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Molecular determinants of cocaine block of hERG potassium channels

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Molecular determinants of cocaine block of hERG

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Abbreviations:

hERG: human ether-a-go-go related gene; WT: wild type; LQTS, long QT syndrome; I_{Kr}, cardiac rapidly activating delayed rectifier K^+ current; E-4031: 1-[2-(6-methyl-2-pyridyl)ethyl]-4-(methylsulfonyl-aminobenzoyl) piperidine.

ABSTRACT

The use of cocaine causes cardiac arrhythmias and sudden death. Blockade of the cardiac potassium channel hERG has been implicated as a mechanism for the pro-arrhythmic action of cocaine. hERG (the human ether-a-go-go-related gene) encodes the pore-forming subunits of the rapidly activating delayed rectifier K^+ channel (I_{Kr}) which is important for cardiac repolarization. Blockade of I_{Kr}/hERG represents a common mechanism for drug-induced long QT syndrome. The mechanisms for many common drugs to block the hERG channel are not well understood. We investigated the molecular determinants of hERG channels in cocaine-hERG interactions using site-targeted mutations and patch clamp method. Wild type and mutant hERG channels were heterologously expressed in HEK 293 cells. We found that there was no correlation between inactivation gating and cocaine block of hERG channels. We also found that consistent with Thr-623, Tyr-652 and Phe-656 being critical for drug binding to hERG channels, mutations in these residues significantly reduced cocaine induced block, and the hydrophobicity of the residues at position 656 dictated the channel's cocaine sensitivity. While the S620T mutation, which removed hERG inactivation, reduced cocaine block by 21 fold, the S620C mutation, which also completely removed hERG inactivation, did not affect the blocking potency of cocaine. Thus, Ser-620 is another pore helix residue whose mutation can interfere with cocaine binding independent of its effect on inactivation.

INTRODUCTION

The use of cocaine causes sudden death in otherwise healthy individuals (Kloner *et al.*, 1992). Clinical studies indicate that cocaine abuse is associated with cardiac repolarization abnormalities such as long QT syndromes (LQTS) and Torsades de pointes arrhythmia in humans (Schrem *et al.*, 1990; Perera *et al.*, 1997; Gamouras *et al.*, 2000; Riaz and McCullough, 2003). This type of arrhythmia is typical during reduction of the rapidly activating delayed rectifier K^+ current (I_{Kr}), which is important for cardiac repolarization. The Human-ether-a-go-go-Related Gene (hERG) encodes the pore forming subunit of channels that conduct I_{Kr} (Sanguinetti *et al.*, 1995; Trudeau *et al.*, 1995). Indeed, it has been found that cocaine blocks native I_{Kr} and heterologously expressed hERG channels (Clarkson *et al.*, 1996; O'Leary, 2001; Zhang *et al.*, 2001).

hERG block represents a common mechanism for drug-induced LQTS, which can potentially lead to a severe cardiac arrhythmia, Torsades de pointes, and sudden cardiac death (Keating and Sanguinetti, 2001). Due to the fatal nature of LQTS side effects, a number of commonly prescribed medications have been removed from the market (Keating and Sanguinetti, 2001). The mechanisms for many therapeutically and structurally diverse compounds preferentially blocking hERG channels are not well understood and are of significant interest for drug safety. Site-directed mutagenesis has identified pore helix residues Thr-623, Ser-624 and Val-625 and aromatic residues Tyr-652 and Phe-656 in the S6 transmembrane segment of hERG subunits to be important molecular determinants for high affinity binding to hERG channels by many drugs such as MK-499, dofetilide, cisapride, terfenadine, quinidine and clofilium (Lees-Miller *et al.*, 2000; Mitcheson *et al.*, 2000; Perry *et al.*, 2004). In addition to the structural determinant residues, the inactivation

gating of hERG appears also important for high affinity drug binding (Ficker *et al.*, 1998; Zhang *et al.*, 1999; Ficker *et al.*, 2001). For example, the structurally related EAG channel, which also has the tyrosine and phenylalanine residues equivalent to Tyr-652 and Phe-656, and has the residues equivalent to Thr-623, Ser-624 and Val-625 of hERG but does not inactivate, is not sensitive to hERG blocker dofetilide. Sensitivity to block by dofetilide was introduced when the EAG channel was made capable of inactivating by mutations (Ficker *et al.*, 2001). In addition, mutations which disrupt inactivation reduce the potency of blockade by various compounds (Ficker *et al.*, 1998; Zhang *et al.*, 1999). Recent studies further suggested that it is the positioning of the aromatic residues in the S6 segment, not inactivation *per se*, that dictates the sensitivity of hERG to cisapride (Chen *et al.*, 2002; Lin *et al.*, 2005).

In the present study, we investigated the role of inactivation and the structural determinants of hERG in cocaine sensitivity of the channel. Whole-cell voltage clamp technique was used to record currents from wild type (WT) and mutant hERG channels heterologously expressed in human embryonic kidney (HEK) 293 cells. Our results demonstrate a lack of correlation between hERG inactivation and block by cocaine. We also show that amino acid residues in pore helix and S6 segment of hERG are critical for cocaine block of the channel. Hydrophobicity of the side chain of residue 656 determines cocaine affinity. We further describe that in addition to affecting hERG inactivation gating, the S620T mutation directly interferes with cocaine binding to hERG channels.

MATERIALS AND METHODS

Molecular Biology

The hERG cDNA in pCDNA3 was obtained from Dr. Gail A. Robertson (University of Wisconsin (Trudeau *et al.*, 1995)). We made mutations in the pore and S6 region of hERG to assess the role of inactivation and the binding domain for cocaine block of the channel. Mutations of hERG were introduced via PCR using overlap extension (Ho *et al.*, 1989). The forward and reverse flanking primers were designed to cover two unique restriction sites (*BstE*II at nucleotide 2038 and *Sbf*I at nucleotide 3093). The first round of PCR was performed using the forward flanking primer-reverse mutant primer, and the reverse flanking primer-forward mutant primer, respectively. The resulting two PCR products were used as templates and amplified by flanking primers in a second round of PCR. The final PCR product was cloned into Zero Blunt Vector (Invitrogen, Carlsbad, CA) and was digested with *BstE*II and *Sbf*I (New England Biolabs, Beverly, MA). Fragments with mutations were used to replace the corresponding WT fragment in the hERG expression plasmid. The S620T, S620C, S631A, G628C:S631C, F656V:S620T and F656V:S631A mutations were made to remove hERG inactivation gating (Smith *et al.*, 1996; Ficker *et al.*, 1998; Zhang *et al.*, 1999). The F627Y and S641A were constructed to accelerate inactivation to assess the role of inactivation gating in cocaine block. The T623A, S624A and Y652A were constructed to assess the role of these amino acid residues in cocaine induced block. The F656M, F656T, F656V, F656W and F656Y mutations were made to assess the correlation between the hydrophobicity of the side chain of residues 656 and cocaine block. The S620A, S620F, S620I, S620V and S620Y were constructed but none of them expressed functional channels. To generate double mutations of S620T:F656V and S631A:F656V, we used *Bgl* II (New England Biolabs, Beverly, MA) to obtain a fragment which contained the S620T or S631A mutant. The fragment was used to replace the equivalent fragment

in the F656V mutant channel. All mutations were verified by using a high-throughput 48 capillary ABI 3730 sequencer (UCDNA Services, University of Calgary, Calgary, AB, Canada).

Mutant hERG channels were transiently expressed in HEK 293 cells (American Type Culture Collection, Manassas, VA). HEK 293 cells were seeded at 5×10^5 cells/60-mm diameter dish. The cells were transiently transfected using 10 µl Lipofectamine with 4 µg hERG mutant cDNA in pCDNA3 vector. After 24-48 h, 50-80% of cells expressed channels. To evaluate the role of KCNE1 in cocaine block of hERG, HEK 293 cells were co-transfected with 4 µg hERG mutant cDNA plus 4 µg KCNE1 cDNA in pCDNA3 vector mixed in 10 µl Lipofectamine. The KCNE1 cDNA in pSP64 vector was obtained from Dr. Michael C. Sanguinetti (University of Utah (Sanguinetti *et al.*, 1996)). Non-transfected HEK 293 cells contain a small-amplitude background current that is usually less than 100 pA upon a depolarizing pulse to 50 mV. Thus, the effects of overlapping endogenous currents of HEK 293 cells on the expressed current are minimal (Lin *et al.*, 2005). A HEK 293 cell line stably expressing hERG channels was obtained from Dr. Craig T. January (University of Wisconsin, (Zhou *et al.*, 1998)) was also used. In this cell line, the hERG cDNA (Trudeau *et al.*, 1995) was subcloned into *Bam*HI/*Eco*RI sites of the pCDNA3 vector (Invitrogen, Carlsbad, CA). The stably transfected cells were cultured in Minimum Essential Medium (MEM) supplemented with 10% fetal bovine serum and contained 400 µg/ml G418 to select for transfected cells. For electrophysiological study, the cells were harvested from the culture dish by trypsinization, and stored in standard MEM medium at room temperature for later use. Cells were studied within 8 h of harvest.

