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Fentanyl-induced block of hERG channels is exacerbated by hypoxia, hypokalemia, alkalosis, and the presence of hERG1b

Jared N. Tschirhart and Shetuan Zhang

Department of Biomedical and Molecular Sciences, Queen's University, Kingston, Ontario, Canada

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Running Title: Augmentation of fentanyl-induced block of hERG channels

Address correspondence to:

Dr. Shetuan Zhang, Department of Biomedical and Molecular Sciences, Queen's University, 18 Stuart Street, Kingston, Ontario, K7L 3N6 Canada.Tel: (613) 533-3348; Fax: (613) 533-6412; E-mail: shetuan.zhang@queensu.ca

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Abbreviations: ANOVA, analysis of variance; APD, action potential duration; FBS, fetal bovine serum; HEK, human embryonic kidney; hERG, human ether a-go-go related gene; IC_{50}, half maximal inhibitory concentration; I_{hERG}, hERG current; I_{Kr}, rapidly activating delayed rectifier potassium current; $[K^+]_o$, extracellular potassium concentration; LQTS, long QT syndrome; MEM, minimum essential medium; S.D., standard deviation; $\tau_{f\text{-}deact}$, fast time constant of deactivation; $\tau_{s\text{-}deact}$, slow time constant of deactivation; WT, wild-type.

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ABSTRACT

Human ether-a-go-go-related gene (*hERG*) encodes the pore-forming subunit of the rapidly activating delayed rectifier potassium channel (I_{Kr}) important for repolarization of cardiac action potentials. Drug-induced disruption of hERG channel function is a main cause of acquired long QT syndrome (LQTS), which can lead to ventricular arrhythmias and sudden death. Illicit fentanyl use is associated with sudden death. We have demonstrated that fentanyl blocks hERG current (I_{hERG}) at concentrations that overlap with the upper range of postmortem blood concentrations in fentanyl-related deaths. Since fentanyl can cause respiratory depression and electrolyte imbalances, in the present study, we investigated whether certain pathological circumstances exacerbate fentanyl-induced block of I_{hERG}. Our results showed that chronic hypoxia or hypokalemia additively reduced I_{hERG} with fentanyl. As well, high pH potentiated the fentanyl-mediated block of hERG channels, with an IC_{50} at pH 8.4 being 7-fold lower than that at pH 7.4. Furthermore, while the full-length hERG variant, hERG1a, has been widely used to study hERG channels, coexpression with the short variant, hERG1b (which does not produce current when expressed alone), produces functional hERG1a/1b channels, which gate more closely resembling native I_{Kr}. Our results showed that fentanyl blocked hERG1a/1b channels with a 3-fold greater potency than hERG1a channels. Thus, in addition to a greater susceptibility due to the presence of hERG1b in the human heart, hERG channel block by fentanyl can be exacerbated by certain conditions such as hypoxia, hypokalemia, or alkalosis, which may increase the risk of fentanyl-induced ventricular arrhythmias and sudden death.

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SIGNIFICANCE STATEMENT

This work demonstrates that heterologously expressed hERG1a/1b channels, which more closely resemble I_{Kr} in the human heart, are blocked by fentanyl with a 3-fold greater potency than the previously studied hERG1a expressed alone. Additionally, chronic hypoxia, hypokalemia, and alkalosis can increase the block of hERG current by fentanyl, potentially increasing the risk of cardiac arrhythmias and sudden death.

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INTRODUCTION

In recent years, fentanyl-associated death has become a serious issue in North America (Alexander *et al.*, 2016; Torralva and Janowsky, 2019). Fentanyl is a synthetic μ-opioid receptor agonist (Raynor *et al.*, 1994). It is used clinically for management of severe pain and combined with other drugs for anesthesia (Stanley, 2014). However, when used illicitly, fentanyl has a propensity to cause sudden death. It is known that fentanyl acts at opioid receptors in the brainstem (Wamsley, 1983), resulting in respiratory depression (McQueen, 1983), which is believed to underlie fentanyl-induced sudden death. However, we have found that fentanyl blocks human ether-a-go-go-related gene (hERG) potassium channels heterologously expressed in human embryonic kidney (HEK) 293 cells (Tschirhart *et al.*, 2019), at sub-micromolar concentrations that are within the upper range of blood concentrations in fentanyl-related deaths (3-383 μg/L or 8.9 nM - 1.14 μM) (Martin *et al.*, 2006).

hERG encodes the pore-forming subunit of the rapidly activating delayed rectifier potassium channel (I_{Kr}), which is important for repolarization of cardiac action potentials (Sanguinetti *et al.*, 1995; Trudeau *et al.*, 1995). A decrease in hERG current (I_{hERG}) caused by mutations or drugs can prolong cardiac action potential duration (APD), leading to long QT syndrome (LQTS) (Curran *et al.*, 1995; Sanguinetti *et al.*, 1995; Keating and Sanguinetti, 2001). Notably, LQTS predisposes affected individuals to a polymorphic ventricular tachycardia known as Torsade de Pointes (Keating and Sanguinetti, 2001), which can lead to sudden death (Chiang and Roden, 2000). In fact, many cases of drug-induced sudden death are caused by blockade of hERG channels, and several drugs have been removed from market due to this reason (Fenichel *et al.*, 2004; Roden, 2004). Fentanyl can prolong cardiac action potentials recorded from isolated canine Purkinje fibers (Blair *et al.*, 1989) and neonatal rat ventricular myocytes (Tschirhart *et al.*,

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2019). Thus, we proposed that blockade of hERG channels may be involved in fentanyl-induced death of individuals after overdose or in the context of compromised cardiac repolarization, which can occur in various conditions. For example, a reduction in serum K^+ concentration, known as hypokalemia, causes LQTS (Curry *et al.*, 1976; Roden *et al.*, 1986). We previously demonstrated that a reduction in extracellular K^+ concentration ($[K^+]_o$) decreases hERG protein density in the plasma membrane, and causes LQTS in a rabbit model (Guo *et al.*, 2009). As well, we have shown that chronic hypoxia reduces I_{hERG} (Lamothe *et al.*, 2017). In addition, acidosis accelerates channel deactivation and decreases amplitude of I_{hERG} (Anumonwo *et al.*, 1999; Terai *et al.*, 2000; Van Slyke *et al.*, 2012; Shi *et al.*, 2019). Since fentanyl may cause respiratory depression and electrolyte imbalances (e.g. hypokalemia, and altered pH). We thus examined the effects of fentanyl on hERG currents under conditions such as hypoxia, hypokalemia, and altered pH.

Like other potassium channels, a single hERG channel is composed of four α-subunits that assemble to form a tetramer (MacKinnon, 1991; Jiang *et al.*, 2003). Two variants of hERG1 have been identified in myocardium of humans and animals, hERG1a and hERG1b (Lees-Miller *et al.*, 1997; London *et al.*, 1997; Jones *et al.*, 2004; Phartiyal *et al.*, 2007; Jones *et al.*, 2014). Since hERG1b (shorter form, containing 820 amino acids) alone does not effectively produce current in expression systems (Phartiyal *et al.*, 2008; McNally *et al.*, 2017), full-length hERG1a, containing 1159 amino acids, has generally been used to characterize the biophysical and pharmacological properties of the channel, defined as the hERG channel. However, when co-expressed, hERG1b and hERG1a can effectively form heterotetramic channels that behave more like native cardiac I_{Kr} (London *et al.*, 1997; Phartiyal *et al.*, 2007; Phartiyal *et al.*, 2008; Sale *et al.*, 2008; Larsen and Olesen, 2010), and display altered sensitivity to certain drugs compared

with hERG1a alone (Abi-Gerges *et al.*, 2011). Thus, we examined the effects of fentanyl on hERG1a/1b heterotetramic channels.

