British Journal of Pharmacology (2006) 149, 481–489

0007–1188/06 $30.00
www.brjpharmacol.org

RESEARCH PAPER

Drug-induced long QT syndrome: hERG K^+ channel block and disruption of protein trafficking by fluoxetine and norfluoxetine

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Background and purpose: Fluoxetine (Prozac®) is a widely prescribed drug in adults and children, and it has an active metabolite, norfluoxetine, with a prolonged elimination time. Although uncommon, Prozac causes QT interval prolongation and arrhythmias; a patient who took an overdose of Prozac exhibited a prolonged QT interval (QTc 625 msec). We looked for possible mechanisms underlying this clinical finding by analysing the effects of fluoxetine and norfluoxetine on ion channels *in vitro*.

Experimental approach: We studied the effects of fluoxetine and norfluoxetine on the electrophysiology and cellular trafficking of hERG K^+ and SCN5A Na^+ channels heterologously expressed in HEK293 cells.

Key results: Voltage clamp analyses employing square pulse or ventricular action potential waveform protocols showed that fluoxetine and norfluoxetine caused direct, concentration-dependent, block of hERG current (I_{hERG}). Biochemical studies showed that both compounds also caused concentration-dependent reductions in the trafficking of hERG channel protein into the cell surface membrane. Fluoxetine had no effect on SCN5A channel or HEK293 cell endogenous current. Mutations in the hERG channel drug binding domain reduced fluoxetine block of I_{hERG} but did not alter fluoxetine's effect on hERG channel protein trafficking.

Conclusions and implications: Our findings show that both fluoxetine and norfluoxetine at similar concentrations selectively reduce I_{hERG} by two mechanisms, (1) direct channel block, and (2) indirectly by disrupting channel protein trafficking. These two effects are not mediated by a single drug binding site. Our findings add complexity to understanding the mechanisms that cause drug-induced long QT syndrome.

British Journal of Pharmacology (2006) **149,** 481–489. doi:10.1038/sj.bjp.0706892; published online 11 September 2006

Keywords: hERG; arrhythmia; electrophysiology; long-QT syndrome; drug safety

Abbreviations: *hERG*, *human ether-a-go-go-related-gene*; HEK293 cells, human embryonic kidney cells; I_{hERG}, hERG K^+ current; I_{Kr}, rapidly activating delayed rectifier potassium current; I_{Na}, SCN5A Na^+ current; LQTS, long QT syndrome; *SCN5A*, *voltage-gated cardiac sodium channel gene*; WT, wild type

Introduction

Acquired long QT syndrome (LQTS) has become an important liability for clinically available drugs and developmental compounds. The mechanism commonly proposed for drug-induced QT interval prolongation is direct block of *human ether-a-go-go-related-gene* (hERG) (Kv11.1) K^+ channels or its native current, rapidly activating delayed rectifier potassium current (I_{Kr}). The drugs bind to a structurally unique receptor domain in the pore-S6 region of the channel to suppress K^+ ion permeation (for review, see Sanguinetti and Mitcheson (2005)). Recently, a second mechanism for drug-induced LQTS has emerged, which is the selective disruption of hERG channel protein trafficking to the cell surface membrane. Fewer mature hERG channels reach the surface membrane, thus reducing hERG K^+ current (I_{hERG}) or I_{Kr}. This indirect mechanism has been shown for only a few uncommonly used drugs, and the drug-binding site mediating this effect is unknown, and the importance of this mechanism is not well understood (for review, see Eckhardt *et al*., 2005b).

This report was prompted by a patient who ingested an overdose of the widely used drug fluoxetine (Prozac) and

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Received 7 June 2006; accepted 6 July 2006; published online 11 September 2006

developed a prolonged QT interval. Fluoxetine is a selective serotonin reuptake inhibitor prescribed for depression, postpartum depression, premenstrual dysphoric syndrome and bulimia. Its popularity is in part owing to the few reported cardiotoxic side effects compared to tricyclic antidepressants (Upward *et al*., 1988). However after initial marketing concerns over the safety of fluoxetine have been raised (Pacher and Kecskemeti, 2004), including reports of QT interval prolongation (Varriale, 2001; Curtis *et al*., 2003), cardiac arrhythmias (Allhoff *et al*., 2001; Abebe-Campino *et al*., 2002) and Torsades de Pointes (Appleby *et al*., 1995; Lherm *et al*., 2000; Wilting *et al*., 2006) as well as synergistic effects when used in combination with other drugs (Michalets *et al*., 1998; Isbister *et al*., 2004; Nykamp *et al*., 2005). The principal metabolite, norfluoxetine, is also active as an antidepressant and has a five-to seven-fold longer elimination half-life compared to the parent drug. Newborns metabolize fluoxetine and norfluoxetine more slowly than adults and toxicity with ventricular arrhythmias has been reported in infants of mothers treated with fluoxetine during pregnancy and while breast feeding (Hale *et al*., 2001; Abebe-Campino *et al*., 2002; Dubnov *et al*., 2005). Previously, fluoxetine was reported to block I_{hERG} studied in *Xenopus* oocytes or Chinese hamster ovary cells with IC_{50} values of 3.1 or 1.5 μM, respectively (Thomas *et al*., 2002; Witchel *et al*., 2002). Norfluoxetine's effect on hERG channels has not been reported previously and nothing is known about possible indirect effects of fluoxetine or norfluoxetine on hERG channel protein trafficking.