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Patch Clamp Recording Method

The whole-cell voltage clamp method was used. The pipette solution contained 135 mM KCl, 5 mM EGTA, 1 mM $MgCl_2$, 10 mM HEPES, and was adjusted to pH 7.2 with KOH. The bath solution contained 135 mM NaCl, 5 mM KCl, 10 mM HEPES, 10 mM glucose, 1 mM $MgCl_2$, 2 mM $CaCl_2$ and was adjusted to pH 7.4 with NaOH. For recordings in the presence of different external K^+ concentrations, the concentration of Na^+ was changed according to K^+ concentration to maintain a constant osmolarity, and the pH was adjusted to 7.4 with NaOH or KOH. E-4031 was purchased from Calbiochem (EMD Biosciences, Inc. San Diego, CA). Cocaine was purchased from Sigma (St. Louis, MO). Cisapride and all other chemicals were from Sigma-Aldrich (Oakville, Ontario). Cocaine was either dissolved in the water to make 10-100 mM stock solutions or added to the bath solutions to make desired concentrations.

Aliquots of cells were allowed to settle on the bottom of a <0.5 mL cell bath mounted on an inverted microscope (TE2000, Nikon, Tokyo, Japan). Cells were superfused with specific bath solutions. The bath solution was constantly flowing through the chamber and the solution was changed by switching the perfusates at the inlet of the chamber. This bath solution change took less than 10 s. Patch electrodes were fabricated using thin-walled borosilicate glass (World Precision Instruments, Sarasota, FL). The pipette had inner diameters of ~1.5 μm and resistances of ~2 MΩ when filled with the pipette solution. An Axopatch 200B amplifier was used to record membrane currents. Computer software (pCLAMP9, Axon Instruments, Foster City, CA) was used to generate voltage clamp protocols, acquire data, and analyze current signals. Data were filtered at 5 to 10 kHz and sampled at 20 to 50 kHz for all protocols. Typically, 80% series resistance (R_s) compensation was used and leak subtraction was not used. Concentration effects were quantified by fitting the data to

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the Hill equation ($I_{drug}/I_{control}=1/[1+(D/IC_{50})^{H}]$, where D is the drug concentration, IC_{50} is the drug concentration for 50% block, and H is the Hill coefficient to the results. Data are given as mean ± S.E.M. Clampfit (Axon Instruments) and Origin (OriginLab Corp., Northampton, MA) were used for data analysis. Curve fitting was done using multiple non-linear least squares regression analysis. All experiments were performed at room temperature ($23 \pm 1^{o}C$).

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RESULTS

External K^+ concentration does not affect cocaine block of hERG channels

Changes in extracellular K^+ concentration (K^+_o) have been shown to influence hERG inactivation gating and the sensitivity of I_{Kr}/hERG to drugs such as dofetilide, E-4031 and cisapride (Yang and Roden, 1996; Wang *et al.*, 1997; Lin *et al.*, 2005). To investigate the effects of K^+_o on cocaine block of hERG, cocaine effects were examined in 0, 5 or 135 mM K^+_o conditions. To record hERG currents the hERG-expressing HEK cells were repetitively depolarized to voltages between -70 and 70 mV for 4 s, followed by a step to -50 mV to record tail currents. The holding potential was -80 mV. We found that the cocaine block of hERG was not K^+_o-dependent. Bath application of 10 μM cocaine blocked ~50% of the currents in 0, 5 or 135 mM K^+_o (data not shown). To obtain concentration-dependent relationships for block of hERG by cocaine, six to eight cells were studied at each drug concentration. The blocking effects were measured by the degree of peak tail current suppressions at each cocaine concentration. The normalized data were plotted against cocaine concentration and fitted to the Hill equation. The half-maximal inhibition concentration (IC_{50}) for cocaine block of hERG current was 11.1 ± 1.4 μM at 0 K^+_o, 8.7 ± 1.6 μM at 5 mM K^+_o, and 14.4 ± 1.9 μM at 135 mM K^+_o. The IC_{50} values are not significantly different from one another (n = 6-8 cells for each data point, $p>0.05$). The corresponding Hill coefficients were 1.2, 1.0 and 0.9, respectively, suggesting that occupation of a single binding site accounts for the block by cocaine.

It has been reported that KCNE1 (minK) may contribute to the formation of native I_{Kr} channels (McDonald *et al.*, 1997). To study whether KCNE1 plays a role in cocaine-induced hERG block, we co-expressed hERG and minK in HEK 293 cells, and analyzed the effects of cocaine. Successful

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expression of KCNE1 in these cells were approved in separate experiments by expressing KCNQ1 (KvLQT1) alone or KCNQ1 plus KCNE1. Expression of KCNQ1 alone generated a fast activating K^+ current. In contrast, co-expression of KCNQ1 + KCNE1 consistently gave rise to a larger, slowly activating current which represents I_{Ks} (Zhang *et al.*, 2001; Zhang, 2005). We found that co-expression of KCNE1 with hERG did not affect cocaine block of the channel. The IC_{50} and Hill coefficient for cocaine block of current due to hERG + KCNE1 co-expression were 6.7 ± 1.0 μM and 1.0 (at 5 mM K^+_o, n = 3). These values are not different from cocaine block of currents due to hERG expression.

Effects of inactivation-modified mutations on cocaine block of hERG channels

hERG inactivation has been shown to play an important role in high affinity drug binding to hERG channels (Ficker *et al.*, 1998; Zhang *et al.*, 1999; Ficker *et al.*, 2001). We assessed the role of inactivation in hERG block by cocaine. Three well documented inactivation-deficient mutant hERG channels, S620T, S631A and G628C:S631C, were constructed. Previous studies have shown that these mutations interfere with hERG C-type inactivation and significantly reduce blocking potency of various drugs (Ficker *et al.*, 1998; Zhang *et al.*, 1999). As shown in Fig. 1, these mutations essentially removed hERG inactivation. Fig. 1A shows families of the mutant currents for control conditions and after exposure to 10 μM cocaine. From a holding potential of -80 mV, depolarizing steps were applied for 4 s to voltages between -70 and +70 mV in 10 mV increments. The depolarizing steps were followed by a repolarization step to -50 mV to record tail currents. In these mutant channels, depolarizing steps induced outward currents that increased in amplitude as depolarizing steps became more positive. The inward rectification seen in WT hERG channels was absent. Application of 10 μM cocaine did not affect the S620T current. However, it blocked the

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S631A and G628C:S631C currents by about half. To quantify cocaine block, the current amplitude at the end of a 4 s depolarizing step to 50 mV under each cocaine concentration was normalized to the control value. Four to nine cells were studied at each concentration and the normalized current amplitudes were plotted against cocaine concentration. The data were fitted to the Hill equation. The IC_{50} for cocaine block of the S620T, S631A or G628C:S631A current was 178.6 ± 55.0 μM (n = 9), 12.4 ± 3.2 μM (n = 7), or 9.2 ± 1.5 μM (n = 4), respectively. The corresponding Hill coefficients were 1.0, 1.0 and 0.9. Thus, among three inactivation-deficient mutants, only the S620T mutation reduced cocaine block (n = 9, $p<0.01$ compared with WT channels). The S631A and G628C:S631C mutations did not affect channel sensitivity to cocaine ($p>0.05$ compared with WT channels).