The present study revealed that chronic hypoxia, hypokalemia, and increased pH exacerbated fentanyl-induced block of hERG channels. Furthermore, heterotetrameric hERG1a/1b channels were blocked with a greater potency than hERG1a (generally referred to as hERG) homotetrameric channels previously studied. By utilizing several hERG N-deletion mutants that accelerate hERG deactivation, we found a correlation between fentanyl block potency and the rate of hERG channel deactivation. Thus, we propose that several conditions may enhance the block of hERG channels by fentanyl, increasing the risk of QT interval prolongation and sudden death.

MATERIALS AND METHODS

Molecular Biology

hERG1a (defined as hERG) cDNA was obtained from Dr. Gail Robertson (University of Wisconsin-Madison, Madison, WI). Point mutations Y652A and F656T, and the Δ2-354 deletion were constructed using polymerase chain reaction (PCR) overlap extension technique, as described previously (Guo *et al.*, 2006; Massaeli *et al.*, 2010). The Δ2-9 and Δ2-25 hERG mutants were also constructed using the PCR method and confirmed by DNA sequencing (GENEWIZ, South Plainfield, NJ). The HEK293 cell line stably expressing wild-type (WT) hERG (hERG1a) channels (hERG-HEK cell line) was provided by Dr. Craig January (University of Wisconsin-Madison). hERG1b cDNA and the HEK 293 cell line stably expressing hERG1a plus pTet-One controlled hERG1b expression (hERG1a/1b-HEK cell line) was obtained from Dr. Gail Robertson (University of Wisconsin-Madison). G418 (5 μg/mL) and puromycin (0.25

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μg/mL) were used to maintain expression of hERG1a and 1b, respectively. Expression of hERG1b was induced by treating cells with 200 ng/mL doxycycline in media for 48 hours before being used for experiments. In addition, transfection of HEK293 cells stably expressing hERG1a with hERG1b plasmid was performed to study hERG1a/1b channels. The HEK293 cell line stably expressing Δ2-354 hERG was created using transfection followed by G418 selection (1 mg/mL) and maintenance (0.4 mg/mL). Mutant hERG Y652A, Δ2-9, and Δ2-25 were transiently expressed in HEK293 cells. For coexpression of WT and Δ2-354 hERG, 2 μg of Δ2-354 hERG plasmid was mixed with 0.5 μg GFP plasmid, and transfected into hERG-HEK293 cells cultured in 35 mm plates. For transient transfection, GFP plasmid (pIRES2-EGFP, Clontech, CA) was co-transfected with the plasmid of interest at a 1:4 ratio for the selection of transfected cells during patch-clamp experiments. HEK293 cells were cultured in Gibco minimum essential medium (MEM) supplemented with 10% fetal bovine serum (FBS), nonessential amino acids (glycine, L-alainine, L-asparagine, L-aspartic acid, L-glutamic acid, L-proline, and L-serine, 100 μM each), and 1 mM sodium pyruvate (Thermo Fisher Scientific, Waltham, MA). Cells were passaged every 2-3 days and were used for experiments 16-24 hours after passage. During passages, cell culture media were removed then the cells were rinsed with phosphate-buffered saline, treated with 0.25% trypsin for 1 min, suspended in MEM supplemented with 10% FBS, collected for experiments, or plated for culture. Cells were maintained in a 37 °C / 5% CO_2 incubator. For electrophysiological recordings, the cells were collected during passaging and kept in MEM with 10% FBS at room temperature until used (within 4 hours).

Electrophysiological Recordings

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Currents were recorded from HEK cells using the whole-cell voltage-clamp method. Pipettes were pulled from thin-walled (1.5 mm) borosilicate glass (TW150-6, World Precision Instruments, Sarasota, CA) with a P-1000 micropipette puller (Sutter Instrument, Novato, CA) and polished with heat to a resistance of ~2.0 MΩ when filled with solution. An Axopatch 200B Amplifier, Digidata 1440A Digitizer, and Clampex version 10.7 software (Molecular Devices, San Jose, CA) were used for data acquisition and analysis. The pipette solution contained the following (in mM): 135 KCl, 5 EGTA, 5 MgATP, and 10 HEPES (pH 7.2 with KOH). The bath solution contained the following (in mM): 5 KCl, 135 NaCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 glucose, and 10 HEPES (pH 7.4 with NaOH). To create pH 6.4 or 8.4 bath solution, concentrated HCl or NaOH was added to the pH 7.4 solution. For acute concentration-response relationships, control current was first recorded in the absence of drug. Increasing concentrations of drug were then added, and the currents after steady state of block (within 1 minute after drug application) were normalized to control and plotted as relative currents against concentrations to construct concentration-response relationships. Recordings were carried out at room temperature (22 ± 1°C).

Peak tail currents upon the repolarizing step to −50 mV following the 50 mV depolarization were used to analyze the amplitude of I_{hERG}. To calculate the IC_{50} values, the currents at individual fentanyl concentrations were normalized to the control current (in the absence of drug), summarized and plotted against concentrations in a log scale. The data were fitted to the Hill equation ($y= (A_1-A_2)/(1+(x/x_o)^p)+A_2$) in Microcal Origin (OriginLab, Northampton, Massachusetts, USA) to obtain half maximal inhibitory concentration (IC_{50}) values and Hill coefficients. The deactivation time course was analyzed in Clampfit. The decay of tail currents was fitted to a double exponential function to obtain the fast ($\tau_{f\text{-}deact}$) and slow

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time constant ($\tau_{s\text{-}deact}$) of hERG deactivation. Each cell was individually fitted and the values are shown in the scatter plots.

Hypoxic Culture

For culture in hypoxic conditions, a C-Chamber incubator subchamber with a ProOx 110 oxygen controller (BioSpherix, Parish, NY) was used. 0.5% O_2 was maintained with an influx of 95% N_2 / 5% CO_2 gas mixture, and temperature was maintained at 37 °C. Cells were passaged, and incubated for 6 hours. Control cells were maintained in the same incubator, but outside of the hypoxic subchamber. Cells were collected for electrophysiological studies by trypsinization, re-suspended in media, and used within 1 hour.

Drugs and Reagents

Fentanyl citrate was purchased from Toronto Research Chemicals (North York, ON, Canada). The powder form of fentanyl citrate was handled in a biological safety cabinet with proper protective equipment to prevent exposure. Doxycycline hyclate was purchased from BioShop Canada Inc. (Burlington, ON, Canada). Drugs were dissolved in double-distilled water, aliquoted and stored at −20°C. For patch-clamp experiments, fentanyl was diluted in bath solution and used within 8 hours. When investigating mutant channels or different conditions, data were always paired with WT or CTL from the same days with the same drug stock solutions. For acute block, control solution was drained from the bath with a suction pump, and drug-containing solution was applied through a gravity-fed perfusion system. MEM, FBS, Lipofectamine 2000, G418 (Geneticin), trypsin, nonessential amino acids, and sodium pyruvate

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were purchased from Thermo Fisher Scientific (Waltham, MA). Electrolytes, EGTA, HEPES, and glucose were purchased from Sigma-Aldrich (St. Louis, MO).

Statistical Analysis

Data are expressed as the mean ± S.D. or scatter plots. Data were analyzed using a one-way analysis of variance (ANOVA) with Tukey's post-hoc test, or a two-tailed Student's *t*-test with GraphPad Prism (GraphPad Software, San Diego, CA). A *P* value of 0.05 or less was considered to be statistically significant.

