within two standard deviations of the mean. Hence, patch-clamp recordings can produce highly repeatable results over long time intervals and many cell passages of a stable cell line.

3.2. Effect of temperature

Although technically more difficult, patch-clamp recording at near-physiological temperature is better suited for drug safety testing than at room temperature testing because

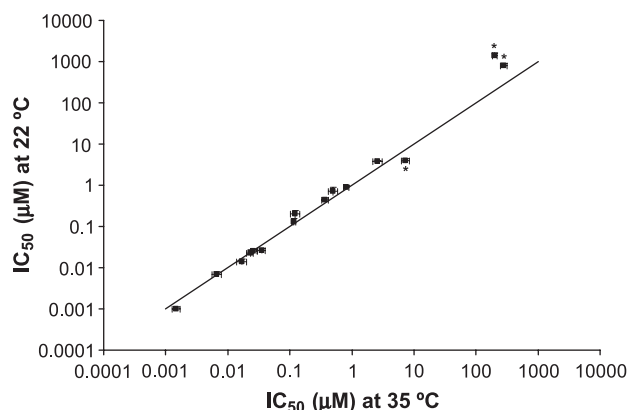


Fig. 2. Comparison of hERG IC_{50} values measured at 22 (ordinate) and 35 °C (abscissa). IC_{50} values were obtained by fitting 12–22 data points at 3–5 concentrations for each of 15 different compounds (listed in Table 1). The error bars represent upper and lower 95% confidence intervals for the estimation of IC_{50} . Data were obtained using a 2-s step-pulse protocol. Asterisks indicate statistically significant differences between the means obtained at 22 vs. 35 °C ($P < 0.05$).

temperature-dependent processes can contribute to the steady-state blockade of hERG K^+ channels, including those associated with channel gating, drug-receptor binding, and drug accumulation near the intracellular active site.

We tested the hypothesis that temperature influences steady-state hERG blockade using a standard 2-s step-pulse test pattern repeated at 10-s intervals to monitor hERG changes in current amplitude during external application of drug. As shown in Fig. 2 and Table 1, of the 15 drugs tested, both D,L-sotalol and erythromycin were markedly temperature sensitive (≥ 2.9 -fold change in IC_{50}). Evaluation at room temperature underestimated the IC_{50} for D,L-sotalol and erythromycin by a factor of 2.9 and 7.1, respectively.

Increased temperature (Fig. 3) accelerated the time course of hERG K^+ channel gating, as well as the steady-

state level of inhibition. Typical hERG voltage-clamp current records acquired during control and after equilibration with erythromycin at 1 mM are superimposed in Fig. 3A and B. Data were acquired in two separate experiments conducted at either 22 (Fig. 3A) or 35 °C (Fig. 3B). Control traces show that activation by the conditioning prepulse (+20 mV), followed by partial repolarization (–50 mV test pulse), evoked large, slowly deactivating tail currents. However, the time course of both activation and deactivation was much faster at 35 °C (Fig. 3B), as indicated by the initial rise of outward current during the prepulse and final decay during the test pulse. The application of erythromycin (1 mM) reduced the amplitude of both the prepulse and test pulse current, and the level of inhibition was much greater at 35 °C. It is noteworthy that at 35 °C (Fig. 3B), a time-dependent onset of inhibition was evident during the prepulse, consistent with the blockade of activated channels. Similar results were obtained in a range of concentrations (0.1–1 mM, Fig. 3C). At each concentration, the level of inhibition was significantly greater ($P < 0.05$) at the higher temperature (filled bars, Fig. 3).

Because drug-induced hERG inhibition often depends on channel activation, the temperature dependence of erythromycin may be secondary to the temperature dependence of gating. However, in many cases, temperature-dependent gating was not associated with a temperature-dependent drug effect. An example is shown in Fig. 3, where E-4031 applied at 22 (open bars, Fig. 3D) or 35 °C (filled bars, Fig. 3D) reduced test pulse current amplitude to the same extent. No significant differences were observed within the tested concentration range (3–100 nM). Thus, the inhibitory effect of 30 nM E-4031 was independent of temperature and the temperature-dependent changes in channel gating.