We have investigated the electrophysiological properties of fluoxetine and norfluoxetine on hERG K^+ and *voltage-gated cardiac sodium channel gene* (SCN5A) Na^+ channels heterologously expressed in a human cell line. We studied hERG and SCN5A channels because they are common targets for drug interaction and proarrhythmia. Both compounds selectively reduced I_{hERG} at similar drug concentrations by two mechanisms, (1) directly by blocking ion permeation, and (2) indirectly by disrupting channel protein trafficking. A preliminary report of this work has appeared (Eckhardt *et al*., 2005a).

Methods

hERG and SCN5A channel expression

Stable transfection of *hERG1 (KCNH2)* wild type (WT) or *SCN5A (hH1c)* WT complementary DNA (cDNA) into human embryonic kidney cells (HEK293) cells has been described previously (Zhou *et al*., 1998b; Valdivia *et al*., 2002). In some experiments, a second hERG WT HEK293 cell line was used. The new cell line differs from the previous HEK293 hERG cell line only in that a bovine growth hormone polyadenylation (poly-A) signal was removed from the pCDNA3 (Invitrogen, Carlsbad, CA, USA) expression vector. This poly-A signal is redundant as the hERG cDNA originally isolated contains a native poly-A signal and tail (Warmke and Ganetzky, 1994). The pCDNA3 poly-A signal was removed through excision by restriction digest at unique sites (*Xba*1–*Bbs*1) upstream and downstream of the signal. The single-strand overhangs of the digested vector were filled-in with Klenow polymerase and ligated together to reform the expression vector. The hERG1 WT cDNA was inserted into the *Bam*HI–*Eco*RI restriction sites of the new vector and native HEK293 cells were stably transfected using Superfect (Qiagen GmbH, Hilden, Germany). The electrophysiological and biochemical properties of both WT hERG HEK293 cells lines were similar.

Two hERG channel engineered mutations, F656A and F656C, were generated using a QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA), and DNA sequencing confirmed the nucleotide changes. These mutations were transiently transfected into HEK293 cells using SuperFect and studied within 24–48 h as described previously (Zhou *et al*., 1998b).

Electrophysiology

Membrane currents were recorded using the whole-cell patch clamp technique (Hamill *et al*., 1981). pCLAMP 8.0 software (Axon Instruments, Union City, CA, USA) was used to generate voltage clamp protocols and acquire data, which were analyzed using pCLAMP 8.0 and Microcal Origin (MicroCal, Northampton, MA, USA) software. For recording I_{hERG}, cells were superfused with Tyrode solution containing (in mM) NaCl 137, KCl 4, $CaCl_2$ 1.8, $MgCl_2$ 1, glucose 10, *N*-2-hydroxylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) 10, pH adjusted to 7.4 with NaOH. Patch pipettes contained (in mM) KCl 130, $MgCl_2$ 1, MgATP 5, ethyleneglycoltetraacetate (EGTA) 5, HEPES 10, pH adjusted to 7.2 with KOH and had resistances of 1.5–2 MΩ. For recording Na^+ current (I_{Na}), cells were superfused with Tyrode solution containing (in mM) NaCl 140, KCl 4.0, $CaCl_2$ 1.8, $MgCl_2$ 0.75, HEPES 5 and pH adjusted to 7.4 with NaOH. Patch pipettes contained (in mM) CsCl 20, CsF 120, EGTA 2, HEPES 5 and pH adjusted to 7.4 with CsOH, and had resistances of 1–1.5 MΩ. Series resistance compensation was 70–80% and leak subtraction was used for experiments with I_{Na}. Membrane currents were recorded using square pulse or ventricular action potential waveform protocols at 23 ± 1 and 37 ± 1°C, respectively.

Western blot analysis

Western blot analysis of hERG protein was performed as described previously (Zhou *et al*., 1998b). Whole-cell lysates were analyzed by solubilizing HEK293 cells in lysis buffer (50 mM Tris-HCl at pH 7.5, 150 mM NaCl, 5 mM EDTA, 1% Nonidet P-40 (NP-40) buffer and 10% glycerol) containing a protease inhibitor mini-tablet (Roche Diagnostics, Mannheim, Germany) for 2 h at 4°C. The insoluble materials were pelleted at 12 000 r.p.m. for 10 min at 4°C. Protein concentration was measured using the BCA method (Pierce Chemical, Rockford, IL, USA) or Bio-Rad DC protein assay (Bio-Rad Laboratories, Hercules, CA, USA). Soluble lysate proteins were then heated with an equal amount of Laemmli sample buffer containing 5 mM dithiothreitol for 30 min at 37°C. The denatured protein lysates were subjected to sodium dodecylsulfate-polyacrylamide gel electrophoresis followed by elecrophoretic transfer onto nitrocellulose membranes. The membranes were incubated with hERG antiserum (1:10 000 dilution) at room temperature

overnight, and the antibody was detected as described previously (Zhou *et al.*, 1998b). Each Western blot shows data scanned from one nitrocellulose membrane and reproduced at constant grayscale intensity.

Image density analysis of hERG protein detected on Western blot analysis was performed using a Bio-Rad GS-700 Imaging Densitometer and Molecular Analyst software (Bio-Rad Laboratories). Using this software, a box of constant size was placed around each control and experimental 155 kDa (mature hERG protein) band, as well as an empty lane (background), and pixel intensities were integrated to yield relative density values. Following background subtraction, these values were normalized against the control value on each Western blot. To validate that this densitometry method yielded a graded response with decreasing protein concentration, serial dilutions of hERG WT lysate (100, 80, 60, 40, 20 and 0% protein concentration) were made with NP-40 lysis buffer followed by Western blot analysis. Example Western blot data are given in Figure 1a and show a graded decrease in the intensity of both the 155 and 135 kDa bands with serial dilution. The 155 kDa band densities were normalized to the 100% protein concentration band (lane 2). Figure 1b summarizes the density analysis of 10 Western blots to show a step-wise, nearly linear, decrease in 155 kDa band density with decreasing protein concentration.