RESULTS

Chronic Hypoxia and Acute Fentanyl Decrease I_{hERG} in an Additive Manner

Chronic hypoxia impairs hERG function (Lamothe *et al.*, 2017) and prolongs QT intervals in patients (Kenigsberg *et al.*, 2007; Mehra *et al.*, 2009). To investigate the concurrent effects of chronic hypoxia and fentanyl on hERG channels, we cultured hERG-HEK cells in hypoxic (0.5% O_2) or normoxic (21% O_2, control) conditions for 6 hours, and then examined the block effect of 1 μM fentanyl on I_{hERG}. As we reported previously (Lamothe *et al.*, 2017; Tschirhart *et al.*, 2019), compared to control culture, 6-hour culture in 0.5% O_2 or acute application of 1 μM fentanyl significantly decreased I_{hERG}. The combination of hypoxic culture and 1 μM fentanyl resulted in I_{hERG} that was significantly less than either condition alone. These results are shown in Fig. 1 and analyzed with one-way ANOVA with Tukey's post-hoc test (**$P < 0.01$, comparisons as indicated). In addition, to determine whether chronic hypoxic culture alters channel sensitivity to fentanyl, we compared the extents of fentanyl-induced block in normoxic and hypoxic cultured cells. In hypoxic cultured cells, 1 μM fentanyl blocked I_{hERG} by

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59 ± 12%, which was not significantly different from 1 μM fentanyl-induced block in normoxic cultured cells (by 52 ± 11%, $P > 0.05$, unpaired student *t* test). Thus, chronic hypoxia and fentanyl have an additive effect in reducing I_{hERG}.

Hypokalemia Augments Fentanyl-induced Blockade of hERG

To study the effects of hypokalemia and fentanyl on I_{hERG}, we cultured hERG-HEK cells in normal medium (control, CTL, 5 mM K^+) or 2 mM K^+ for 72 hours. In hypokalemic patients, serum potassium as low as 1.2 mM K^+ was reported (Garcia *et al.*, 2008). Thus, investigating the effects of 2 mM K^+_o plus fentanyl is of clinical relevance. After culture, we examined the blocking effects of fentanyl on I_{hERG} recorded in standard, 5 mM K^+-containing bath solution. Compared to CTL, culture in 2 mM K^+ medium for 72 hours or acute application of 1 μM fentanyl significantly decreased I_{hERG}. The combination of 2 mM K^+ culture and 1 μM fentanyl resulted in I_{hERG} that was significantly less than either condition alone. These results are shown in Fig. 2A and analyzed with one-way ANOVA with Tukey's post-hoc test (*$P < 0.05$, **$P < 0.01$, comparisons as indicated). When recorded in standard, 5 mM bath solution, 1 μM fentanyl blocked I_{hERG} from cells cultured in 2 mM K^+ medium by 59 ± 8%, which was not significantly different than block from cells cultured in normal, 5 mM K^+ medium (59 ± 10%, $P > 0.05$). Therefore, chronic hypokalemia and fentanyl additively reduce I_{hERG}.

To further study the effects of acute extracellular K^+ concentration on fentanyl-induced block, we examined fentanyl block of I_{hERG} recorded in 5 or 2 mM K^+-containing bath solution from cells cultured in normal medium. To clearly demonstrate the degree of block, we normalized current after fentanyl block to control current in each recording condition (Fig. 2B). While 1 μM fentanyl blocked I_{hERG} by 54 ± 9% in 5 K^+ bath solution, it blocked I_{hERG} by 60 ±

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6% in 2 mM K^+ bath solution ($P < 0.05$, unpaired student *t* test). Thus, hypokalemia augmented fentanyl-induced reduction of I_{hERG} in additive (due to chronic hypokalemia) and synergistic (due to acute hypokalemia) manners.

Fentanyl Block is Modified by Extracellular pH

Altered extracellular pH affects hERG channel properties (Anumonwo *et al.*, 1999; Terai *et al.*, 2000; Van Slyke *et al.*, 2012; Shi *et al.*, 2019) and drug-channel interactions (Zhang *et al.*, 1999; Wang *et al.*, 2016). In addition, like most hERG channel blockers such as verapamil (Zhang *et al.*, 1999), fentanyl binds to the internal cavity of the channel from the intracellular side of the membrane (Tschirhart *et al.*, 2019). Fentanyl has a pKa of 8.12, thus it is mostly positively charged at pH 7.4 (Thurlkill *et al.*, 2005). Since the neutral form, but not the charged form can freely cross the cell membrane, alterations in pH would change the proportion of fentanyl molecules in the neutral form and thus affect accessibility of fentanyl to the binding sites to achieve block.

Our results showed that hERG channels were blocked with a lesser potency at pH 6.4 and a greater potency at pH 8.4 compared to pH 7.4 (Fig. 3A&B). The IC_{50} values were 3220 ± 314 nM with a Hill coefficient of 0.8 ± 0.1 for pH 6.4, 832 ± 53 nM with a Hill coefficient of 0.9 ± 0.1 for pH 7.4, and 127 ± 4 nM with Hill coefficient of 0.8 ± 0.1 for pH 8.4. Our results further showed that the reversibility of fentanyl block was affected by pH. At pH 7.4, 3 μM fentanyl blocked I_{hERG} by 74 ± 5%. After 2 minutes of washout, current recovered to 86 ± 7% of control (Fig. 3C). At pH 8.4, 3 μM decreased I_{hERG} by 90 ± 4% *(*$P < 0.01$ compared to pH 7.4). However, the block was only partially reversible. After 2 minutes of washout, the current recovered and stabilized at 50 ± 12% of the control current *(*$P < 0.01$ compared to pH 7.4

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washout) (Fig. 3C). The delayed current recovery upon washout is not due to the extent of block; when the solution was subsequently changed to pH 7.4, I_{hERG} recovered to near control levels (data not shown).

hERG Drug-Binding-Site Mutant Channels Display Altered pH-Dependence Compared to WT Channels

We previously showed that two aromatic residues Tyr652 and Phe656 are involved in fentanyl block of hERG channels (Tschirhart *et al.*, 2019). Our results indicate that these residues also played a role in high pH-potentiated channel block. As shown in Fig. 4A&B, Y652A hERG channels were also blocked with a greater potency at extracellular pH 8.4. The IC_{50} values were 24.7 ± 1.5 μM with a Hill coefficient of 1.1 ± 0.1 for pH 7.4, and 7.1 ± 1.0 μM with a Hill coefficient of 1.1 ± 0.2 for pH 8.4. However, in contrast to WT hERG channels, $I_{Y652A\text{-}hERG}$ completely recovered upon fentanyl washout. At pH 7.4, 50 μM fentanyl decreased $I_{Y652A\text{-}hERG}$ by 70 ± 7%. After 2 minutes of washout, current recovered to 93 ± 3% of control (Fig. 4C). At pH 8.4, 50 μM decreased $I_{Y652A\text{-}hERG}$ by 75 ± 10% *(P* > 0.05 compared to pH 7.4). After 2 minutes of washout, current recovered to 103 ± 14% compared to control current *(P* > 0.05 compared to pH 7.4 washout).

Deletion of the hERG N-terminus Enhances Fentanyl Block Potency

As there is evidence suggesting that native hERG channels are composed of 1a and 1b isoforms (Lees-Miller *et al.*, 1997; London *et al.*, 1997; Jones *et al.*, 2004; Phartiyal *et al.*, 2007; Jones *et al.*, 2014), we were interested in examining whether these channels are blocked by fentanyl with a different potency than the homotetrameric 1a channels (hERG) previously

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studied. Since hERG1b alone does not produce current (Phartiyal *et al.*, 2008), we examined fentanyl block of homotetrameric Δ2-354 hERG channels stably expressed in HEK cells (Δ2-354 hERG-HEK cells). These channels resemble hERG1b, as the 1b isoform lacks the first 376 amino acids of 1a (Lees-Miller *et al.*, 1997; London *et al.*, 1997). Δ2-354 hERG channels were blocked with a greater potency than WT hERG channels (Fig. 5A&B). The IC_{50} values were 869 ± 17 nM (Hill coefficient of 1.0 ± 0.1) for WT hERG, and 247 ± 10 nM (Hill coefficient of 0.9 ± 0.1) for Δ2-354 hERG channels ($P < 0.01$).

To establish the physiological significance of this increased block potency, we transiently transfected Δ2-354 hERG into hERG-HEK cells to produce hERG1a/Δ2-354 hERG channels in order to resemble native hERG1a/1b channels. Interestingly, these channels were also more sensitive to fentanyl than hERG1a (Fig. 5A&B). The IC_{50} was 294 ± 10 nM (Hill coefficient of 1.0 ± 0.1).