A difference in the time course of the effect of erythromycin on hERG K^+ channels offers a more likely

Table 1
Effect of temperature and pulse pattern on the concentration-dependence of hERG blockade

Drug ^a	Class	hERG IC_{50} (μ M)			
		At 22 °C 2-s step pulse	At 35 °C 2-s step pulse	At 35 °C 0.5-s step pulse	At 35 °C step ramp
Erythromycin	Antibiotic	1410	199*	–	115* [†]
d,l-Sotalol	Antiarrhythmic	810	278*	320	268*
Loratadine	Antihistamine	3.9	7.3*	–	2.3* [†]
Diphenhydramine	Antihistamine	3.8	2.6*	–	–
Quinidine	Antiarrhythmic	0.89	0.82	0.86	1.07* [†]
Fluoxetine	Antidepressant	0.71	0.50	0.55	0.46
Verapamil	Antianginal	0.444	0.37	–	0.136* [†]
Thioridazine	Antipsychotic	0.200	0.116*	–	0.096*
Ketanserin	Antihypertensive	0.128	0.121	–	0.107
Cisapride	Prokinetic	0.026	0.023	–	0.027
Bepidil	Antianginal	0.023	0.035*	0.023	0.023
Haloperidol	Antipsychotic	0.025	0.026	–	0.019
E-4031	Antiarrhythmic	0.014	0.017	–	0.012
Terfenadine	Antihistamine	0.0070	0.0066	0.0066	0.0084
Pimozide	Antipsychotic	0.0010	0.0015	0.0026*	0.0010

^a Listed in rank order of increasing potency.

* Significantly different from results obtained at 22 °C ($P < 0.05$).

[†] Significantly different from results obtained at 35 °C using a 2-s step-pulse procedure ($P < 0.05$).