Statistical methods

Data are given as mean ± s.e.m. Concentration–response relations for block of I_{hERG} by fluoxetine or norfluoxetine were determined using the Hill equation,

$$I_{drug}/I_{control} = 1/[1 + (D/IC_{50})^n]$$

where $I_{drug}/I_{control}$ is fractional block, D is drug concentration, IC_{50} is drug concentration for 50% block and n is the Hill coefficient (Zhou *et al.*, 1998b). Concentration–response relations for the effect of fluoxetine or norfluoxetine on normalized Western blot density values of the 155 kDa protein band were determined using a modified Hill equation,

$$d_{drug}/d_{control} = 1/[1 + (D/dC_{50})^n]$$

where $d_{drug}/d_{control}$ is fractional reduction in density compared to control, D is drug concentration, dC_{50} is the drug concentration for a 50% decrease in density and n is the Hill coefficient (see Ficker *et al.*, 2002, 2004). Statistical significance was determined using Student's paired *t*-test and a *P*-value of <0.05 was considered significant.

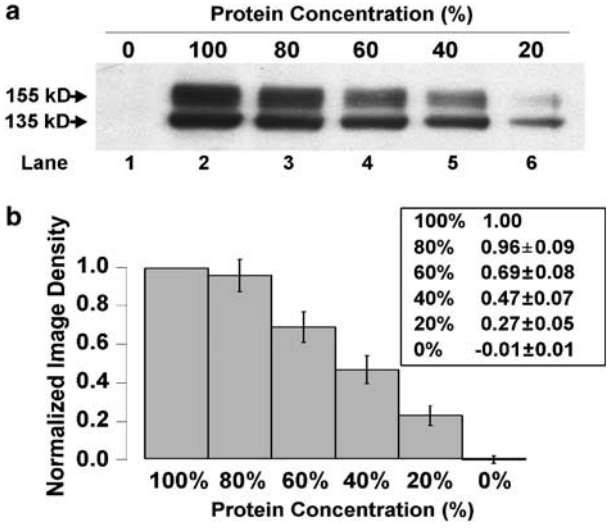

Figure 1 Serial dilution densitometry. (**a**) Typical Western blot of serial dilutions of hERG protein lysates. (**b**) Analysis of the normalized 155 kDa band densities of Western blots ($n = 10$). Individual normalized densitometry values (mean ± s.e.m.) are given in the table (inset).

Drugs

Fluoxetine, norfluoxetine, E4031 and cisapride were purchased from Sigma Chemicals (St Louis, MO, USA) or Biomol International LP (Plymouth Meeting, PA, USA). Each was dissolved in distilled water to give a 10 mM stock solution and further dilutions were made in Tyrode solution.

Results

Clinical history

A 19-year-old female with a history of depression, anorexia and bulimia, was admitted to the University of Wisconsin Hospital and Clinics following the intentional overdose of ~400 mg of Prozac. Her initial ECG showed sinus rhythm of 88 b.p.m. with a prolonged QT interval of 515 ms and a QTc (Bazett method) of 625 ms (Figure 2). There was no family history of cardiac disease, palpitations, syncope, seizure, deafness or sudden death. Her physical examination was unremarkable. Urine toxin screen showed no abnormalities. Serum blood levels of fluoxetine and norfluoxetine were not obtained. A follow-up ECG 3 weeks later showed a QTc of 400 ms (Figure 2). We hypothesized that Prozac caused her LQTS by reducing I_{hERG}.

Direct block of I_{hERG} by fluoxetine and norfluoxetine

The effects of fluoxetine and norfluoxetine on I_{hERG} in HEK293 cells were studied using two voltage clamp proto-

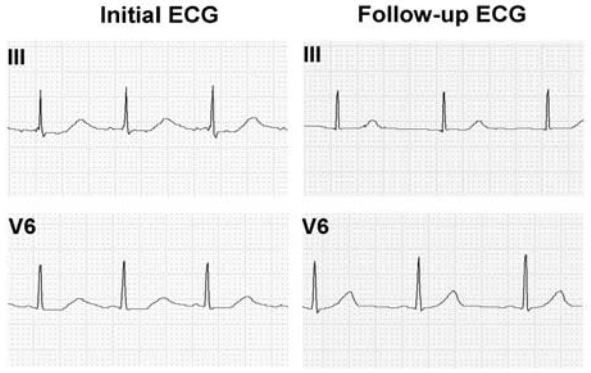

Figure 2 ECG tracings (normal standard calibration) of leads III and V6 of the patient with Prozac overdose.