To further investigate the role of the inactivation in cocaine block we constructed the F627Y and S641A mutations. The S641A mutation has been reported to accelerate the onset of hERG inactivation (Bian *et al.*, 2004). We discovered that the F627Y mutation also significantly accelerates hERG inactivation. Due to the accelerated inactivation, the outward current in 5 mM K^+_o was very small. Therefore, the F627Y and S641A mutant currents were recorded in 135 mM K^+_o. To examine the inactivation time course of these currents, mutant hERG channels were fully activated and inactivated by a depolarizing step to +60 mV for 500 ms. The cell was then repolarized to −100 mV for 10 ms to allow channel recovery from inactivation to open state but not enough for channel deactivation (Smith *et al.*, 1996; Spector *et al.*, 1996; Zhang *et al.*, 2003). A test step was then applied to different voltages to observe inactivation time courses (Fig. 2A). The inactivation time constant (τ_{inact}) was obtained by fitting the current decay to a single exponential function. Fig. 2A shows WT hERG currents during the test steps to voltages between 10 and 60 mV

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in 10 mV increments in 135 mM K^+_o. Fig. 2B shows the F627Y hERG currents under the same conditions. The averaged τ_{inact} for WT, F627Y and S641A currents in five to eight cells are plotted as a function of test potentials in Fig. 2C. Both the F627Y and S641A significantly accelerated the onset of inactivation at all voltages tested. For example, at 20 mV, the τ_{inact} for WT was 17.9 ± 1.2 ms (n = 6, in 135 mM K^+_o), whereas it was 2.3 ± 0.2 ms for F627Y channels (n = 6, $p<0.01$, in 135 mM K^+_o), and it was 2.4 ± 0.1 ms for S641A channels (n = 4, $p<0.01$). In contrast to the accelerated inactivation, the activation properties of both F627Y and S641A mutant channels were only slightly different from WT channels. The voltages for half maximal activation ($V_{1/2}$) and the slope factor (*k*) of WT channels were −10.7 ± 1.1 mV and 8.4 ± 1.0 mV (n = 6). The $V_{1/2}$ and *k* of the F627Y were −18.5 ± 1.1 mV (n = 6, $p<0.05$) and 10.2 ± 0.4 mV ($p>0.05$). The $V_{1/2}$ and *k* of the S641A were −9.7 ± 2.0 mV and 7.9 ± 0.3 mV ($p>0.05$, n = 4). Fig. 2D and E show families of F627Y currents in control (D) and in the presence of 10 μM cocaine (E). Fig. 2F compares the concentration-dependent block of WT, F627Y and S641A currents by cocaine. The IC_{50} and Hill coefficient of cocaine block of F627Y were 7.7 ± 1.4 μM and 0.9, and for the S641A, they were 12.9 ± 0.6 μM and 0.8 (n = 4-7 cells for each data point). Thus, IC_{50} of cocaine block of F627Y or S641A was not different from that on WT channels (IC_{50} = 14.4 ± 1.9 μM, H = 0.9, in 135 mM K^+_o, $p>0.05$). The fact that F627Y and S641A accelerated hERG inactivation but did not affect the potency of cocaine block suggests that inactivation plays no role in cocaine-hERG channel interactions.

Cocaine shares the binding domain with cisapride and E-4031 in hERG channels

Previous studies have suggested that the binding domain of cocaine on hERG channel is on the internal side of the membrane (Zhang *et al.*, 2001). Most hERG channel blockers access the binding sites from the cell interior (Ficker *et al.*, 1998; Zhang *et al.*, 1999; Mitcheson *et al.*, 2000).

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We addressed whether the binding domain for cocaine is the same as for other hERG blockers. Cisapride and E-4031 are well studied hERG blockers which bind to a domain that involves the aromatic residues at positions 652 and 656 (Mitcheson *et al.*, 2000; Lin *et al.*, 2005). We tested whether cocaine could compete with these compounds for binding by taking advantage of the fact that upon washout of cocaine hERG current recovery is extremely fast whereas that upon washout of cisapride or E-4031 is slow. In these experiments, hERG channels were activated from the holding potential of -80 mV by a depolarizing step to 50 mV for 4 seconds, and followed by a repolarizing step to -50 mV to record tail currents. The cells were depolarized repeatedly every 15 seconds. Fig. 3A and B show examples of hERG tail current peak amplitude plotted against time during control, application of and washout of drugs. As shown in Fig. 3A, application of 1 μM cisapride resulted in a complete block of the hERG current. Current recovery upon washout was slow. The current recovered to 11.5±1.8% of its control after 2 minutes of washout (n = 4 cells, Fig. 3C). We then tested for competitive binding between cocaine and cisapride. In Fig. 3B, hERG current was first blocked by 1 μM cisapride for a same period of time as in Fig. 3A. Cisapride was then replaced by 100 μM cocaine for 1 minute prior to washout. In contrast to the washout of cisapride, washout of cocaine which had replaced cisapride, resulted in an almost complete recovery of the hERG current from drug block in 2 minutes (87.5 ± 7.8%, n = 5). Thus, replacement of cisapride by cocaine significantly accelerated hERG current recovery upon washout. We also examined the competitive binding between cocaine and E-4031 (Fig. 3D-F). As shown in Fig. 3D, application of 0.5 μM E-4031 resulted in a complete inhibition of the hERG current. Washout for 2 minutes recovered the current to 12.4 ± 2.3% of its control (n = 5). In Fig 3E, following the complete current inhibition by 0.5 μM E-4031, 100 μM cocaine was used to replace E-4031 for 1 minute and cocaine was then washed out. Replacement of E-4031 by cocaine and then washout of drug resulted in a much faster current recovery than that from washout of E-4031 (55.7

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± 8.8%, n = 4, p<0.01). These results suggest that cocaine may compete with cisapride and E-4031 for a common receptor site.

Pore helix residues and aromatic residues of S6 are involved in cocaine binding to hERG channels

Residues in pore helix and S6 of each hERG subunit are involved in high affinity binding of various compounds to hERG channels (Lees-Miller *et al.*, 2000; Mitcheson *et al.*, 2000; Perry *et al.*, 2004). To determine whether these residues are also important for cocaine block of hERG, T623A, S624A and Y652A mutations were constructed and the effects of cocaine were examined (Fig. 4). While S624A and Y652A displayed a gating behavior similar to WT channels, T623A mutation significantly accelerated inactivation. Since we have showed that K^+_o has no effect on cocaine block of hERG channels, the effects of cocaine on T623A were examined in 135 mM K^+_o whereas the effects of cocaine on S624A and Y652A were assessed in 5 mM K^+_o (Fig. 4A). We found that these mutations significantly reduced the channel affinity to cocaine (Fig. 4B). The IC_{50} for cocaine block of T623A, S624A and Y652A were 166.8 ± 19 μM, 45.5 ± 4.4 μM and 309.6 ± 49 μM. The corresponding Hill coefficients were 1.0, 1.0 and 1.1, respectively (n = 4-6 cells for each data point). These IC_{50} values are all significantly larger than those in WT channels (8.7 ± 1.6 μM in 5 mM K^+_o, and 14.4 ± 1.9 μM in 135 mM K^+_o, p<0.01). Again, the reduced cocaine sensitivity was not associated with hERG inactivation gating since inactivation was accelerated in T623A, slowed in S624A, and remained the same in Y652A. Specifically, at 20 mV, the τ_{inact} was 1.4 ± 0.2 ms for T623A (n = 7, p<0.01, compared to 17.9 ± 1.2 ms of WT channels in 135 mM K^+_o). The τ_{inact} was 18.2 ± 3.5 for S624A (n = 4, P<0.01) and 9.4 ± 0.9 ms for Y652A (n = 4, P>0.05 compared to 12.9 ± 1.1 ms for WT channels in 5 mM K^+_o n = 6). The reduced cocaine affinity of S623A, S624A and

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Y652A were also not associated with the altered channel activation gating. For example, the $V_{1/2}$ and *k* were 3.4 ± 0.5 mV and 10.0 ± 0.4 mV for T623A (n = 7), –8.6 ± 2.9 mV and 8.0 ± 0.5 mV for S624A (n = 4), 4.1 ± 1.8 mV and 9.3 ± 0.6 mV for Y652A (n = 4). Thus, $V_{1/2}$ of T623A and Y652A were more positive ($p<0.01$), and $V_{1/2}$ of S624A was similar to that of WT channels which has a $V_{1/2}$ of −10.7 ± 1.1 mV and a *k* of 8.4 ± 1.0 mV (n = 6).