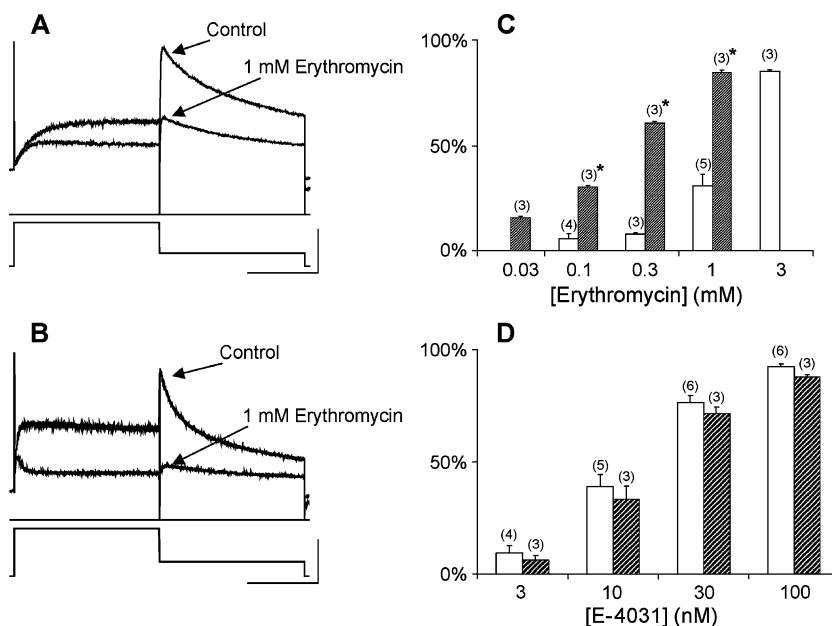


Fig. 3. Effects of temperature on drug-induced inhibition of hERG currents. (A) Superimposed current traces (upper panel) evoked by voltage steps (lower panel) before and after equilibration in 1 mM erythromycin at 22 °C. Horizontal calibration, 1 s; vertical calibration, 100 mV and 466 pA. (B) Currents recorded at 35 °C. Horizontal calibration, 1 s; vertical calibration, 100 mV and 341 pA. (C) Erythromycin concentration response. Mean hERG inhibition (%)±S.E.M. vs. concentration (mM) at 35 (shaded bars) and 22 °C (open bars). Numbers in parentheses are cells per observation. Asterisks indicate statistically significant differences between the means obtained at 22 vs. 35 °C ($P<0.05$). (D) E-4031 concentration response. Mean hERG inhibition (%)±S.E.M. vs. concentration (nM) at 35 (shaded bars) and 22 °C (open bars). Numbers in parentheses are cells per observation.

explanation for temperature-dependent inhibition. As shown in Fig. 4A, the onset of block by 1 mM erythromycin was extremely slow; a clear steady state was not achieved after

10 min of exposure. The time required to reach one half of the maximal effect ($t_{0.5}$) at 1 mM erythromycin was 156 ± 45 s (mean±S.E.M., $n=5$ cells) at 22 °C. Increased temperature

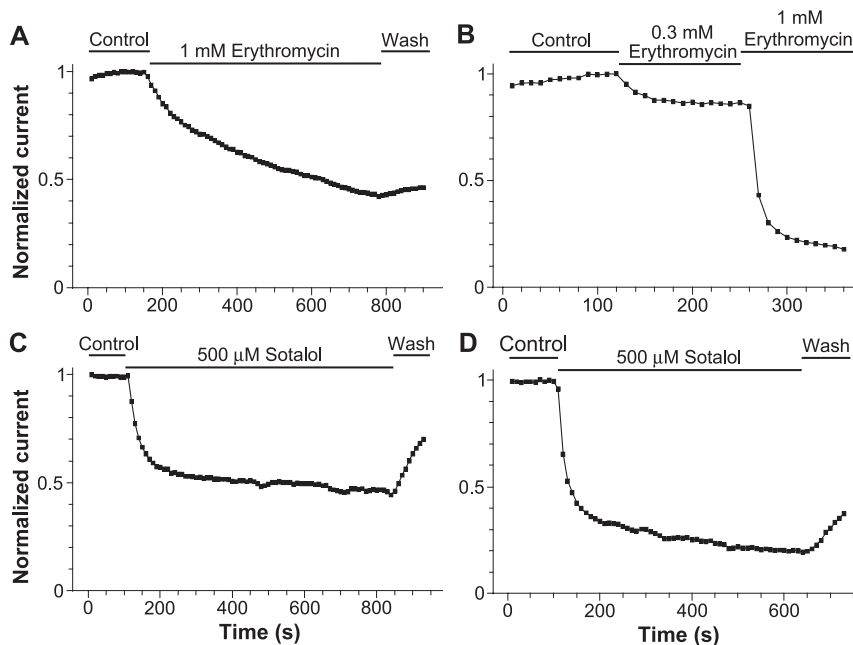


Fig. 4. Typical time course of drug-induced hERG inhibition. (A) Effect of erythromycin. Peak test pulse current normalized maximum amplitude recorded at 22 °C. Cell was exposed sequentially to vehicle alone (control), 1 mM erythromycin, and vehicle (wash) during repetitive stimulation (2-s step pulse at 10-s interval). (B) Peak normalized current amplitude at 35 °C during sequential application of 0.3 and 1 mM erythromycin. (C) Effect of *d,l*-sotalol. Peak test pulse current normalized maximum amplitude recorded at 22 °C. Cell was exposed sequentially to vehicle alone (control), 500 μM *d,l*-sotalol, and vehicle (wash) during repetitive stimulation (2-s step pulse at 10-s interval). (D) Peak normalized current amplitude at 35 °C during the application of 500 μM *d,l*-sotalol.