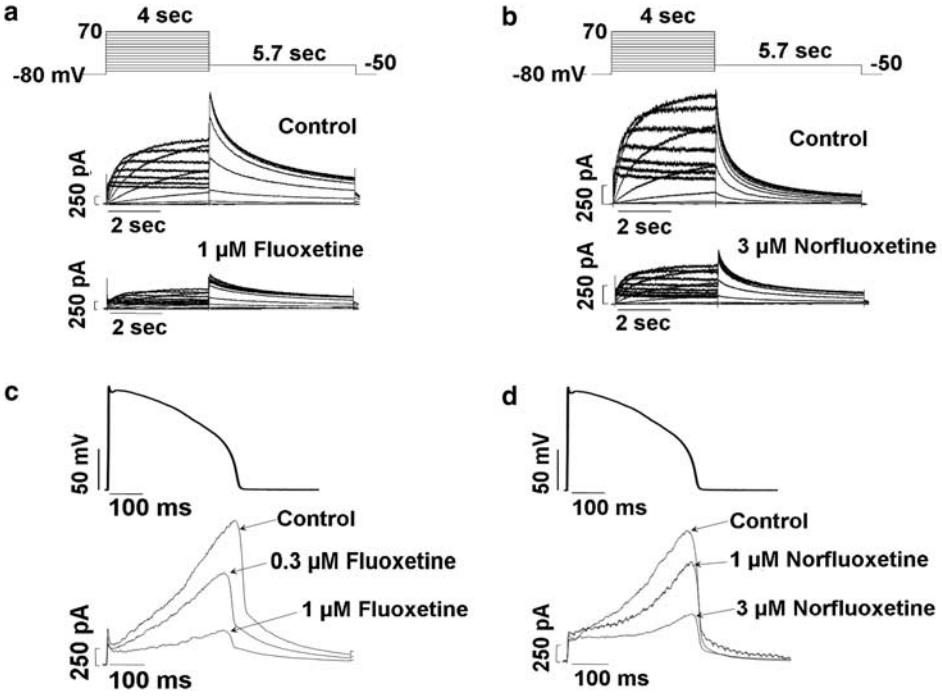

Figure 3 Effect of fluoxetine and norfluoxetine on I_{hERG}. (**a** and **b**) Square pulse protocol along with families of I_{hERG} traces for control conditions and after 1 μM fluoxetine or 3 μM norfluoxetine. (**c** and **d**) Action potential waveform protocol along with I_{hERG} traces for control conditions, after 0.3 and 1 μM fluoxetine or 1 and 3 μM norfluoxetine.

cols, a square pulse protocol and an action potential waveform protocol. Figure 3 shows these protocols along with typical I_{hERG} traces. For the square pulse protocol, cells were depolarized from a holding potential of −80 to 70 mV in 10 mV increments for 4 s, followed by a step to –50 mV for 5.7 s to record tail I_{hERG}, with the protocol applied every 15 s. Figure 3a shows representative I_{hERG} traces from one cell for control conditions and after 10 min of exposure to fluoxetine (1 μM). Figure 3b shows representative I_{hERG} traces from a different cell for control conditions and following norfluoxetine (3 μM). Both compounds directly reduce I_{hERG}. For the action potential waveform protocol, cells were depolarized from a holding potential of −82 mV to a peak voltage of 42 mV and repolarization was 90% complete (APD_{90}) in 380 ms (Zhou *et al*., 1998b), with the waveform applied at 5 s intervals. Because of its channel gating properties, I_{hERG} reaches its maximum amplitude during phases 2 and 3 of the ventricular action potential waveform (Zhou *et al*., 1998b). Fluoxetine (0.3 and 1 μM, Figure 3c) and norfluoxetine (1 and 3 μM, Figure 3d) caused a concentration-dependent reduction in I_{hERG}. Using these voltage clamp protocols, block of I_{hERG} was determined for fluoxetine (0.1, 0.3, 1, 3 and 10 μM, $n = 5$–6 cells at each concentration) or norfluoxetine (0.3, 1, 3, 10 and 30 μM, $n = 3$–5 cells at each concentration). Block of I_{hERG} developed rapidly and was reversible following drug washout (data not shown). Peak tail I_{hERG} (square pulse protocol, voltage step from 70 to −50 mV) or the maximum outward I_{hERG} (action potential waveform protocol) at steady-state block for each fluoxetine or norfluoxetine concentration was normalized to its respective control I_{hERG} value obtained before drug exposure. The averaged normalized I_{hERG} values at each drug concentration were fitted to the Hill equation. The IC_{50} value for fluoxetine was 0.7 ± 0.1 μM with a slope factor of 1.0 ± 0.2 obtained using the square pulse protocol and 0.7 ± 0.1 μM with a slope factor of 1.4 ± 0.2 obtained with the action potential waveform protocol. The IC_{50} values for norfluoxetine were 2.3 ± 0.2 μM with a slope factor of 1.3 ± 0.1 and 2.5 ± 0.1 μM with a slope factor of 1.3 ± 0.1 for the two different protocols, respectively. Thus, the two voltage clamp protocols generated nearly identical concentration–response relations, suggesting that both compounds rapidly achieve steady-state block following cell depolarization.

Disruption of hERG protein trafficking

We studied the effect of fluoxetine and norfluoxetine on hERG channel protein trafficking. hERG channel biogenesis involves complex co- and post-translational protein processing through the secretory pathway (Zhou *et al*., 1998a, b; Jones *et al*., 2004), and its disruption is an important mechanism in congenital type-2 LQTS (see Delisle *et al*., 2004; Anderson *et al*., 2006). Cells expressing hERG WT channel protein were incubated with fluoxetine or norfluoxetine for 24 h as described previously (Zhou *et al*., 1999). Cells were then subjected to Western blot analysis. Figure 4a shows representative results. WT channels (lane 1) show protein bands at ~135 kDa (immature, core glycosylated protein in the endoplasmic reticulum) and at ~155 kDa (mature, complexly glycosylated channel protein in the surface membrane). The intensity of the 155 kDa protein band decreased with increasing cell culture concentrations of fluoxetine (1, 3 and 10 μM; lanes 2–4) or norfluoxetine (3