Phe-656 has been shown to be critical for drug binding to hERG channels (Mitcheson *et al.*, 2000). It is shown that the hydrophobicity of the side chain at this position dictates the potency for block of hERG channels by MK-499, cisapride and terfenadine (Fernandez *et al.*, 2004). To test whether Phe-656 is also involved in cocaine binding, we constructed five mutant channels F656M, F656T, F656V, F656W and F656Y. The biophysical and pharmacological consequences of these mutations were determined. All of these mutations retained relatively normal biophysical properties. Fig. 5A a and b show families of the mutant currents in the absence and presence of 10, 50 or 200 μM cocaine. Currents were elicited by 4-s pulses to various voltages between -70 mV and 70 mV from a holding potential of –80 mV. The depolarizing steps were followed by a 5-s repolarizing pulse to record tail currents. To construct the current-voltage (I-V) relationships currents at the end of 4-s depolarizing pulses were measured and normalized to the peak value in control recordings (Fig. 5Ac). These I-V relationships were bell-shaped and peaked at voltages between –30 mV and 20 mV. It is known that the rectification (bell-shape) of the I-V relationship of WT hERG channels results from rapid inactivation. These results indicate that these mutant channels retain a relatively normal voltage dependence of inactivation. The voltage dependence of activation of these channels was examined by determining the potentials for half-maximal activation ($V_{1/2}$) and slopes (*k*) of each mutant channel. The tail current amplitude at -50 mV were plotted against the depolarizing

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voltages and fitted to the Boltzmann function (Table 1). The $V_{1/2}$ and *k* of WT channels were –10.7 ± 1.1 mV and 8.4 ± 1.0 mV (n = 6). While the F656W mutation caused a significant shift of $V_{1/2}$ to the negative voltages, other F656 mutations only had minor effects on $V_{1/2}$, and none of the mutations significantly changed the slope factor *k* (Table 1). The time constants for the onset of channel activation were analyzed at -10 mV and the time constants for deactivation were analyzed at -50 mV (Table 1). While the deactivation was significantly accelerated by some mutations such as the F656V, there was no significant change in the onset of activation in most mutations except for F656W. The accelerated activation of F656W was probably due to the negatively shifted activation curve. The K^+ selectivity was analyzed by measuring reversal potential of the current in each F656 mutation. The reversal potential was not altered by any of these mutations (Table 1).

Consistent with the Phe-656 being a key residue for cocaine binding, the cocaine sensitivity was altered in Phe-656 mutant channels. As can be seen in Fig. 5Ab, whereas 10 μM cocaine reduced the F656W and F656Y currents by half, 50 or 200 μM cocaine were required to achieve a similar effect on the F656M, F656V or F656T currents. The summarized I-V relationships of each mutant channel for control (○) and in the presence of cocaine (●) are shown in Fig. 5Ac. Concentration-dependent relationships for cocaine block of these channels were determined by the tail current inhibition and data are summarized in Fig. 5B. The IC_{50} and Hill coefficient were 8.4 ± 0.8 μM and 1.0 for the F656W (n = 4); 12.2 ± 1.6 μM and 1.1 for the F656Y (n = 5); 31.4 ± 3.6 μM and 0.8 for the F656M (n = 7); 88.9 ± 11.8 μM and 0.9 for the F656V (n = 5); 161.8 ± 24.2 μM and 0.8 for the F656T (n = 7). To compare the effect of each mutation on cocaine sensitivity of the channel, the IC_{50} for each mutation was compared with that of WT in Fig. 5C. While F656W and F656Y did not affect and F656M only slightly decreased cocaine sensitivity, F656V and F656T significantly

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decreased cocaine sensitivity of the channel. It has been shown that the magnitude of two-dimensional van der Waals hydrophobic surface area of these mutant side chains follows the order of W ≈ F ≈ Y ≈ M > V > T. A close correlation between the van der Waals hydrophobic surface area for the side chain of residue 656 and channel block by cisapride, terfenadine, and MK-499 has been described (Fernandez *et al.*, 2004). Our results indicate that the hydrophobicity of the side chain of residue 656 also determines the channel sensitivity to cocaine. Thus, the Phe-656 is a critical site for cocaine binding to hERG channels.

Ser 620 is involved in cocaine binding to hERG channels

We have shown that among the inactivation-deficient mutant channels S631A, G628C:S631C and S620T, only the S620T significantly decreased cocaine sensitivity (Fig. 1). S620 is located on the pore helix and contributes to the lining of the inner pore region (Ficker *et al.*, 1998; Mitcheson *et al.*, 2000). In addition to Y652 and F656 in S6, residues in pore helix have also been shown to contribute to the formation of the drug binding domain (Mitcheson *et al.*, 2000) and we have shown that T623A mutation, which faces the central cavity of the channels and is located next to S620 by one turn of helix, disrupts cocaine binding to hERG channels. Thus we hypothesized that S620T mutation, in addition to its well known effect of removal of hERG inactivation, may directly disrupt cocaine binding to the channel. To examine this possibility, the inactivation-deficient mutations were introduced to hERG F656V channels. Both hERG F656V:S620T and F656V:S631A channels displayed inactivation deficiency. Depolarizations to 50 mV for 4 s induced a sustained outward current with no sign of inactivation (data not shown). The cocaine block was measured at the end of 4 s depolarization and normalized to the control values. For each drug concentration, five to eleven cells were studied. Whereas removal of C-type inactivation from F656V by S631A had little

effect on the channel sensitivity to cocaine, introduction of S620T mutation to the F656V significantly reduced cocaine sensitivity. The IC_{50} for cocaine block of F656V:S631A was 56.1 ± 7 μM with a Hill coefficient of 0.7 (n = 4; $p>0.05$ compared with 88.9 ± 11.8 μM for F656V). The IC_{50} for cocaine block of F656V:S620T was 1547.1 ± 266.2 μM with a Hill coefficient of 1.0 (n = 4, $p<0.01$ compared with 88.9 ± 11.8 μM for F656V). These results suggest that inactivation gating plays little role in cocaine binding to HERG channels. The S620T induced reduction of cocaine block may be through interfering with cocaine binding.

To assess mutations of Ser-620 in interfering cocaine binding to hERG channels, additional mutations were constructed. The S620A was made to decrease the size of the side chain. The S620V and S620I were made because either valine or isoleucine is present in most other voltage-gated K^+ channels at the equivalent position. The S620F and S620Y were made because aromatic residues of phenylalanine and tyrosine on S6 are important for high affinity drug binding. Unfortunately, none of these mutants expressed functional channels. However, the S620C did express robust currents. Importantly, the S620C completely removed hERG inactivation but retained cocaine sensitivity similar to WT channels. As can be seen in Fig. 6A, S620C currents showed no sign of inactivation. In contrast to the S620T mutation that considerably decreased cocaine block (Fig. 1A), the S620C channel did not affect the channel sensitivity to cocaine very similar to WT channels (Fig. 6B & C). The IC_{50} for cocaine block of the S620C channel was 7.7 ± 1.7 μM (n = 5, Hill coefficient of 0.7 ± 0.1), which is very close to the IC_{50} of 8.7 ± 1.6 μM for cocaine block of WT channels ($p>0.05$). Since S620C completely removed inactivation without affecting cocaine block, it indicates that cocaine block of hERG is not dependent on inactivation, and the S620T may directly interfere with cocaine binding independent of inactivation gating.

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To further assess the role of the S620T mutation in reducing drug sensitivity of hERG, the effects of E-4031, a typical hERG channel blocker, on inactivation-deficient mutant channels, S631A, G628C:S631C, S620C and S620T, were studied (Fig. 7). As can be seen in Fig. 7, while all of these mutant channels displayed reduced E-4031 sensitivity, the S620T produced a significantly larger effect than other mutants. The IC_{50} and Hill coefficient for E-4031 block of WT hERG channels were 39.4 ± 5.5 nM and 1.0 (n = 9). The IC_{50} and Hill coefficient were 152.8 ± 25.5 nM and 0.9 for S631A (n = 5), 180.7 ± 32.0 nM and 0.8 for G628C:S631C (n = 6), 233.6 ± 27.2 nM and 0.8 for S620C (n = 4), and 3054.3 ± 238.5 nM and 1.0 for S620T (n = 4). The IC_{50} values of E-4031 were not statistically significant among channels S631A, G628C:S631C and S620C ($p>0.05$), but they were significantly larger than that for WT channels. Importantly, the IC_{50} for E-4031 block of S620T was significantly larger than those for E-4031 block of other inactivation-deficient channels, S631A, G628C:S631C and S620C ($p<0.01$).

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DISCUSSION

The present study demonstrated inactivation gating and cocaine block of hERG channels are not coupled. First, changing extracellular K^+ concentration, which is known to regulate hERG inactivation (Zhang *et al.*, 2003), did not affect cocaine affinity. Second, accelerating hERG inactivation by the F627Y and S641A mutations did not change the IC_{50} for cocaine block. Third, disrupting hERG inactivation by mutations, S631A, G628C:S631A, and S620C did not affect cocaine block of the channel. Inactivation gating has been suggested to play an obligatory role in high affinity drug binding to hERG channels (Ficker *et al.*, 1998; Ficker *et al.*, 2001). Removal of hERG inactivation has been shown to reduce hERG sensitivity to many drugs such as dofetilide, verapamil, E-4031, haloparidol and clofilium (Wang *et al.*, 1997; Ficker *et al.*, 1998; Zhang *et al.*, 1999; Suessbrich *et al.*, 1997). Our recent data and that presented by Chen et al have suggested that inactivation per se is not a determinant for cisapride block of hERG channels (Chen *et al.*, 2002; Lin *et al.*, 2005). Instead, inactivation induced a conformation that favors drug binding, and Phe-656 is involved in the inactivation-facilitated drug block (Lin *et al.*, 2005). In the present study, we showed that removal of hERG inactivation by each of the mutations S631A, G628C:S631C and S620C increased IC_{50} of E-4031 by 4-6 folds. In contrast, the channel inactivation did not affect cocaine block of hERG channels. The reason for this discrepancy is not known but may be related to the chemical structure of cocaine.