(Fig. 4B) produced a marked acceleration, such that 0.3 and 1 mM erythromycin reached steady state within 1 min. The $t_{0.5}$ for 1 mM erythromycin at 35 °C, 18 ± 4 s ($n=3$ cells), was significantly shorter ($P < .5$) than that observed at 22 °C. Thus, the room temperature evaluation of erythromycin would be expected to overestimate the IC_{50} (Fig. 5A). A comparison of the concentration–response curves at 22 versus 35 °C revealed a sevenfold overestimation of IC_{50} at the lower temperature (Table 1). Moreover, the slow onset of block at room temperature (Fig. 4A) may account for the extremely steep concentration dependence of the concentration–response curve (slope coefficient=2.2; Fig. 5A, \square) derived from the 10-min exposure to drug.

The concentration–response curves for D,L-sotalol (Fig. 5B) show a similar pattern. The IC_{50} estimated from data obtained at 22 °C was threefold higher than that obtained at 35 °C, and the slope of the concentration–response curve was steeper at the lower temperature. However, unlike erythromycin, the onset of D,L-sotalol inhibition was insensitive to temperature, as shown in Fig. 4C and D. A comparison of the time course of the effect of 500 μ M D,L-sotalol at either 22 (Fig. 4C) or 35 °C (Fig. 4D) revealed a similar pattern: In both conditions, the onset of inhibition was biphasic, with initial rapid blockade during the first 5 min of exposure, followed by a slow approach to steady state after approximately 10 min of exposure. The time required to reach one half of the maximal effect ($t_{0.5}$) at 500

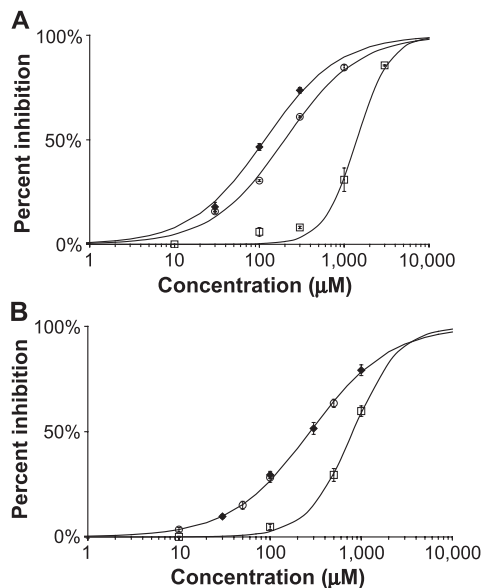


Fig. 5. Effect of temperature on concentration–response relationships. (A) Percent hERG inhibition (mean \pm S.E.M.) as a function of erythromycin concentration at 35 °C (\blacklozenge : step-ramp protocol, $n=3-5$; \circ : 2-s step-pulse protocol, $n=3$) and 22 °C (\square : 2-s step-pulse protocol, $n=3-5$). Data were fit to binding functions with IC_{50} =115 (Hill coefficient=1.1, \blacklozenge), 199 (Hill coefficient=1.0, \circ), and 1410 μ M (Hill coefficient=2.2, \square). (B) Percent hERG inhibition (mean \pm S.E.M.) as a function of D,L-sotalol concentration at 35 °C (\circ : 2-s step-pulse protocol, $n=3-12$; \blacklozenge : step-ramp protocol, $n=3-12$) and 22 °C (\square , $n=4-6$). Data were fit to a Hill function with IC_{50} =278 (Hill coefficient=1.0) and 810 μ M (Hill coefficient=1.7), respectively, at 35 and 22 °C.