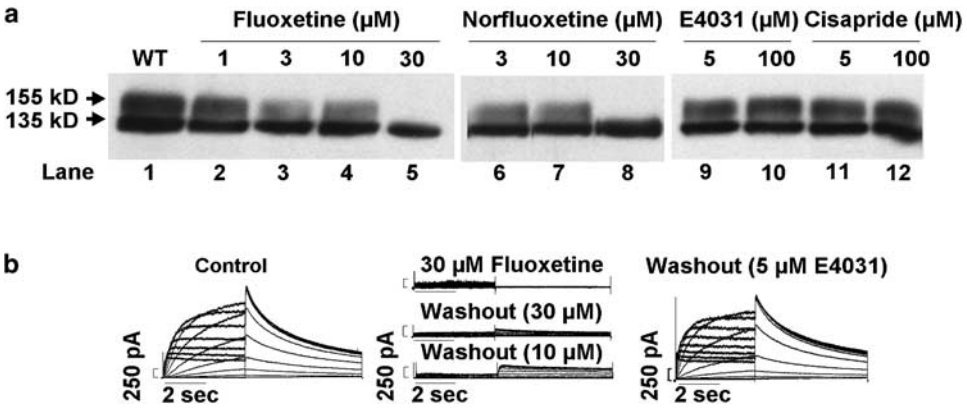

Figure 4 (**a**) Western blot analysis of hERG WT channel protein with increasing concentrations of fluoxetine, norfluoxetine, E4031 and cisapride. (**b**) I_{hERG} traces (square pulse protocol, see Figure 3a) for control conditions (left panel), following 24 h incubation without drug washout or after 1 h drug-free conditions for 10 or 30 μM fluoxetine (middle panels), and I_{hERG} 1 h following washout of 5 μM E4031 (right panel).

and 10 μM; lanes 6 and 7), whereas the 135 kDa protein band remained intact, and at 30 μM fluoxetine (lane 5) or 30 μM norfluoxetine (lane 8) only the 135 kDa protein band was detected. Similar findings of a concentration-dependent decrease in the 155 kDa band were obtained in six Western blot analyses with fluoxetine (1, 3, 10 and 30 μM, 4–6 blots at each concentration) and in four Western blot analyses with norfluoxetine (1, 3, 10 and 30 μM, 2–4 blots at each concentration). Two additional drugs, E4031 and cisapride (5 or 100 μM), which block hERG channels with even higher affinities (IC_{50} values 7.7 and 4.3–6.5 nM, respectively (Mohammad *et al*., 1997; Zhou *et al*., 1998b; Anson *et al*., 2004)) were studied similarly. Western blot analysis detected both the 135 and 155 kDa protein bands with E4031 (lanes 9 and 10) and cisapride (lanes 11 and 12). These bands were similar in intensity to control conditions (lane 1) and the same results were obtained in five additional Western blots. Thus, E4031 and cisapride at concentrations >10 000-fold above their IC_{50} values for hERG channel block had no effect on the trafficking of the channel protein.

We confirmed for fluoxetine that disruption of trafficking of the 155 kDa protein band abolished I_{hERG}. Representative I_{hERG} traces are shown in Figure 4b. For control conditions (left panel) the peak tail I_{hERG} was 103.4 ± 4.4 pA/pF for the voltage step from 70 to -50 mV ($n=4$ cells). The middle panel shows that I_{hERG} was virtually absent when the cells were incubated for 24 h and studied in 30 μM fluoxetine (peak tail $I_{hERG} = 2.7 \pm 1.5$ pA/pF, $n=4$ cells), conditions that both disrupt trafficking and block the channel. Fluoxetine (30 μM) washout for 1 h resulted in little additional I_{hERG} (peak tail $I_{hERG} = 11.8 \pm 3.8$ pA/pF, $n=4$ cells). Incubation for 24 h in 10 μM fluoxetine, which incompletely disrupts hERG protein trafficking (Figure 4a, lane 4), followed by drug washout for 1 h resulted in a larger peak tail I_{hERG} (35.2 ± 5.6 pA/pF, $n=4$ cells, $P<0.05$ compared to peak tail I_{hERG} after culture in and washout of 30 μM fluoxetine). The right panel shows I_{hERG} traces recorded after incubation of cells in 5 μM E4031 for 24 h followed by 1 h of drug washout. Peak tail I_{hERG} was 100.2 ± 21.5 pA/pF ($n=4$ cells), similar to control ($P>0.05$). These data confirm electrophysiologically the biochemical finding that fluoxetine disrupts hERG protein trafficking whereas the higher affinity I_{hERG} blocking drug E4031 did not.

We tested whether E4031 (5 μM) could prevent fluoxetine or norfluoxetine-mediated disruption of hERG protein trafficking, as E4031 has been shown to cause 'pharmacological rescue' of some LQT2 trafficking-defective channels (Zhou *et al*., 1999). Cells expressing hERG WT channels were incubated for 24 h with (1) 5 μM E4031, (2) 5 μM E4031 + 30 μM fluoxetine or (3) 5 μM E4031 + 30 μM norfluoxetine, and were then subjected to Western blot analysis. E4031 did not prevent fluoxetine or norfluoxetine-mediated disruption of the 155 kDa protein band ($n=2$ blots, data not shown), whereas E4031 alone had no effect (see Figure 4a, lane 9).

Time course of disruption and recovery of hERG protein trafficking

Drug-induced alteration in hERG channel protein biogenesis should develop and recover gradually. Western blot analysis (Figure 5a) shows the time-dependent loss of the mature hERG protein band with cell incubation in 30 μM fluoxetine and its recovery following drug washout from the culture medium. Similar findings were obtained in four additional Western blot analyses. Patch clamp recordings (Figure 5b) show the time-dependent loss of I_{hERG} with cell incubation in 30 μM fluoxetine (followed by 1 h of culture in drug-free conditions) and its recovery following drug washout from the culture medium. Peak tail I_{hERG} densities (voltage step from 70 to -50 mV, $n=4$–7 cells at each time) normalized to the control data showed a 58.1% reduction at 4 h and a 94.2% reduction at 24 h of fluoxetine incubation. Following 4 and 24 h of washout of the drug from the culture medium, normalized peak tail I_{hERG} densities increased to 57.8 and 137.8% of control.