Heterotetrameric hERG1a/1b Channels are Blocked by Fentanyl with a Greater Potency

To ensure that the enhanced block observed with Δ2-354 hERG channels also applies to the physiological hERG1a/1b tetramers, we examined fentanyl block of hERG1a/1b channels expressed in HEK cells. hERG1a/1b channels were blocked with a greater potency than hERG1a (generally referred to as hERG) (Fig. 6). The IC_{50} values were 862 ± 28 nM (Hill coefficient of 1.0 ± 0.1) for hERG1a, and 332 ± 5 nM (Hill coefficient of 1.0 ± 0.1) for hERG1a/1b.

Fentanyl Block Potency Correlates with Channel Deactivation Time Constants

Since fentanyl is trapped by channel closure (Tschirhart *et al.*, 2019), we posited that accelerated deactivation may contribute to the increased sensitivity of Δ2-354 and hERG1a/1b

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channels to fentanyl block. To this end, we examined Δ2-9 and Δ2-25 hERG channels transiently expressed in HEK cells. Like Δ2-354 hERG, Δ2-9 and Δ2-25 hERG mutants also display accelerated deactivation (Ng *et al.*, 2011). Since smaller deletions may have less impact on channel structure, they would therefore allow us to investigate the role of accelerated deactivation more directly. Our results showed that Δ2-9 hERG channels were blocked with a greater potency than WT hERG, with an IC_{50} of 386 ± 10 nM and a Hill coefficient of 1.1 ± 0.1 (Fig, 7A). As well, Δ2-25 hERG current were blocked with an IC_{50} of 203 ± 2 nM and a Hill coefficient of 1.0 ± 0.1 (Fig. 7B). We then plotted the IC_{50} values for each cell in all channel types (hERG1a, Δ2-9, Δ2-25, Δ2-354, hERG1a/Δ2-354, and hERG1a/1b studied in Fig. 5, 6, and 7) against the corresponding fast ($\tau_{f\text{-}deact}$, Fig. 7C) and slow deactivation time constants ($\tau_{s\text{-}deact}$, Fig. 7D) of tail currents upon −50 mV repolarizing step. There was a correlation between each deactivation time constant and IC_{50}. The correlation coefficient (r) determined by linear regression analysis was 0.76 for $\tau_{f\text{-}deact}$ ($P < 0.01$) and 0.68 for $\tau_{s\text{-}deact}$ ($P < 0.01$). These data indicate that accelerated deactivation increases the block of hERG by fentanyl.

Hypoxia, Hypokalemia, and pH Affect Fentanyl Block of hERG 1a/1b Channels in a Manner Similar to hERG1a

To investigate whether hERG 1a/1b channels respond to hypoxia, hypokalemia, and pH along with fentanyl in a manner similar to hERG1a channels, we cultured hERG 1a/1b-expressing HEK cells in normoxic (Control, CTL) or hypoxic (0.5% O_2) conditions for 6 hours and recorded currents in the absence and presence of 0.5 μM fentanyl. Compared to control, 6-hour culture in 0.5% O_2 or acute application of 0.5 μM fentanyl significantly decreased $I_{hERG1a/1b}$.

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The combination of hypoxic culture and 0.5 μM fentanyl resulted in $I_{hERG1a/1b}$ that was significantly less than either treatment alone (Fig. 8A).

To study the effects of hypokalemia and fentanyl on $I_{hERG1a/1b}$, we cultured channel-expressing HEK cells in normal medium (control, CTL, 5 mM K^+) or 2 mM K^+ for 72 hours. After culture, we examined the blocking effects of fentanyl on $I_{hERG1a/1b}$ recorded in standard, 5 mM K^+-containing bath solution in the absence or presence of 0.5 μM fentanyl. Compared to CTL, culture in 2 mM K^+ medium for 72 hours or acute application of 0.5 μM fentanyl significantly decreased $I_{hERG1a/1b}$. The combination of 2 mM K^+ culture and 0.5 μM fentanyl resulted in $I_{hERG1a/1b}$ that was significantly less than either condition alone (Fig. 8B).

To study the effects of pH on fentanyl-induced block of $I_{hERG1a/1b}$, we examined 0.5 μM fentanyl-mediated block of currents recorded in standard bath solutions with pH 6.4, 7.4, or 8.4. $I_{hERG1a/1b}$ was blocked with a lesser potency at pH 6.4 and a greater potency at pH 8.4 compared to pH 7.4 ($P < 0.01$ for each, one-way ANOVA with Tukey's post hoc test). While 0.5 μM fentanyl blocked $I_{hERG1a/1b}$ by 58 ± 13 % at pH 7.4, it blocked $I_{hERG1a/1b}$ by 19 ± 10 % at pH 6.4, and by 84 ± 4 % at pH 8.4 (Fig. 8C).

DISCUSSION

Fentanyl-related death is a serious issue in North America (Alexander *et al.*, 2016; Torralva and Janowsky, 2019). After finding that fentanyl blocks the hERG potassium channel and reduces I_{Kr} (Tschirhart *et al.*, 2019), the present work examined conditions that may interact with or exacerbate fentanyl-induced channel block. First, chronic hypoxia reduces mature hERG density and I_{hERG} (Lamothe *et al.*, 2017). We found that chronic hypoxia and acute fentanyl reduced I_{hERG} in an additive manner (Fig. 1). Since fentanyl-mediated respiratory depression

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(McQueen, 1983) may result in hypoxia, the additive impact of fentanyl-induced channel block and hypoxia-associated channel density reduction would further impair the function of hERG channels.

Fentanyl abusers may experience conditions such as diarrhea, vomiting, or malnourishment that can lead to hypokalemia (a reduced serum K^+ concentration), which decreases cell-surface hERG expression (Guo *et al.*, 2009; Massaeli *et al.*, 2010), and causes LQTS (Curry *et al.*, 1976; Roden *et al.*, 1986). Indeed, our results showed that chronic hypokalemia and acute fentanyl additively reduced I_{hERG} (Fig. 2A). In addition, we found that hERG channels were more sensitive to fentanyl when I_{hERG} was recorded in 2 mM K^+-containing bath solution compared to fentanyl-induced block of I_{hERG} recorded in standard 5 mM K^+-containing bath solution (Fig. 2B). These findings are consistent with previous reports that reduced extracellular K^+ concentration potentiates drug-mediated block of hERG channels (Yang and Roden, 1996; Wang *et al.*, 1997; Lin *et al.*, 2007) and drug-induced LQTS (Zehender *et al.*, 1991; Wenzel-Seifert *et al.*, 2011). Thus, low K^+ levels (hypokalemia) may decrease I_{hERG} through chronically decreasing hERG protein expression as well as acutely potentiating fentanyl-induced block.

Drug abuse can cause electrolyte and acid-base disturbances. In particular, hypokalemia is often associated with metabolic alkalosis. Our results demonstrate that hERG channels were blocked with a 3.9-fold less potency at extracellular pH 6.4 (Fig. 3A&B), and a 6.6-fold greater potency at extracellular pH 8.4, compared to pH 7.4 (Fig. 3A&B). There are a couple of potential explanations for this phenomenon. First, high pH may increase the membrane permeability of fentanyl. Fentanyl has a pK_a of 8.12 (Thurlkill *et al.*, 2005), and thus is predominately positively charged at a pH of 7.4. At a pH of 8.4, the portion of fentanyl in the neutral form would be more

than half. Since the neutral form of fentanyl may more readily cross the cell membrane to block hERG channels from internal side of the cell (Tschirhart *et al.*, 2019), enhanced membrane permeability would be expected to increase block potency.