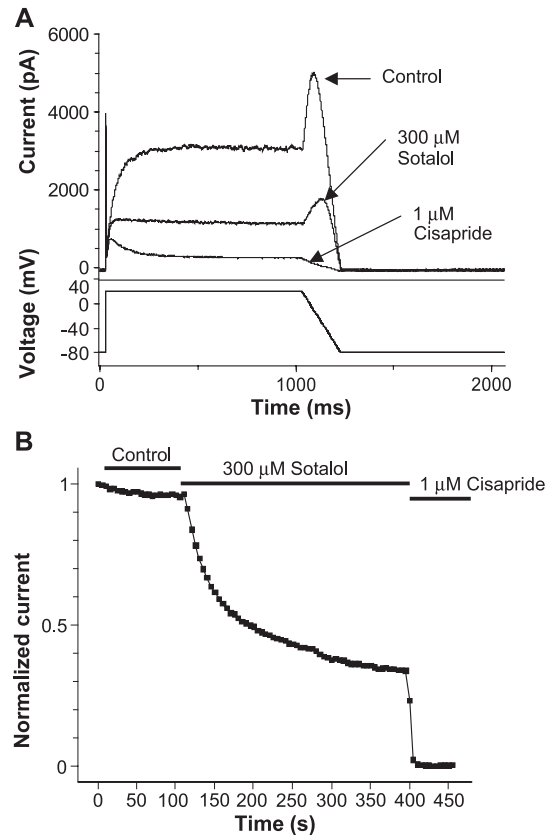


Fig. 6. *d,l*-Sotalol blockade measured using a step-ramp pulse protocol 300 μ M *d,l*-sotalol at 35 °C. (A) Typical current traces recorded before and after equilibration in *d,l*-sotalol and following the application of a supramaximal dose of cisapride are superimposed. (B) The time course of the effects of blocker application on peak hERG K^+ current.

μ M D,L-sotalol was 80 ± 15 ($n=5$ cells) and 52 ± 7 s ($n=6$ cells) at 22 and 35 °C, respectively. These $t_{0.5}$ values were not significantly different ($P < .05$).

3.3. Effect of pulse protocol

Blockade of voltage-gated hERG K^+ channels occurs primarily when channels are in either the open (activated) or the inactivated states. Therefore, variability between the results obtained in different laboratories may stem, in part, from the use of different amplitudes or durations of stimulus voltages. We tested two patterns of stimulation: a step-pulse pattern consisting of a conditioning prepulse (+20 mV amplitude) to activate channels, followed by a test pulse (–50 mV amplitude) to assess blockade repeated at 10-s intervals from a holding potential of –80 mV (e.g., Fig. 3A); or a step-ramp pattern consisting of a conditioning step (+20 mV amplitude, 1-s duration), followed by a repolarizing test ramp (+20 to –80 mV at –0.5 V/s) to assess blockade repeated at 5-s intervals from a holding potential of –80 mV (Fig. 6A and B). The effect of pulse duration was evaluated using either 0.5- or 2-s conditioning and test steps in the step-pulse pattern.

A comparison of the data obtained at 35 °C (Table 1) indicates good agreement between most IC₅₀ values obtained using either a 2-s step-pulse or a step-ramp pattern. However, as shown in Table 1, the IC₅₀ values acquired using a step-pulse pattern significantly ($P < 0.05$) underestimated hERG inhibition: The IC₅₀ ratio (step-pulse/step-ramp) was 1.7, 2.7, and 3.2 for erythromycin, verapamil, and loratadine, respectively. Similarly, a 0.5-s step-pulse pattern underestimated the blocking potency of pimoizide by twofold (Table 1).

4. Discussion

4.1. Variability of published data

As shown in Fig. 7, published IC₅₀ values obtained either in cloned hERG K⁺ channels, expressed either in a clonal mammalian cell line or in native I_{Kr} in mammalian myocytes, vary greatly. For instance, differences in expression systems (myocytes, Sanguinetti & Jurkiewicz, 1990, vs. transfected cells, Zhou et al., 1998) may explain the reported 50-fold difference in sensitivity to E-4031. However, even when drugs are tested using mammalian heterologous systems, IC₅₀ values obtained in different laboratories may vary by more than an order of magnitude. For instance, cisapride IC₅₀ values ranging from 0.24 (Potet, Bouyssou, Escande, & Baro, 2001) to 0.0065 μM