Fluoxetine does not alter SCN5A Na^+ or HEK293 cell endogenous currents

To understand whether fluoxetine is selective for hERG channels, or whether it can affect similarly other ion channels, we tested its effects on I_{Na}. Cells expressing SCN5A

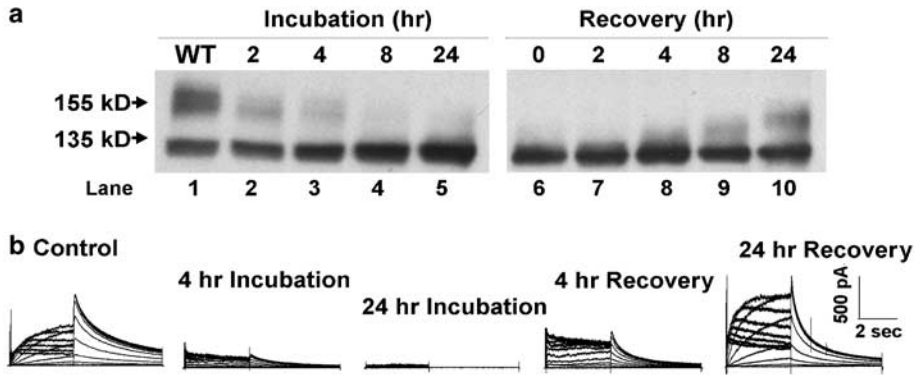

Figure 5 (**a**) Western blot analysis of the time-dependence of the development and recovery of fluoxetine-induced disruption of hERG protein trafficking. (**b**) Families of I_{hERG} traces for control conditions, and at different times of incubation in 30 μM fluoxetine, and after removal of fluoxetine from the culture medium.

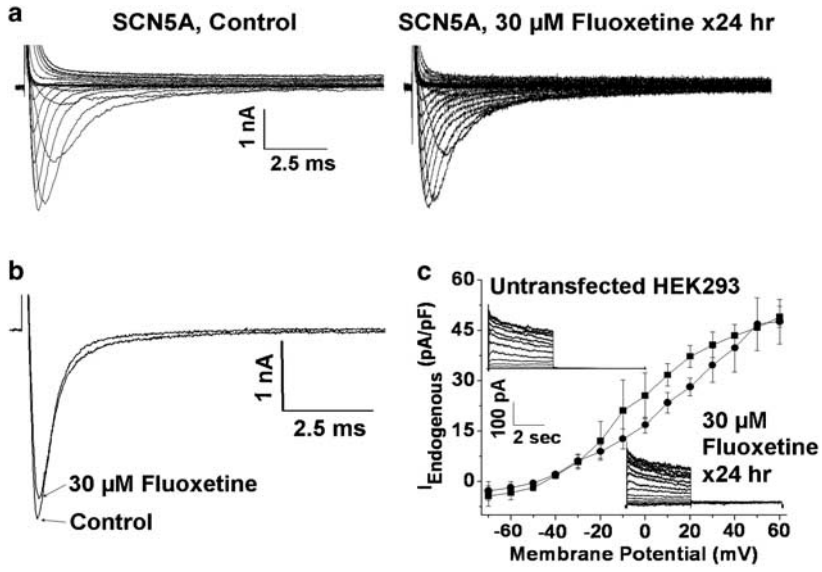

Figure 6 (**a**) Families of I_{Na} recorded for control conditions and following 24 h incubation in 30 μM fluoxetine. (**b**) I_{Na} recordings for control conditions and after 10 min superfusion of 30 μM fluoxetine. (**c**) *I–V* plots of $I_{Endogenous}$ for control conditions and after 24 h incubation in 30 μM fluoxetine. Insets show representative families of current traces.

channels were depolarized from a holding potential of −140 mV to between −120 to 80 mV in 10 mV increments for 24 ms. Figure 6a shows representative families of I_{Na} traces recorded for control conditions and after 24 h of culture in 30 μM fluoxetine (followed by 1 h of culture in drug-free conditions). Peak inward I_{Na} for control conditions was -230 ± 79 pA/pF ($n = 6$ cells) and following 24 h culture in fluoxetine was -280 ± 50 pA/pF ($n = 6$ cells, $P > 0.05$ compared to control). We also tested whether fluoxetine directly blocked $I_{Na.}$ As shown in Figure 6b, I_{Na} was recorded for control conditions and following 30 μM fluoxetine treatment for 10 min (voltage step −140 to −20 mV). Peak inward I_{Na} for control conditions was -194 ± 18 pA/pF and in fluoxetine it was -171 ± 15 pA/pF ($n = 5$ cells, $P > 0.05$ compared to control). Thus, fluoxetine did not significantly alter I_{Na}. We also studied the effect of fluoxetine on the small amplitude endogenous current found in untransfected HEK293 cells. Representative families of current traces are shown in the insets in Figure 6c (voltage clamp protocol as in Figure 3a). Endogenous current ($I_{Endogenous}$) was measured at the end of depolarizing steps to between –70 and 60 mV for cells in control conditions ($n = 3$ cells) and following 24 h incubation in 30 μM fluoxetine (followed by 1 h of culture in drug-free conditions, $n = 3$ cells), and are plotted in Figure 6c. Cell culture in fluoxetine for 24 h had minimal effect on $I_{Endogenous}$.