Another possible explanation for the extracellular pH-dependence of fentanyl block is that the neutral form of fentanyl has a higher affinity for the hERG channel than the positively charged form. This possibility has merit, as fentanyl interacts with hydrophobic residues lining the pore of the channel (Tschirhart *et al.*, 2019). Indeed, at pH 8.4, fentanyl-mediated block of WT channels was only partially reversed upon washout of the drug (Fig. 3C), however, fentanyl-mediated block of Y652A channels was completely reversed upon washout of the drug (Fig. 4C). Therefore, high affinity binding to aromatic residues Tyr652 and Phe656 may explain the enhanced potency of fentanyl at increased extracellular pH. A potential flaw in this theory is the pipette solution remained at pH 7.2 for these experiments; therefore, once inside the cell the majority of fentanyl would be positively charged. However, the pore of hERG is open to the extracellular side of the membrane (Wang and MacKinnon, 2017). Thus, it is possible that the high pH of the extracellular solution is conferred to the pore of the channel. Once fentanyl molecules are in the pore, they may become unionized and bind to aromatic residues with the proposed high affinity. This scenario is supported by evidence suggesting that the pore of hERG can become blocked by protons at lower pH values (Bett and Rasmusson, 2003; Van Slyke *et al.*, 2012). The alteration of pH within the channel pore presents another possibility. Such a change in pH may alter channel structure and ionization state of residues, which could create a higher affinity binding site for fentanyl. While future studies would be required to elucidate the molecular mechanisms for pH dependence of fentanyl-mediated block, our finding that alkalosis increases the block of hERG by fentanyl has clinical implications. Conditions that cause

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alkalosis such as repetitive vomiting, dehydration, or endocrine disorders may increase the risk of fentanyl-associated arrhythmias. However, it should be noted that in the context of fentanyl-induced respiratory depression, hypercapnia and resulting acidosis (Agrafiotis *et al.*, 2015) may decrease hERG block potency. Nonetheless, alterations in drug-block potency during acid-base disturbances should be considered when assessing the cardiac safety of drugs.

Two transcript variants of hERG exist, hERG1a and the shorter hERG1b (Lees-Miller *et al.*, 1997; London *et al.*, 1997). Early investigations of native hERG channels from adult rat, mouse, and human heart tissues only resulted in the detection of hERG1a protein, and therefore it was proposed that hERG1b does not contribute to cardiac I_{Kr} (Pond *et al.*, 2000). However, further research has detected hERG1b proteins in rat, canine, human (Jones *et al.*, 2004), and equine (Finley *et al.*, 2002) heart tissue, suggesting that native I_{Kr} contains hERG1a and 1b isoforms. Furthermore, electrophysiological evidence suggests the presence of hERG1b in native channels. First, when hERG1a and 1b are coexpressed in heterologous expression systems, the currents resemble I_{Kr} more closely than hERG1a alone (London *et al.*, 1997; Sale *et al.*, 2008; Larsen and Olesen, 2010). Second, the functional role of hERG1b was elucidated using human induced pluripotent stem cell-cardiomyocytes, in which knockdown of hERG1b with short hairpin RNA decreases I_{Kr}, prolongs APD, and increases APD variability (Jones *et al.*, 2014). Third, mutations in hERG1b have been linked to LQTS in patients (Sale *et al.*, 2008).

To investigate fentanyl blockade of hERG1a/1b channels, we first examined fentanyl block of homotetrameric Δ2-354 hERG channels as an alternative to hERG1b since hERG1b does not produce current when expressed alone in HEK cells (data not shown). Δ2-354 hERG channels resemble hERG1b, as the 1b isoform lacks the first 376 amino acids of 1a (Lees-Miller *et al.*, 1997; London *et al.*, 1997). Our results showed that Δ2-354 hERG channels were blocked

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by fentanyl with a 3.5-fold greater potency than WT (hERG1a) channels (Fig. 5). When Δ2-354 hERG was expressed in hERG-HEK cells, currents were blocked with a 3.0-fold greater potency than WT channels (Fig. 5).

After this proof of concept, we obtained hERG1b plasmid as well as a stable hERG1a/1b cell line from Dr. Gail Robertson at University of Wisconsin - Madison. We also transfected hERG1b plasmid to hERG1a-HEK cells. Our results showed that heterotetrameric hERG1a/1b channels were blocked to a similar degree as hERG1a/Δ2-354 hERG channels, with a 2.6-fold greater potency than homotetrameric hERG1a channels (Fig. 6). Since hERG1a and hERG1b share an amino acid sequence for the S1 segment to the C-terminus including the pore region, the altered block potency may be due to changes in channel gating. One feature of hERG1a/1b channels is accelerated deactivation gating compared to hERG1a channels (Sale *et al.*, 2008). We previously demonstrated that fentanyl is an open-channel blocker and is trapped by channel closure (Tschirhart *et al.*, 2019). Furthermore, fentanyl block of hERG is strongly voltage-dependent, being greater at positive voltages and weaker at negative voltages. Thus, following block during a depolarizing step, a certain degree of unblock occurs upon repolarizing steps (e.g. −50 mV) before channels deactivate, which then traps fentanyl molecules that occupy the pore within the closed channels (Tschirhart *et al.*, 2019). Thus, accelerated deactivation would result in increased trapping of drugs, leading to enhanced block. This notion is supported by our results obtained in several hERG N-terminal deletion mutants, all of which demonstrated accelerated deactivation and enhanced sensitivity to fentanyl (Fig. 5 - 7). Furthermore, plotting the IC_{50} for fentanyl block versus $\tau_{f\text{-}deact}$ and $\tau_{s\text{-}deact}$ resulted in a statistically significant correlation between deactivation time constants and block potency (Fig. 7C&D). In addition to accelerated deactivation, due to faster activation and recovery from inactivation (Sale *et al.*, 2008),

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hERG1a/1b channels occupy the open state for longer than hERG1a channels. Thus, the possibility that a greater affinity for the open state of channels contributes to the greater sensitivity of hERG1a/1b channels to fentanyl cannot be excluded (Abi-Gerges *et al.*, 2011; Sale *et al.*, 2008).

Finally, our results showed that hERG1a/1b channels responses to hypoxia, hypokalemia, and pH along with fentanyl are similar to hERG (hERG1a) channels (Fig. 8).

In summary, we have uncovered several circumstances where the block of hERG by fentanyl may become more significant. First, hERG1a/1b channels are more susceptible to block, raising the possibility that fentanyl block may be more clinically significant than previously thought since both hERG1a and hERG1b are present in the human heart. In addition, conditions such as hypoxia, hypokalemia, and alkalosis can exacerbate fentanyl-mediated impairment of hERG current, creating situations where arrhythmias due to hERG blockade may underlie fentanyl-related sudden death.

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Conflict of Interest: The authors declare that they have no conflicts of interest with the contents of this article.

Authorship Contributions:

Participated in research design: Tschirhart and Zhang

Conducted experiments: Tschirhart

Performed data analysis: Tschirhart

Wrote or contributed to the writing of the manuscript: Tschirhart and Zhang

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FOOTNOTES

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LEGENDS FOR FIGURES

Fig. 1. Combined effects of chronic hypoxia and acute fentanyl on I_{hERG}. hERG-HEK cells were cultured in normoxia (CTL, 21% O_2) or hypoxia (Hx, 0.5% O_2) for 6 hours. For each cell, I_{hERG} was recorded in the absence or presence of 1 μM fentanyl (FENT). Representative current traces elicited with the voltage protocol are shown (top). The start-to-start interval was 15 seconds. Peak tail currents upon −50 mV repolarization after the 50 mV depolarizing step were used for analysis and are summarized (bottom). Lines represent the mean for each group. CTL, n=12; FENT, n=12; Hx, n=10; Hx+FENT, n=10 from 3 independent experiments. $^{**}P < 0.01$, one-way ANOVA with Tukey's post-hoc test.