Based on a pharmacophore model derived from a comparative molecular field analysis (CoMFA) (Cavalli *et al.*, 2002), a high affinity hERG blocking compound is generally a flexible molecule consisting of three aromatic moieties connected through a nitrogen function that is a tertiary amine

throughout the whole set of molecules. The tertiary amine renders the compounds protonated at physiological pH which may be important in conferring biological activity. In addition, polar groups attached to the phenyl ring at one end of the drug molecule can favor the activity. Cocaine is a methyl-benzoyl-ecgonine. Its nitrogenated base can be considered as a tertiary amine (pK_a ~8.6; (Crumb, Jr. and Clarkson, 1990)) which is incorporated into a rigid ring system. While polar groups do exist, there is only one aromatic ring in the cocaine structure. Therefore, cocaine does not possess chemical features for high affinity hERG inhibition. Indeed, while many hERG blockers inhibit hERG current with IC_{50} in nanomolar range, cocaine blocks hERG with an IC_{50} of 8.7 μM.

Despite of the uncoupling between cocaine block and hERG inactivation, we found that cocaine competes with cisapride and E-4031 for binding. Consistently, the residues important for hERG blockers such as methanesulfonanilides were also involved in cocaine binding. It has been shown that the drug binding domain of hERG is comprised of amino acids located on the S6 transmembrane segment (Y652 and F656) and pore helix (T623 and S624) of the channel subunit that face the cavity of the channel (Mitcheson *et al.*, 2000; Perry *et al.*, 2004). We found that mutations at these positions reduced cocaine block of the channel. For Phe-656, we found that the F656W, F656Y did not change and the F656M only slightly reduced cocaine sensitivity. The F656V mutation reduced cocaine block by 10 fold, and the F656T mutation reduced hERG block by 19 fold. The different cocaine affinity among these mutations at 656 could be due to the fact that the van der Waals hydrophobic surface areas of tryptophan, tyrosine and methionine closely resemble that of phenylalanine, while valine is less hydrophobic and threonine is a hydrophilic molecule. Thus, our results suggest that measures of hydrophobicity at 656 dictate cocaine sensitivity, similar to that described for other drugs (Fernandez *et al.*, 2004).

Based on our data and a three-dimensional quantitative structure-activity relationship model (Cavalli *et al.*, 2002), we propose that the protonated nitrogen and polar groups of cocaine are oriented toward the selectivity filter end of the central cavity, and the aromatic ring of the drugs are pointed toward the intracellular opening of the inner helices. The protonated nitrogen of cocaine may interact with Y652 by cation-π interactions, the polar groups of cocaine may interact with the T623 and S624 via polar interactions, and the aromatic ring of cocaine may interact with F656 by hydrophobic interactions. However, because cocaine is rigid and does not possess typical high affinity structure for hERG block, its interaction with F656 may be weak. For example, while the F656T mutation reduced the potency of MK-499 and cisapride for hERG block by 209 and 145 folds, the same mutation F656T only reduced cocaine block by 19 folds. We have previously shown that the inactivation associated conformational change of F656 is involved in the inactivation-facilitated cisapride block (Lin *et al.*, 2005). Therefore, it seems possible that the binding stereometry of rigid cocaine molecule to hERG channel does not allow inactivation associated conformational change to increase cocaine affinity.

The relatively weak interaction between cocaine and hERG may also explain the very rapid blocking and unblocking kinetics (Zhang *et al.*, 2001). For many drugs the development of hERG channel block is slow (Snyders and Chaudhary, 1996; Zhang *et al.*, 1999). Recovery from block by drugs such as methanesulfonanilides (e.g., E-4031, dofetilide, MK-499) is extremely slow and is thought to be due to trapping of the charged drug moiety within the inner vestibule of the channel (Mitcheson *et al.*, 2000b). If unblocking of cocaine is faster than channel closing, such kinetics would minimize trapping of cocaine.

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While we showed that cocaine block of hERG is independent of hERG inactivation, our results showed that the S620T mutation, which removes inactivation, reduced cocaine block by 21 folds. We also found that the 620T produced a much stronger effect on reduction of E-4031 block than other inactivation-deficient mutant channels. Notably, although both S620C and S620T did not inactivate, S620T reduced E-4031 block 13-fold more than S620C. The effect of S620C mutation on reduction of E-4031 potency was similar to those of S631A and G628C:S631C inactivation-deficient mutants. These results indicate that in addition to via inactivation, S620T also reduces drug block by direct interference with drug binding. This deliberation is consistent with previous studies in EAG channels. EAG channels are not sensitive to hERG blockers. In bEAG, the residues equivalent to hERG S620 and S631 are T432 and A443. Thus, in respect to the serine residues, the WT bEAG is equivalent to the hERG S620T:S631A double mutation. Ficker et al has shown that introducing Ser at 432 (T432S) in bEAG (equivalent to position 620 in hERG) increased dofetilide sensitivity by 4 folds. Although the 4-fold increase of dofetilide block induced by the T432S was not dramatic, the fact that this mutation increased drug sensitivity but did not change the channel gating suggests that Ser at 432 (S620 in hERG) was involved in the dofetilide binding to EAG channels independent of inactivation gating (Ficker *et al.*, 2001).

Since S620 is located in the pore helix, just one turn of helix next to T623. It is possible that S620T disrupted drug binding by changing the conformation of the binding domain. The fact that S620T reduced cocaine sensitivity whereas S620C did not suggests the size of the side chain at this position is critical for the proper shape/size for drug-hERG interaction.

The concentrations for cocaine to block hERG channels are within the range achieved in humans. For example, in cocaine-associated sudden death, average post mortem plasma cocaine concentrations of 20 μM have been reported (Mittleman and Wetli, 1984). It has been assumed that KCNE2 (MiRP1) and KCNE1 (minK) contribute to the formation of native I_{Kr} channels. However, we found that co-expression of KCNE1 with hERG did not affect cocaine block of the channel. As for KCNE2, it has been reported that the pharmacological sensitivity of hERG currents in CHO cells was indistinguishable from that of I_{Kr} and was unaffected by KCNE2 (MiRP1) co-expression (Kamiya *et al.*, 2001; Weerapura *et al.*, 2002). Since the values of IC_{50} for cocaine to block I_{Kr} and hERG are very close (Clarkson *et al.*, 1996; O'Leary, 2001; Zhang *et al.*, 2001), it seems that both KCNE1 and KCNE2 do not play a significant role in cocaine-induced block of hERG currents.

In summary, we have identified critical amino acid residues of hERG that contribute to cocaine binding and demonstrated dissociation between inactivation gating and cocaine block of hERG channels. The finding that mutations in S620 residue affect drug binding prompts caution using the S620T mutation to evaluate the role of inactivation in drug block of hERG channels.

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Footnotes

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LEGENDS FOR FIGURES

Fig. 1. Effects of inactivation-deficient mutations on the cocaine block of hERG channels. A: The S620T, S631A and G628C:S631C hERG currents in control conditions and after exposure to 10 μM cocaine. The intracellular solution contained 135 mM K^+ and the bath solution contained 5 mM K^+. **B**: Concentration dependence of cocaine block of S620T, S631A and G628C:S631C channels. Each data point represents the average from 4-7 cells. Cocaine block of WT channels was also shown for comparison.

Fig. 2. F627Y and S641A accelerated inactivation but did not affect the cocaine block of hERG channels. Whole-cell hERG currents were recorded in symmetric K^+ concentrations (135 mM). **A:** The voltage protocol and WT hERG inactivation time courses. **B:** The F627Y inactivation time courses. Dotted lines in A and B indicate the zero current level. **C:** The inactivation time constant (τ_{inact})-voltage relationships in WT (•), F627Y (Δ) and S641A hERG channels (■). **D & E:** The voltage protocol and families of F627Y currents recorded in the absence (D) and presence of 10 μM cocaine (E). **F:** Concentration-dependent block of WT (•), F627Y (Δ) and S641A hERG channels (■). The block was calculated based on the degree of the tail current suppression at -50 mV relative to the control value (n = 4-7, $p>0.05$).