Concentration–response relations: block of I_{hERG} *versus disruption of hERG protein trafficking*

The concentration–response relations for block of peak tail I_{hERG} by fluoxetine and norfluoxetine are shown in Figure 7a and b, respectively, determined with the square pulse and action potential waveform protocols (Figure 3a and b). Block was concentration-dependent, had similar IC_{50} values, and the slope factors were close to unity, consistent with drug interaction at a single site. Figure 7a and b also show the concentration–response relations for fluoxetine and nor-

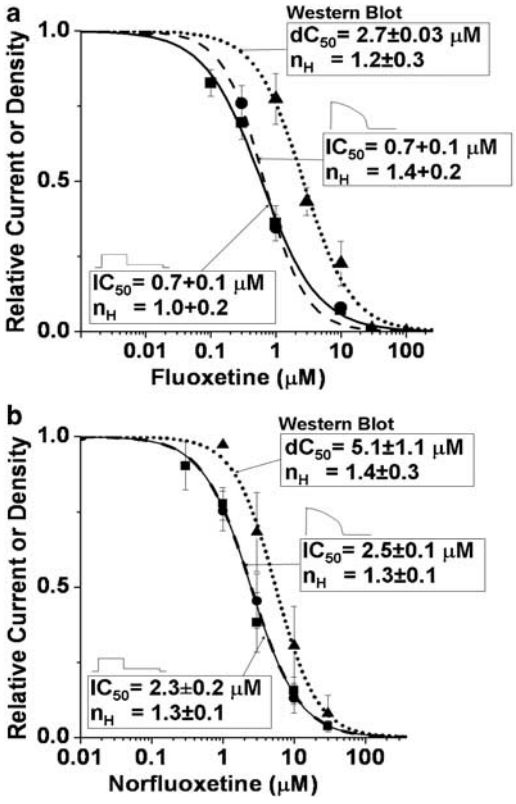

Figure 7 (**a** and **b**) Concentration–response relations for fluoxetine and norfluoxetine, obtained using square pulse protocol, action potential waveform protocol and from normalized Western blot image density measurements fitted to Hill equation (see text for detail).

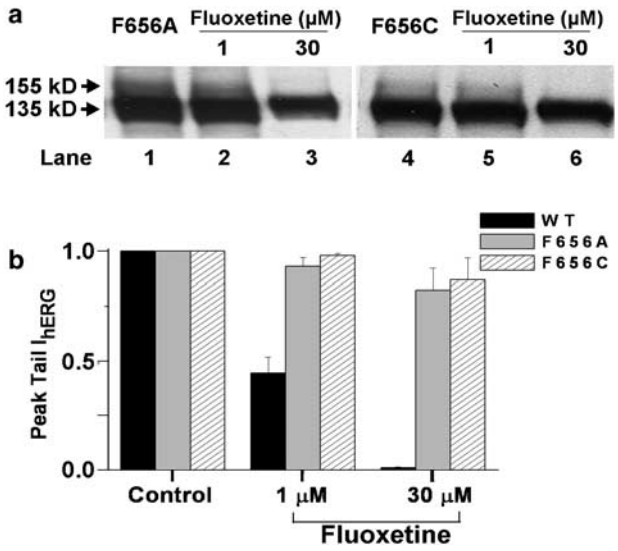

Figure 8 (**a**) Western blot analysis of F656A and F656C mutations for control conditions and following 24 h incubation with 1 and 30 μM fluoxetine. (**b**) Plots of normalized peak tail I_{hERG} (square pulse protocol) for WT, and the F656A and F656C mutations, for control conditions and following 1 or 30 μM fluoxetine exposure.

fluoxetine derived from normalized Western blot image density measurements of the 155 kDa band. When fitted to the Hill equation, the dC_{50} values for disruption of the mature hERG protein band were 2.7 ± 0.03 and 5.1 ± 1.1 μM, with slope factors of 1.2 ± 0.3 and 1.4 ± 0.3, respectively. These values are close to those obtained for direct block of I_{hERG}.

One explanation for the similar drug sensitivities and slope values is that fluoxetine and norfluoxetine bind to a common site that mediates both drug-induced hERG channel block and disruption of its protein trafficking. In the hERG channel, aromatic residues of the S6 domain (Y652 and F656) form part of a drug-binding site for high-affinity channel block (Lees-Miller *et al*., 2000; Mitcheson *et al*., 2000). We used site-directed mutagenesis to substitute the phenylalanine at position 656 with alanine (F656A) or cysteine (F656C), and tested whether these engineered mutations altered fluoxetine-induced drug block and disruption of protein trafficking. Figure 8a shows Western blot analysis of the effect of fluoxetine. Cells were transiently transfected with the F656A and F656C channel mutations. For control conditions, F656A (lane 1) or F656C (lane 4) showed both the 135 and 155 kDa protein bands, similar to hERG WT protein (see Figure 3a, lane 1). Incubation with 1 μM fluoxetine for 24 h (lanes 2 and 5) showed both the 135 and 155 kDa protein bands, whereas, incubation with 30 μM fluoxetine for 24 h resulted in the disappearance of the 155 kDa protein bands (lanes 3 and 6). Similar results were obtained with three additional Western blot analyses. Figure 8b shows plots of normalized peak tail I_{hERG} recorded from cells transiently transfected with hERG WT, F656A and F656C constructs. Cells were depolarized from a holding potential of -80 to 20 mV for 4 s, followed by a repolarizing step to -120 mV for 5.7 s to record tail current with the protocol applied every 15 s. Following control recordings, perfusion was switched to Tyrode solution containing 1 or 30 μM fluoxetine. Steady-state block of peak tail I_{hERG} was recorded and normalized to the control value. With the F656A mutation, 1 or 30 μM fluoxetine reduced peak tail I_{hERG} by only $7.0 \pm 4.2\%$ ($n = 4$ cells) and $17.8 \pm 9.0\%$ ($n = 4$ cells), respectively. With the F656C mutation, 1 or 30 μM fluoxetine reduced peak tail I_{hERG} by $2.0 \pm 1.0\%$ ($n = 4$ cells) and $13.1 \pm 9.0\%$ ($n = 4$ cells), respectively. With hERG WT channels, 1 or 30 μM fluoxetine reduced I_{hERG} by $56.0 \pm 7.0\%$ ($n = 5$ cells) and $99.0 \pm 4.0\%$ ($n = 6$ cells), respectively. These data show that engineered mutations (F656A and F656C) in the hERG drug-binding domain markedly reduced channel sensitivity to block by fluoxetine, but did not alter fluoxetine-mediated disruption of hERG channel protein trafficking.