Fig. 2. Combined effects of 2 mM K^+_o and fentanyl on I_{hERG}. (A) hERG-HEK cells were cultured in normal, 5 mM K^+_o (CTL) or 2 mM K^+_o (2K) for 72 hours. I_{hERG} was then recorded in the absence or presence of 1 μM fentanyl (FENT) in normal, 5 mM K^+ bath solution. Representative currents and summarized I_{hERG} amplitudes (peak tail currents after 50 mV depolarization) are shown. Lines represent the mean. CTL, n=21, and FENT, n=21 from 6 independent experiments; $2K^+$, n=12, and $2K^+$+FENT, n=12 from 3 independent experiments. (B) hERG-HEK cells were cultured in normal media (5 mM K^+_o). I_{hERG} elicited with the protocol above the current traces was recorded in 5 mM K^+_o ($5K^+$) or 2 mM K^+_o solution ($2K^+$) in the absence or presence of 1 μM fentanyl. The start-to-start interval was 15 seconds. Currents in the presence of fentanyl were normalized to the currents before drug and summarized. Lines represent the mean. $5K^+$, n=18; $2K^+$, n=18. $^{*}P < 0.05$, $^{**}P < 0.01$, one-way ANOVA with Tukey's post-hoc test (A) or 2-tailed unpaired *t* test (B).

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Fig. 3. Effects of extracellular pH on fentanyl-mediated block of hERG channels. (A) Representative hERG currents recorded in extracellular solution with a pH of 6.4, 7.4, or 8.4 in the absence (control, CTL) or presence of 500 nM or 2500 nM fentanyl. The voltage protocol is the same as shown in Fig. 2B. (B) Concentration-response relationships for fentanyl-mediated block of I_{hERG}. Peak tail currents upon −50 mV repolarization at each concentration of fentanyl were normalized to CTL and plotted as relative I_{hERG} (I_{hERG}-Rel) against fentanyl concentrations. The data were fitted to the Hill equation to determine IC_{50} values. pH 6.4, n= 5; pH 7.4, n=7; pH 8.4, n=5 from 3 independent experiments. (C) I_{hERG} recorded during control, 3 μM of fentanyl, and upon washout in bath solutions with pH of 7.4 or 8.4. The voltage protocol is the same as shown in Fig. 2B with a start-to-start interval of 15 seconds. I_{hERG} from each cell upon repetitive pulses was normalized to that upon the first pulse, and summarized. pH 7.4, n=7; pH 8.4, n=8 from 3 independent experiments. Error bars represent S.D.

Fig. 4. Effects of extracellular pH on fentanyl-mediated block of Y652A hERG channels. Representative Y652A hERG currents recorded in extracellular solution with a pH of 7.4 or 8.4 in the absence (control, CTL) or presence of 10 or 50 μM fentanyl. The voltage protocol is the same as shown in Fig. 2B. (B) Concentration-response relationships for fentanyl-mediated block of $I_{Y652A\text{-}hERG}$. Peak tail currents upon −50 mV repolarization at each concentration of fentanyl were normalized to CTL and plotted against fentanyl concentrations. The data were fitted to the Hill equation to determine IC_{50} values. pH 7.4, n=7; pH 8.4, n=5 from 3 independent experiments. (C) $I_{Y652A\text{-}hERG}$ recorded during control, 50 μM of fentanyl, and upon washout in bath solutions with pH of 7.4 or 8.4. The voltage protocol is the same as shown in Fig. 2B with a

start-to-start interval of 15 seconds. $I_{Y652A\text{-}hERG}$ upon repetitive pulses from each cell was normalized to CTL ($I_{Y652A\text{-}hERG}$ upon the first pulse). pH 7.4, n=3 from 2 independent experiments; pH 8.4, n=6 from 3 independent experiments. Error bars represent S.D.

Fig. 5. Block of N-terminal deletion hERG mutant by fentanyl. (A) Voltage protocol and representative currents recorded from WT hERG1a-HEK cells (1a), Δ2-354 hERG-HEK cells (ΔN), and HEK cells expressing hERG1a and Δ2-354 hERG (1a+ΔN) in the absence (control, CTL) or presence of 500 nM or 2500 nM of fentanyl. The start-to-start interval was 15 seconds. Peak tail currents upon −50 mV repolarization were used for analysis. (B) Concentration-response relationships for fentanyl-mediated block of hERG1a (1a), Δ2-354 hERG (ΔN), and hERG1a/Δ2-354 hERG (1a+ΔN) currents. The currents in the presence of each concentration of fentanyl were normalized relative to CTL and plotted as relative current (Current-Rel) against fentanyl concentrations. Data were fitted to the Hill equation to determine IC_{50} values. hERG1a, n=12 from 5 independent experiments; Δ2-354 hERG, n=12 from 3 independent experiments; hERG1a + Δ2-354 hERG, n=12 from 3 independent experiments. Error bars represent S.D.

Fig. 6. **Block of hERG1a/1b currents by fentanyl.** Representative hERG1a and hERG1a/1b currents in the absence (CTL) or presence of 500 nM or 2500 nM fentanyl. The voltage protocol was the same as in Fig. 5A. Peak tail currents upon −50 mV repolarization at each concentration of fentanyl were normalized to CTL and plotted against fentanyl concentrations. Data were fitted to the Hill equation to determine IC_{50} values. hERG1a, n=7 from 3 independent experiments; hERG1a/1b, n=6 from 2 independent experiments.

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Fig. 7. Higher fentanyl block potency is associated with accelerated deactivation of hERG channels. Representative Δ2-9 hERG (A) and Δ2-25 hERG (B) currents in the absence (CTL) or presence of 500 nM or 2500 nM fentanyl. The voltage protocol was the same as in Fig. 5A. Tail currents upon −50 mV repolarization were used for analysis. The concentration-response curves are shown beneath the currents for Δ2-9 hERG (A) and Δ2-25 hERG (B), respectively. Data were fitted to the Hill equation to determine IC_{50} values. For Δ2-9 hERG, n=5 from 2 independent experiments; for Δ2-25 hERG, n=8 from 3 independent experiments. Error bars represent S.D. (C and D) IC_{50} values plotted against the respective fast ($\tau_{f\text{-}deact}$, C) and slow time constant of deactivation ($\tau_{s\text{-}deact}$, D) from data obtained in A and B, as well as in Fig. 5 and 6. The plotted data were fitted with linear regression analysis. For Δ2-9 hERG, n=5 from 2 independent experiments; Δ2-25 hERG, n= 8 from 3 independent experiments; Δ2-354 hERG, n=12 from 3 independent experiments; WT hERG1a/Δ2-354 hERG, n=12 from 3 independent experiments; hERG1a/1b, n=6 from 2 independent experiments; WT hERG1a, n=19 from 7 independent experiments.

Fig. 8. Effects of chronic hypoxia, hypokalemia and external pH on fentanyl-induced block of $I_{hERG1a/1b}$. (A) hERG1a/1b-HEK cells were cultured in normoxia (CTL, 21% O_2) or hypoxia (Hx, 0.5% O_2) for 6 hours. For each cell, $I_{hERG1a/1b}$ was recorded in the absence or presence of 0.5 μM fentanyl (FENT). Representative current traces elicited with the voltage protocol shown in Fig. 1 are shown above scatter plots of data from each condition. CTL, n=14, and FENT, n=14 from 4 independent experiments; Hx, n=13, and Hx+FENT, n=13 from 3 independent experiments. (B) hERG1a/1b-HEK cells were cultured in normal (5 mM K^+, CTL) or 2 mM K^+ ($2K^+$) medium for 72 hours. $I_{hERG1a/1b}$ was then recorded in the absence or presence of 0.5 μM

fentanyl (FENT) in standard bath solution. Representative currents and scatter plot of $I_{hERG1a/1b}$ amplitudes at −50 mV repolarization after 50 mV depolarization are shown. CTL, n=11; FENT, n=11; $2K^+$, n=16; $2K^+$+FENT, n=16 from 3 independent experiments. (C). Representative hERG1a/1b currents and scatter plots of the current amplitudes recorded in bath solutions with a pH of 6.4, 7.4, or 8.4 in the absence (control, CTL) or presence of 0.5 μM fentanyl. pH 6.4, n= 7 from 2 independent experiments; pH 7.4, n=7 from 3 independent experiments; pH 8.4, n=7 from 2 independent experiments. In A-C, $*P < 0.05$, $**P < 0.01$, one-way ANOVA with Tukey's post-hoc test.