Fig. 3. Competitive binding of cocaine with cisapride and E-4031. A: Amplitudes of hERG tail currents during cisapride (1 μM) block and upon washout. **B:** Amplitudes of hERG tail currents during cisapride block followed by cocaine (100 μM) application and upon washout. **C:** Recovery of the hERG current in 2 minutes of washout after 1 μM cisapride or after 1 μM cisapride followed by 100 μM cocaine. **D:** Amplitudes of hERG tail currents during E-4031 (0.5 μM) block and upon

washout. **E:** Amplitudes of hERG tail currents during E-4031 block followed by cocaine (100 μM) application and upon washout. **F:** Recovery of the hERG current in 2 minutes of washout after 0.5 μM E-4031 or after 0.5 μM E-4031 followed by 100 μM cocaine. While the current recovery after 1 μM cisapride or 0.5 μM E-4031 blockade was slow and incomplete, additional exposure to cocaine (100 μM) resulted in a significantly faster recovery of hERG currents upon drug washout (** $P<0.01$ compared with the current recovery upon washout from cisapride or E-4031).

Fig. 4. The T623A, S624A and Y652A mutation reduced cocaine sensitivity of hERG channels. **A**: Families of T623A (135 mM K^+_i and 135 mM K^+_o), S624A and Y652A hERG K^+ currents (135 mM K^+_i and 5 mM K^+_o) for control and in the presence of 50 or 200 μM cocaine. **B**: Concentration-dependent block of the hERG mutant currents by cocaine. The data for WT channel (135 mM K^+_i and 5 mM K^+_o) were also shown for comparison.

Fig. 5. The effects of F656 mutations on cocaine block of hERG channels. Aa&b: Families of the mutant hERG K^+ currents (135 mM K^+_i and 5 mM K^+_o) for control and in the presence of 10, 50 or 200 μM cocaine. **Ac**: I-V relationships for control and in the presence of 10, 50 or 200 μM cocaine for each mutant channel. **B**: Concentration-dependent block of mutant hERG currents by cocaine. The data for WT channels were also shown for comparison. **C**: IC_{50} of cocaine for each of the WT and mutant hERG channels (** $p<0.01$ compared to that of WT channels).

Fig. 6. Dissociation between inactivation gating and cocaine block by S620 mutations. A&B: Families of the S620C mutant channel in the absence (A) and presence of 10 μM cocaine (B). **C:**

Concentration-dependent block of S620C by cocaine (●) is shown in comparison with those of S620T (□) and WT channels (○).

Fig. 7. Effects of inactivation-deficient mutations on E-4031 block of hERG channels. Concentration-dependence of E-4031 block of S631A, G628C:S631C, S620C and S620T are shown along with data of WT channels for comparison.

Table 1. Activation properties of mutant hERG channels. Currents were measured in 135 mM K^+_i / 5 mM K^+_o. $V_{1/2}$, voltage of half-maximal activation; *k*, slope of the activation curve; τ_{act}, activation time constant measured at -10mV; τ_{fast} and τ_{slow}, fast and slow deactivation time constants measured at -50 mV; A_{relf}, relative amplitude of the fast component of deactivation measured at -50 mV; E_{rev}, reversal potential. *, p <0.05; **, p<0.01.

hERG channel	Activation			Deactivation			E_{rev}	n
	$V_{1/2}$ (mV)	*k* (mV)	τ_{act} (ms)	τ_{fast} (ms)	τ_{slow} (ms)	A_{relf}		
WT	-10.7±1.1	8.4±1.0	1898±260	386±38	2002±172	0.37±0.03	-77.8±0.4	6
F656M	-11.0±1.2	7.2±0.4	2058.3±203.3	438.4±42.6	2214.0±179.5	0.42±0.03	-76.5±2.5	7
F656T	-16.8±3.39*	6.6±0.2	1646.5±369	432.2±16	2434.6±57.7	0.34±0.01	-82±0.2	5
F656V	-12.7±1.96	6.0±0.5	1552.1±377	177.3±5.5**	767.6±70.4**	0.69±1.40**	-79.7±1.4	4
F656Y	-18.15±2.7*	6.1±0.6	1971.3±654.5	228.3±32.6*	1172.0±268.1*	0.55±0.06*	-77.8±0.67	5
F656W	-42.35±3.0**	8.2±0.5	375.9±89.4**	597.7±50*	2351.5±322.3	0.54±0.07*	-80.7±1.1	6