Discussion

Drug-induced QT interval prolongation occurs with many types of cardiovascular and non-cardiovascular drugs (Roden, 2004). The mechanism proposed for most has been direct block of I_{Kr} by drug binding to specific amino-acid residues in the pore-S6 region of hERG channels (Lees-Miller *et al*., 2000; Mitcheson *et al*., 2000). A second mechanism recently proposed is disruption of post-translational hERG channel protein processing to reduce channel density in the

surface membrane. This indirect mechanism was first reported for the anti-cancer drug arsenic trioxide (Ficker *et al*., 2004), however, direct block of I_{hERG} by arsenic trioxide has also been reported (Drolet *et al*., 2004). The antiprotozoal agent pentamidine also disrupts hERG channel protein trafficking (Cordes *et al*., 2005; Kuryshev *et al*., 2005). These investigators showed that culturing cells in pentamidine selectively reduced expression of hERG (but not KCNQ1, Kv1.5 and Kv4.3) K^+ channels, and caused action potential prolongation in guinea pig ventricular myocytes. Direct block of I_{hERG} occurred only at much higher ($\sim$250-fold) pentamidine concentrations (Cordes *et al*., 2005). Recently, celastrol, a plant extract, was also reported to inhibit the hERG channel biogenesis at concentrations below those that block directly I_{hERG} (Sun *et al*., 2006).

The present report is the first to show that both fluoxetine and norfluoxetine reduce I_{hERG} at similar drug concentrations, and this is caused by both direct channel block and indirectly by disruption on channel protein trafficking. This supports the idea that both mechanisms may contribute to LQTS. Furthermore, block of I_{hERG} and disruption of hERG protein trafficking was reversible, and fluoxetine did not alter I_{Na} and $I_{Endogenous}$ in HEK293 cells. These findings support the conclusion that fluoxetine and norfluoxetine mediated the reduction in I_{hERG} and disruption of hERG channel trafficking is by a non-apoptotic process that appears to be selective for hERG channels, at least with respect to SCN5A and endogenous current channels. We did not study, however, other ion channels or I_{Kr} in native heart cells.

Drug binding to a pore-S6 domain of the hERG channel (Lees-Miller *et al*., 2000; Mitcheson *et al*., 2000) is thought to mediate direct block of I_{hERG}, whereas the drug-binding site for the selective disruption of hERG channel protein trafficking is unknown. We studied whether the F656A and F656C engineered mutations in the drug-binding domain could alter similarly the drug sensitivity of both the direct and indirect mechanisms. These mutations reduced the sensitivity of I_{hERG} to drug block by fluoxetine, but did not alter the sensitivity to drug-induced disruption of hERG channel protein trafficking. We conclude that direct block of I_{hERG} by fluoxetine is mediated by the pore-S6 drug-binding domain, whereas fluoxetine-induced disruption of hERG channel protein trafficking is mediated by drug binding to a different site on the channel or to a different protein in the secretory pathway.

Despite its widespread use, reports linking QT interval prolongation and cardiac arrhythmias to fluoxetine have been uncommon. The therapeutic steady-state serum concentrations of fluoxetine and norfluoxetine found in humans have been reported to be 0.14–1.36 and 0.22–1.86 μM, respectively (Orsulak *et al*., 1988), and both compounds are highly protein bound which should reduce free drug levels. Our concentration–response relations suggest that, under normal conditions, serum drug levels are likely to have minimal effects on I_{hERG} and its protein trafficking. However, several clinical settings could potentiate the effect of fluoxetine and norfluoxetine on hERG channels to prolong action potentials and the QT interval. These include drug overdose, underlying ion channel diseases (e.g., congenital LQTS), concomitant administration of other QT prolonging drugs, structural heart disease, hypokalemia, altered hepatic drug metabolism via P450 2D6 (e.g., infants, cirrhosis, etc) or conditions that lower serum protein concentrations.

In summary, fluoxetine and its metabolite, norfluoxetine, act to selectively reduce I_{hERG} by direct and indirect mechanisms. There are several clinical implications to these findings: First, fluoxetine is a widely used antidepressant drug with Prozac having been the sixth best-selling drug worldwide in 2000 before expiration of its patent (http://www.imshealth.com), and in 1999 it was the fourth most commonly prescribed drug in the United States with a potential for QT interval prolongation (Curtis *et al*., 2003). Thus, disruption of hERG channel protein trafficking may be a more commonly occurring mechanism contributing to acquired LQTS than was previously understood. Second, direct block of I_{hERG} develops rapidly, whereas disruption of protein trafficking develops slowly, thus the development of drug-induced QT interval prolongation *in vivo* could have different time courses based upon its underlying mechanism. Finally, *in vitro* drug safety testing to assess hERG channel block usually employs acute screening protocols, which may be inadequate to detect the indirect mechanism where hERG channel density is reduced slowly over time.

Acknowledgements

This work was supported by National Institutes of Health Grants R01 HL60723 (CTJ), R01 HL71092 (JCM) and T32 HL07936 (LLE), and an American Heart Association Scientist Development Grant (BPD).

Conflict of interest

The authors state no conflict of interest.

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