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Figure 1

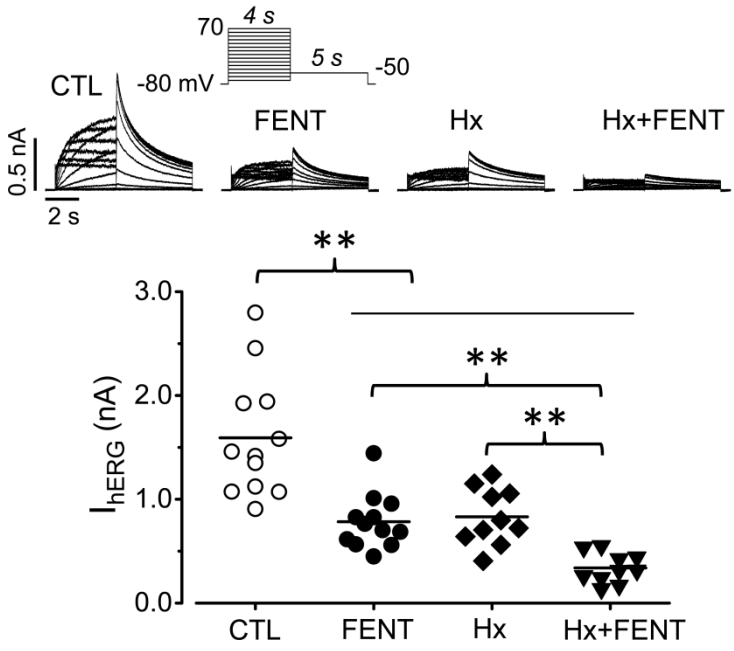

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Figure 2

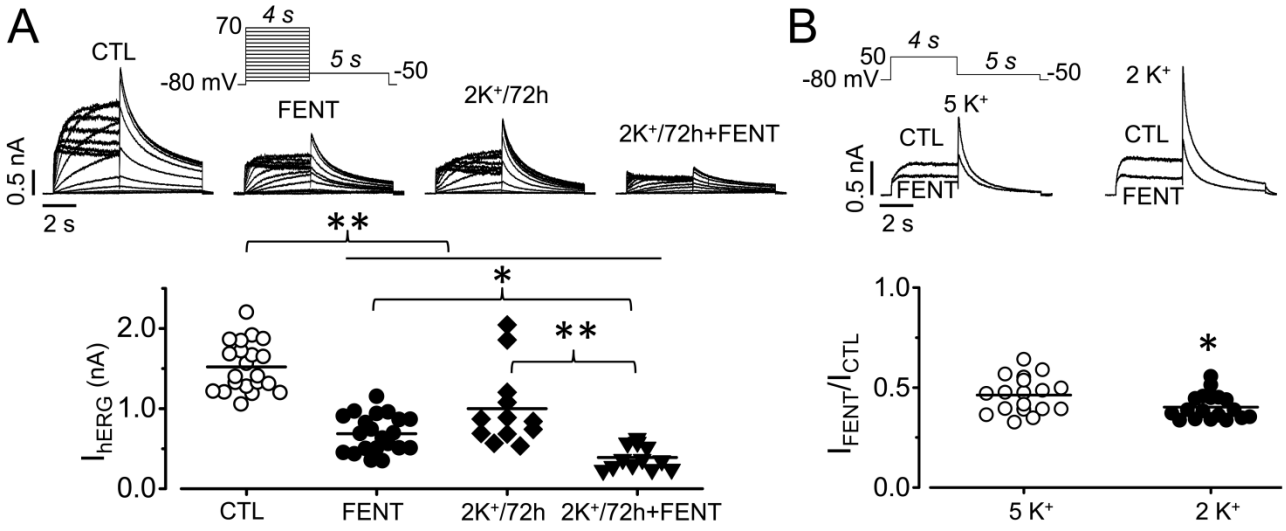

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Figure 3

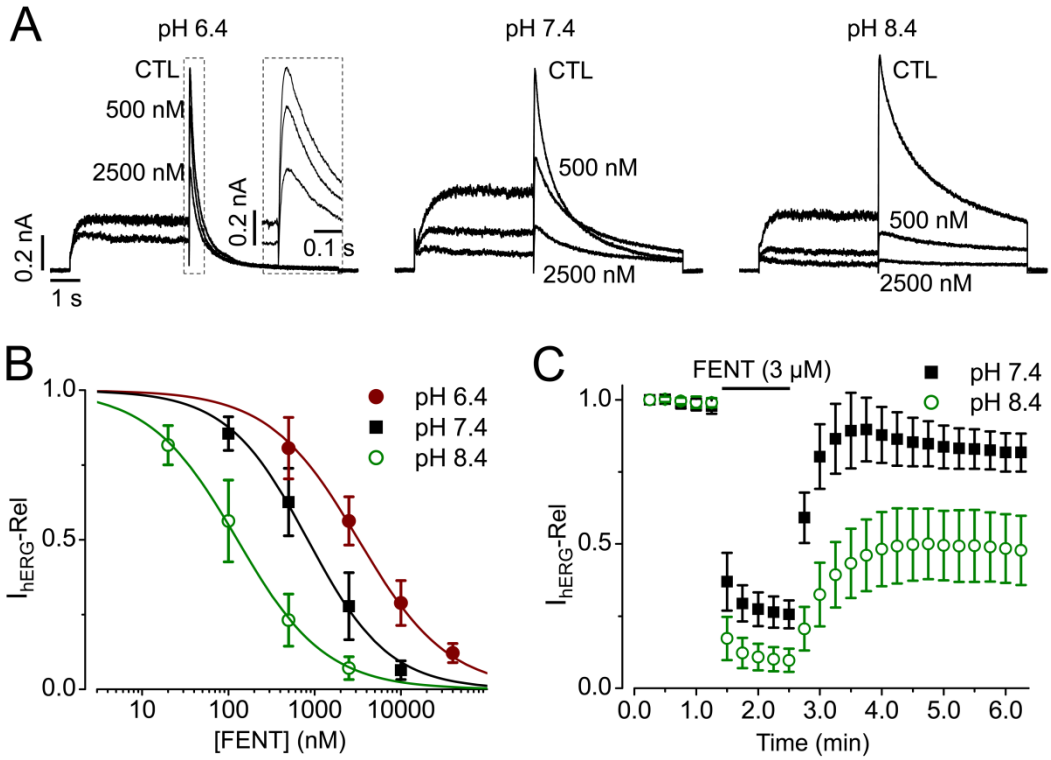

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Figure 4