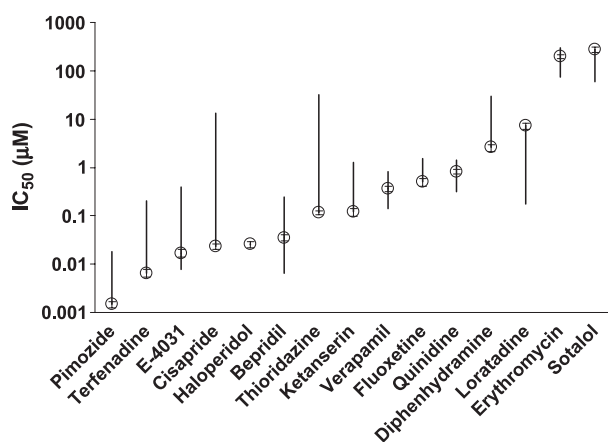


Fig. 7. Reported IC₅₀ values for I_{Kr} and hERG obtained in mammalian cells. Vertical lines show spread between the high and low of published IC₅₀ values. Open circles (O) show IC₅₀ values obtained in the present paper at 35 °C (horizontal hash marks indicate 95% confidence intervals). References for published data: Chouabe, Drici, Romey, and Barhanin (2000), Crumb (2000), Daleau, Lessard, Groleau, and Turgeon (1995), Drolet et al. (1999), Ekins, Crumb, Sarazan, Wickel, and Wrighton (2002), Hanada et al. (2003), Khalifa et al. (1999), Kongsamut, Kang, Chen, Roehr, and Rampe (2002), Kupersmidt et al. (2003), Lacerda et al. (2001), Le Grand et al. (1995), Mohammed et al. (1997), Numaguchi et al. (2000), Po et al. (1999), Potet et al. (2001), Sanguinetti and Jurkiewicz (1990), Wang, Kiyosue, Kiriyama, and Arita (1999), Wang et al. (2003), Weerapura, Nattel, Chartier, Caballero, and Hebert (2002), Witchel et al. (2002a), Witchel, Pabbathi, Hofmann, Paul, and Hancox (2002b), Zhang et al. (1999), Zhou et al. (1998).

Table 2

Comparison of hERG IC₅₀ and therapeutic plasma concentrations

Drug ^a	hERG IC ₅₀ (μM) 35 °C	hERG IC ₅₀ (μM) Published ^c	ETPC _{unbound} ^p (μM)
<i>Bepridil</i>	0.023	0.55 ^d	0.01–0.03
<i>Cisapride</i>	0.027	0.014 ^e	0.002–0.005
<i>Diphenhydramine</i>	2.6	30 ^f	0.02–0.04
<i>E-4031</i>	0.012	0.008 ^g	–
<i>Erythromycin</i>	115	72.2 ^h	3–10
<i>Fluoxetine</i>	0.46	1.5 ⁱ	0.002–0.009
<i>Haloperidol</i>	0.019	0.025 ^j	0.001–0.003
<i>Ketanserin</i>	0.107	–	0.029
<i>Loratadine</i>	2.31	0.173 ^k	0.002
<i>Pimoizide</i>	0.001	0.018 ^l	0.0003–0.001
<i>Quinidine</i>	1.068	0.44 ^m	3.7
<i>d,l-Sotalol</i>	269	–	11
<i>Terfenadine</i>	0.008	0.009 ⁿ	0.001–0.008
<i>Thioridazine</i>	0.096	0.03 ^o	0.2–0.9
<i>Verapamil</i>	0.136	0.143 ^p	0.02–0.09

^a Italicized: known to have QT prolongation risk in man.

^b Effective therapeutic plasma concentration corrected for protein binding (Redfern et al., 2003, package inserts).

^c IC₅₀ values from studies conducted in transfected mammalian cells.

^d Chouabe (2000).

^e Wang et al. (2003).

^f Khalifa et al. (1999).

^g Zhou et al. (1998).

^h Volberg, Koci, Su, Lin, and Zhou (2002).

ⁱ Witchel et al. (2002b).

^j Ekins et al. (2002).

^k Crumb (2000).

^l Kang, Wang, Cai, and Rampe (2000).

^m Paul, Witchel, and Hancox (2002).

ⁿ Wang et al. (2003).