Figure 1

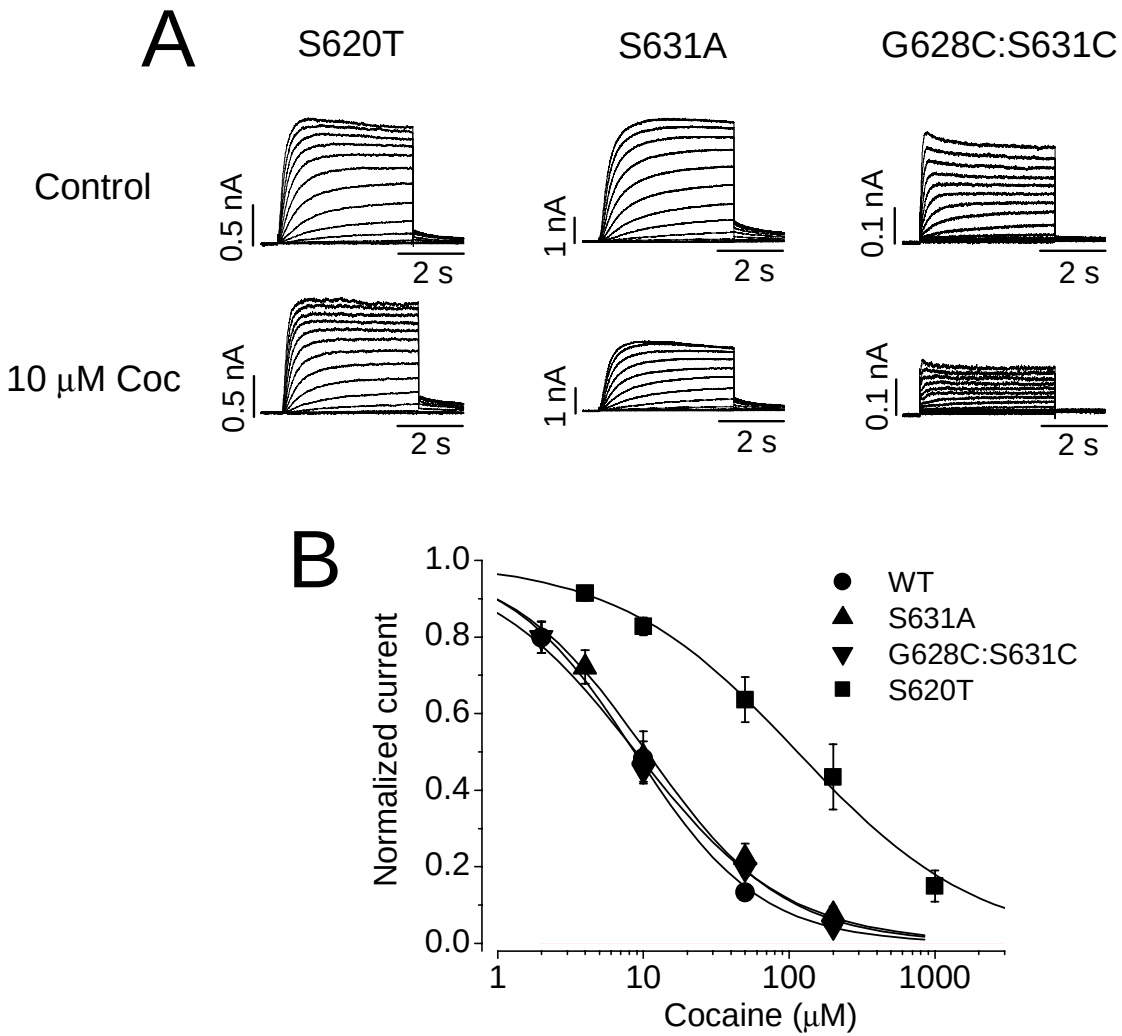

Figure 2

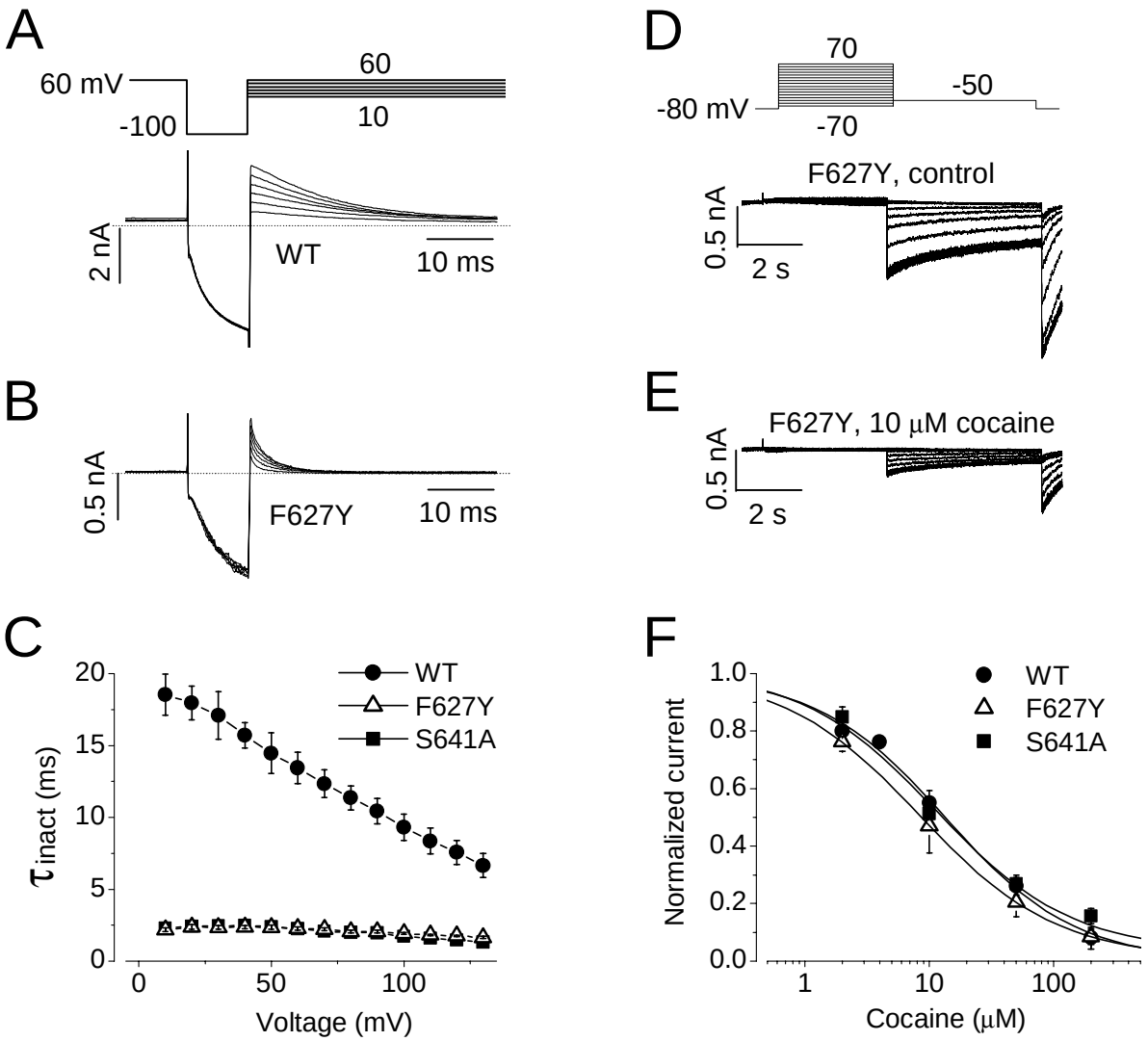

Figure 3

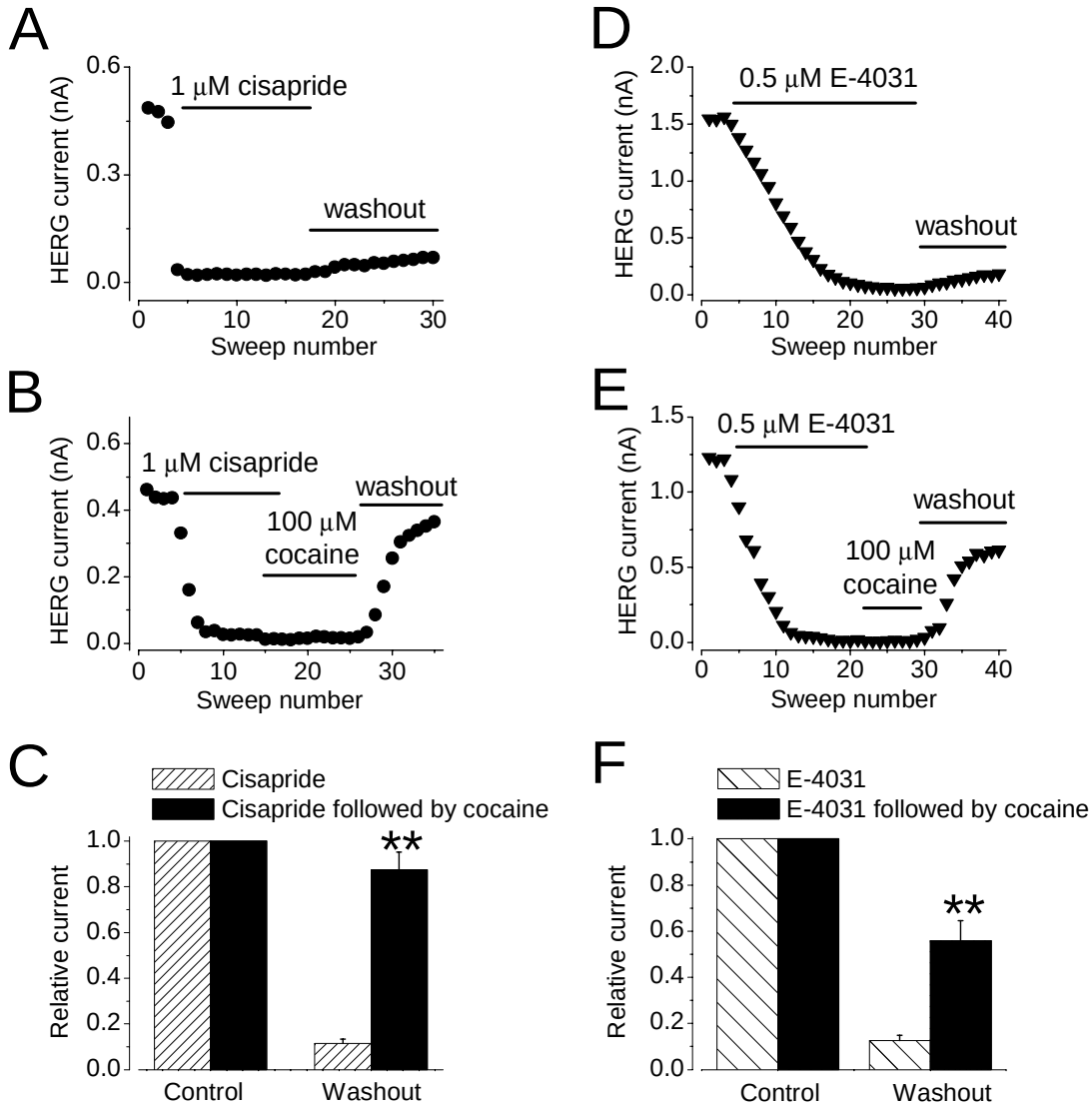