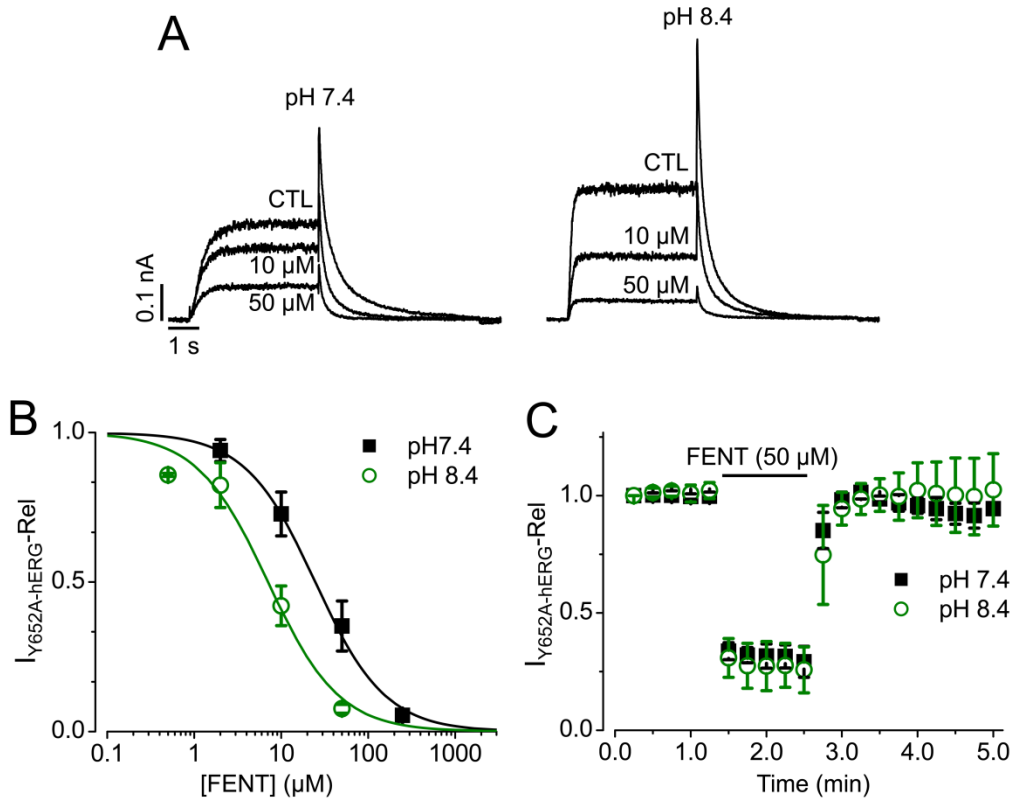

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Figure 5

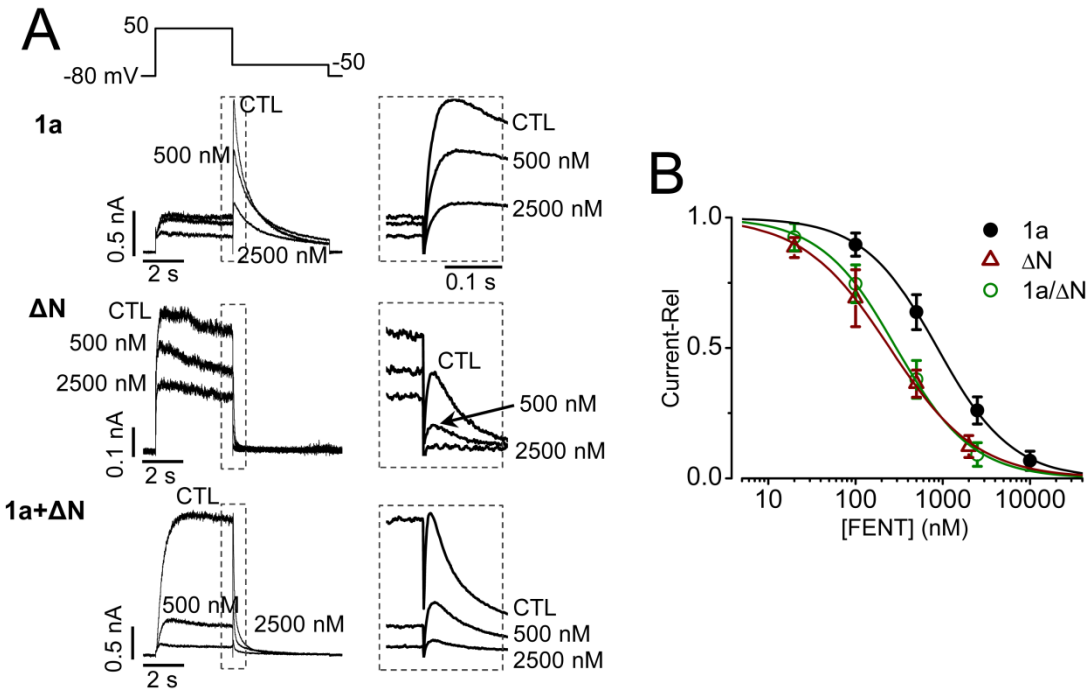

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Figure 6

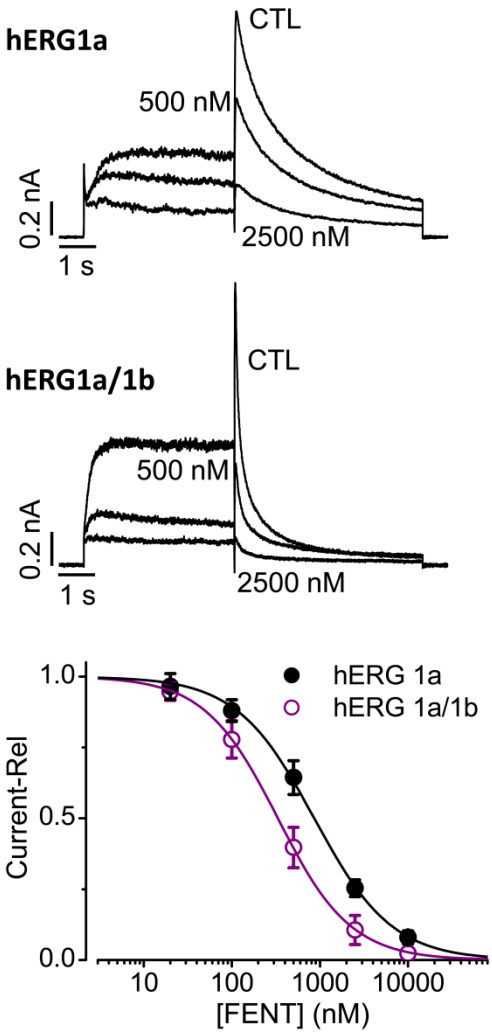

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Figure 7

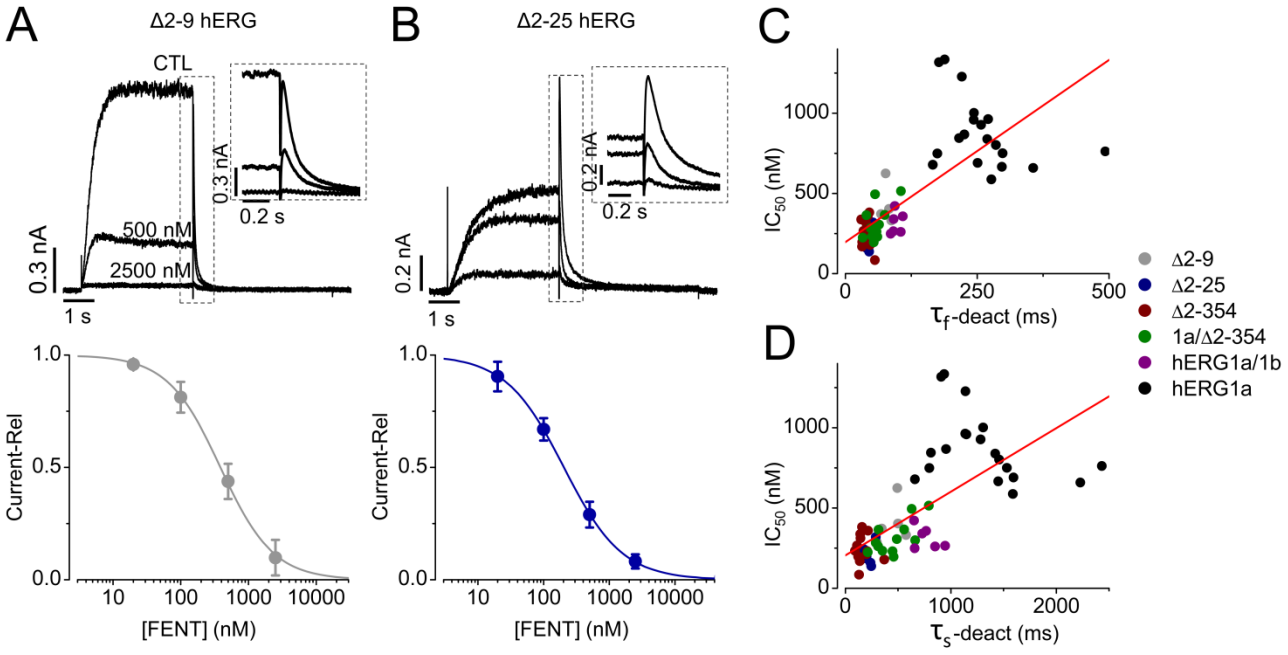

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Figure 8