^o Drolet et al. (1999).

^p Zhang et al. (1999).

(Mohammed, Zhou, Gong, & January, 1997) have been reported. Similarly, 23- and 16-fold differences in hERG IC₅₀ values for terfenadine (Crumb, 2000; Wang et al., 2003) and loratadine (Crumb, 2000; Lacerda et al., 2001) appear in the literature.

Clearly, for the patch-clamp method to serve as a “gold standard” for drug-induced hERG inhibition, both repeatability and accuracy must be demonstrated. Towards this end, we determined the high and low 95% confidence limits of each of our IC₅₀ estimates (e.g., Fig. 7 illustrates data obtained at 35 °C) and calculated the high/low ratio. The mean ratio was 1.33 ± 0.02 ($n=50$ IC₅₀ measurements), with a range of 1.11 to 1.79. Thus, the hERG assay can provide a high level of precision. Therefore, it was important to establish whether experimental conditions, such as temperature or stimulus pattern, can affect the accuracy of the measurement.

4.2. Temperature sensitivity of the hERG assay

The inhibitory effects of D,L-sotalol and erythromycin were temperature sensitive; measurements obtained at near-physiological temperature yielded the lowest IC₅₀ concen-

trations. Moreover, the effects of erythromycin, thioridazine, verapamil, and loratadine were sensitive to pulse protocol, such that a step-ramp pattern repeated at 5-s intervals yielded lower IC_{50} values than a step-pulse pattern at 10-s intervals did. As shown in Table 2, the combination of step-ramp protocol and near-physiological temperature recording conditions provided a good correlation between hERG IC_{50} and therapeutic unbound drug levels associated with cardiac risk. It is particularly noteworthy that IC_{50} values (Table 2, Column 2) obtained for bepridil and pimoziide were 18- to 24-fold lower than those previously reported (Table 2, Column 3) but were in good agreement with therapeutic levels (Table 2, Column 4).

4.3. Pulse protocol sensitivity of the hERG assay

The temperature and pulse dependences of hERG IC_{50} values are interrelated because of the gating-dependent characteristics of hERG blockade. Without exception, hERG blockers bind most effectively to channels in the activated/inactivated gating conformations and dissociate with variable time course from resting conformations. Because transitions into the activated states are markedly accelerated by increased temperature, and the activation voltage range is shifted to more negative potentials (Zhou et al., 1998), the prolonged duration of activating step pulses and increased repetition rate with shortened resting intervals would be expected to promote channel blockade. Verapamil block, for example, has been shown to require channel activation to dissipate rapidly upon repolarization and to increase with increased stimulation frequency (Zhang, Zhou, Gong, Makielski, & January, 1999). Thus, prolonged activating prepulses, combined with more rapid pulsing, would facilitate the accumulation of blocked channels and produce an apparently greater level of steady-state blockade. These characteristics may explain the temperature sensitivity of D,L-sotalol and the pulse dependence of verapamil, pimoziide, loratadine, and erythromycin. However, additional factors may underlie the temperature sensitivity of erythromycin. Unlike D,L-sotalol, the time course of onset of erythromycin blockade was strongly accelerated at near-physiological temperature and failed to reach a steady state after prolonged exposure at room temperature. This result is consistent with the notion that the erythromycin site of action is intracellular and that transmembrane movement of erythromycin is temperature sensitive. In fact, the intracellular accumulation of erythromycin in mammalian cells is markedly inhibited by either low temperature or metabolic inhibitors (Ishiguro et al., 1989; Raghoobar, Lindeyer, Van den Berg, & Van Ginneken, 1988). However, because the actual concentrations in solution were not determined in this study, the possibility of a temperature-dependent increase in solubility and a consequent increase in the effective drug concentration cannot be ruled out.

In conclusion, our results show that patch-clamp recordings are highly repeatable in stably transfected hERG cells over many passages and that recordings obtained at near-physiological temperatures using a step-ramp pulse pattern provide a conservative safety evaluation of hERG inhibition within a diverse panel of drugs.

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