Figure 4

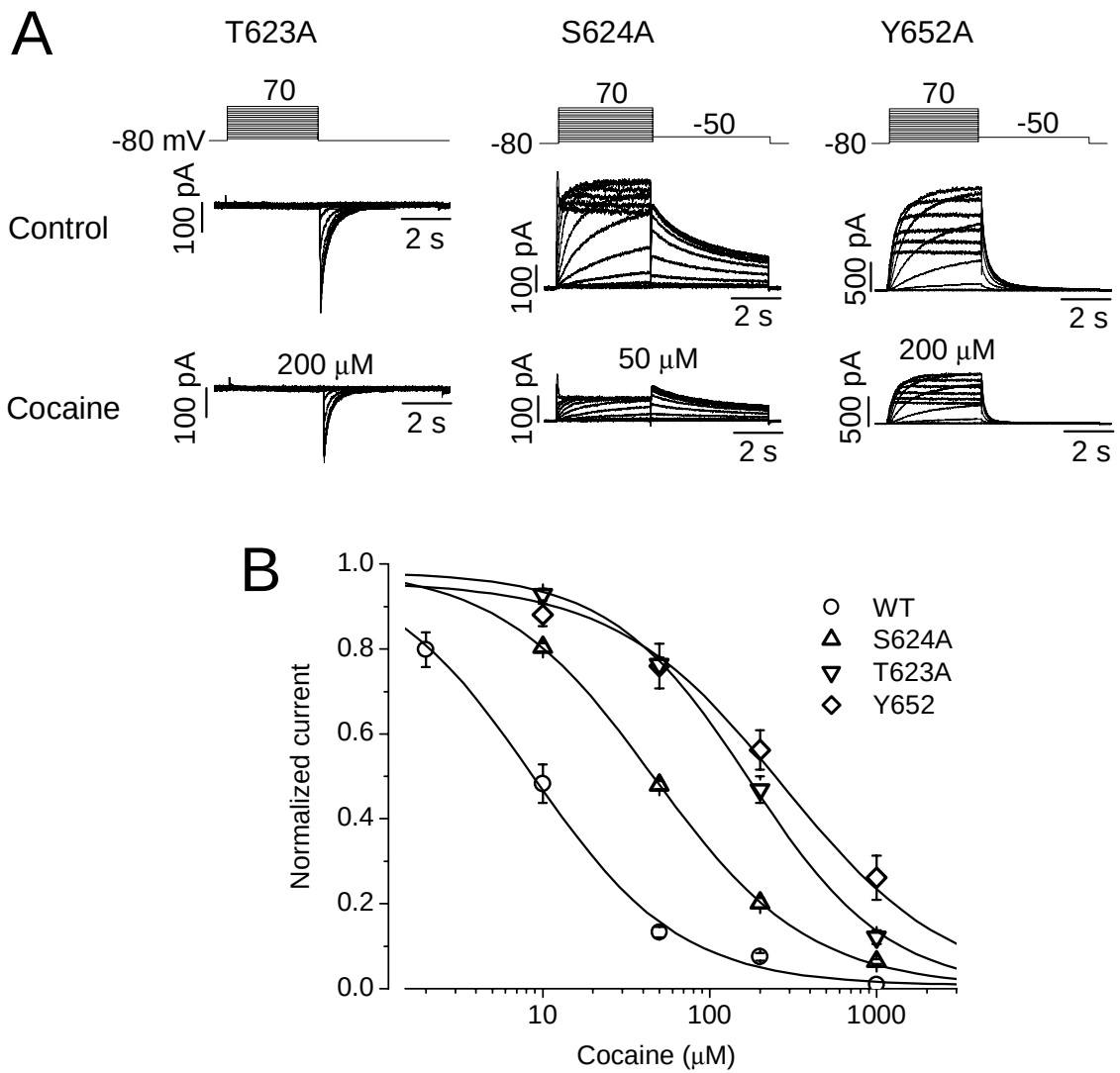

Figure 5

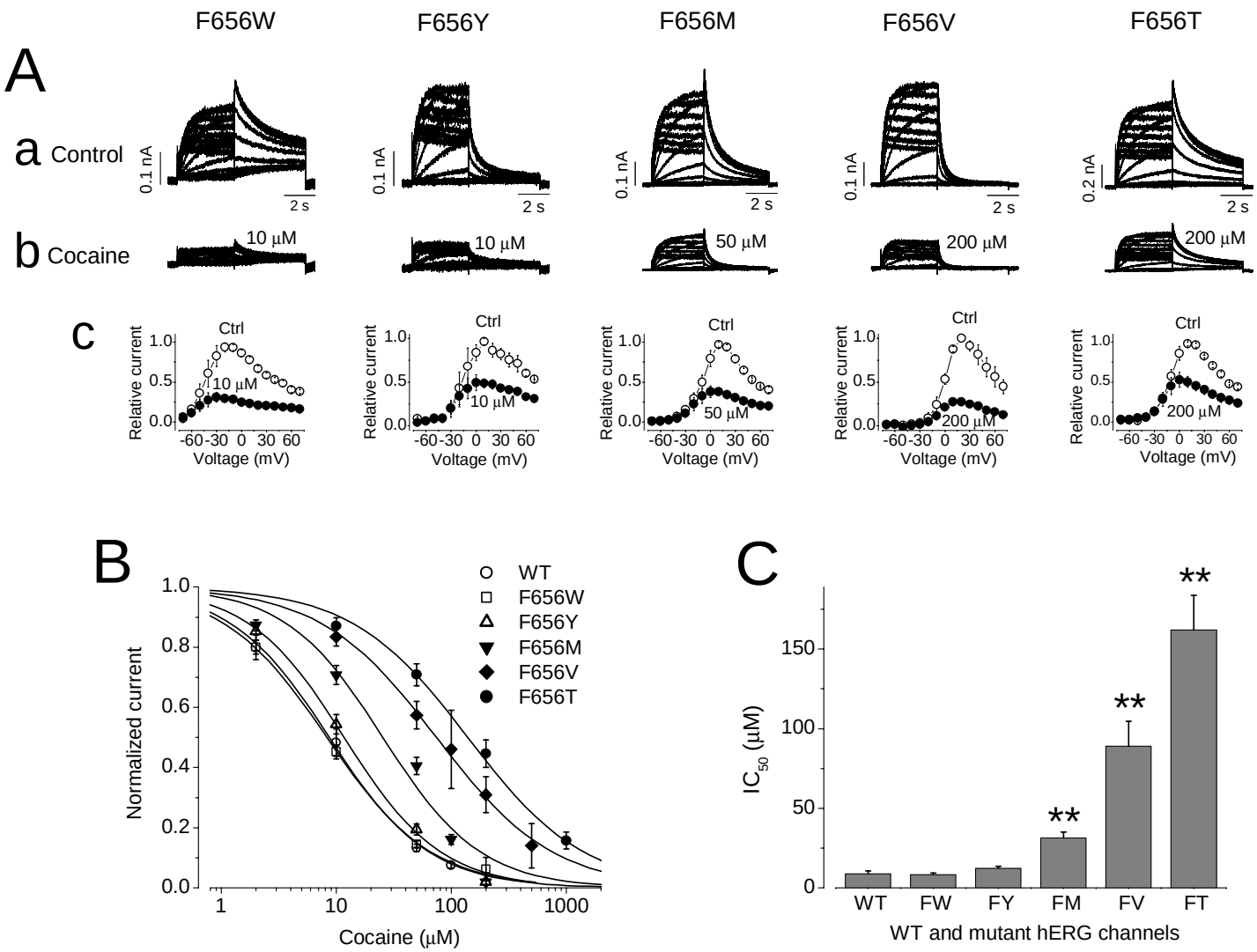

Figure 6

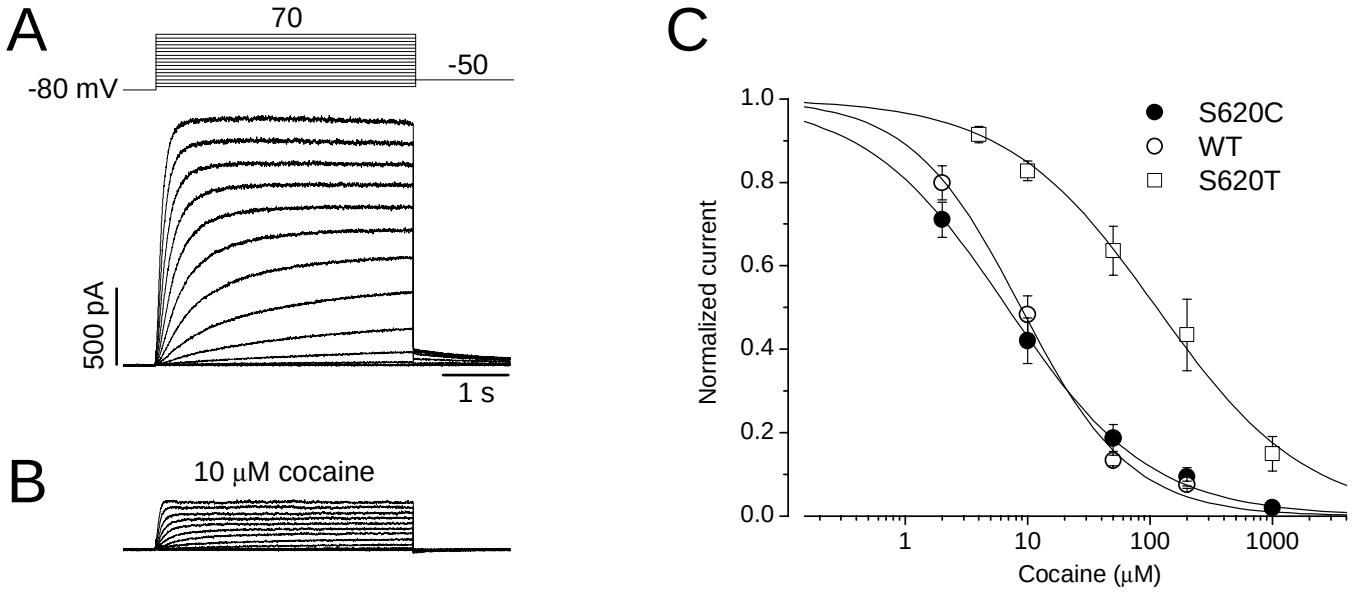

Figure 7