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2-[2-(3,4-Dichloro-phenyl)-2,3-dihydro-1*H*-isoindol-5-ylamino]-nicotinic Acid (PD-307243) Causes Instantaneous Current through Human *Ether-a-go-go*-Related Gene Potassium Channels[S]

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ABSTRACT

Long and short QT syndromes associated with loss and gain of human *ether-a-go-go*-related gene (hERG) channel activity, respectively, can cause life-threatening arrhythmias. As such, modulation of hERG channel activity is an important consideration in the development of all new therapeutic agents. In the present study, we investigated the mechanisms of action of 2-[2-(3,4-dichloro-phenyl)-2,3-dihydro-1*H*-isoindol-5-ylamino]-nicotinic acid (PD-307243), a known hERG channel activator, on hERG channels stably expressed in Chinese hamster ovary (CHO) cells using the patch-clamp technique. In the whole-cell recordings, the extracellular application of PD-307243 concentration-dependently increased the hERG current and markedly slowed hERG channel deactivation and inactivation. PD-307243 had no effect on the selectivity filter of hERG channels. The activity of PD-307243 was use-dependent. PD-307243 (3 and 10 μM) induced instantaneous hERG current with little decay at membrane potentials from −120 to −40 mV. At more positive voltages, PD-307243 induced an I_{to}-like upstroke of hERG current. The actions of PD-307243 on the rapid component of delayed rectifier K^+ current (I_{Kr}) in rabbit ventricular myocytes were similar to those observed in hERG channel-transfected CHO cells. Inside-out patch experiments revealed that PD-307243 increased hERG tail currents by 2.1 ± 0.6 (n = 7) and 3.4 ± 0.3-fold (n = 4) at 3 and 10 μM, respectively, by slowing the channel deactivation but had no effect on channel activation. During a voltage-clamp protocol using a prerecorded cardiac action potential, 3 μM PD-307243 increased the total potassium ions passed through hERG channels by 8.8 ± 1.0-fold (n = 5). Docking studies suggest that PD-307243 interacts with residues in the S5-P region of the channel.

Ion channels may represent chemically tractable and biologically attractive targets, but they are just as likely to present off-target challenges that portend serious safety liabilities for new therapeutic agents. Thus, an understanding of ion channel selectivity and mechanism of action is an important goal in the optimization of drug efficacy and safety. Particularly troublesome are activities at the hERG channel that normally pass the rapid component of the delayed rectifier K^+ current (I_{Kr}), which plays a critical role in phase III repolarization of ventricular action potentials (Abbott et al., 1999). In fact, it is now commonplace to screen compounds for their activity against hERG channels because it is known that inhibition of this channel is associated with the development of long QT syndrome (LQTS) (Brown, 2005; Wible et al., 2005). LQTS can lead to the development of life-threatening arrhythmias; this is clearly an unwanted effect, in particular for noncardiac drugs.

Until recently (Brugada et al., 2004), there was a paucity of information on the link between the hERG channel and the

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ABBREVIATIONS: LQTS, long QT syndrome; CHO, Chinese hamster ovary; hERG, human *ether-a-go-go*-related gene; I, instantaneous current; I_{to}, transient outward K^+ current; SQTS, short QT syndromes; *S*, step current; *T*, tail current; NS3623, *N*-(4-bromo-2-(1*H*-tetrazol-5-yl)-phenyl)-N′-(3′-trifluoromethylphenyl)urea; NS1643, 1,3-bis-(2-hydroxy-5-trifluoromethyl-phenyl)-urea; RPR260243, (3*R*,4*R*)-4-[3-(6-methoxy-quinolin-4-yl)-3-oxo-propyl]-1-[3-(2,3,5-trifluoro-phenyl)-prop-2-ynyl]-piperidine-3-carboxylic acid; PD-118057, 2-{4-[2-(3,4-dichloro-phenyl)-ethyl]-phenylamino}-benzoic acid.

development of short QT syndrome (SQTS). However, it is now known that some instances of SQTS are linked to gain-of-function mutations in hERG channels (Brugada et al., 2004). We also speculate that certain small molecules that are activators of hERG channels could result in modification of the QT interval and lead to the development of SQTS. On the other hand, hERG channel activators might also be of therapeutic use in individuals with conditions of delayed repolarization, with LQTS being the most notable example (Seebohm, 2005). LQTS is a condition that has been shown to be underlined by mutations in a number of ion channel genes, for example, hERG, KCNQ1, and $Na_v1.5$ among others, and by mutations in channel-related genes, such as members of the KNCE family (Modell and Lehmann, 2006). Having detailed knowledge of the mechanisms by which these compounds activate the hERG channel will therefore provide a useful starting point for the development of lead compounds that could potentially be useful in the treatment of such conditions.

At present, only five hERG activators have been studied in great detail: RPR260243 (Kang et al., 2005), NS1643 (Casis et al., 2006; Hansen et al., 2006a), NS3623 (Hansen et al., 2006b), PD-118057 (Zhou et al., 2005), and mallotoxin (Zeng et al., 2006). PD-307243 is a member of a series of compounds that have been reported previously to be activators of hERG channels (Zhou et al., 2005). The previous report is centered on the effects of PD-118057, which causes shortening of the action potential duration and QT interval and prevents early after-depolarizations caused by dofetilide in arterially perfused rabbit ventricular wedge preparation (Seebohm, 2005; Zhou et al., 2005). PD-307243 has been shown to increase hERG tail current by 58% at 1 μM; this is more potent than PD-118057 (Zhou et al., 2005). However, the concentration-dependent effects and the possible mechanisms by which this activation occurs have not been characterized. Herein, we report the putative mechanisms of action of PD-307243 on hERG channels expressed in CHO cells. We found that this drug increased hERG current mainly by slowing hERG channel deactivation and inactivation. In addition, we demonstrated the activating effects of PD-307243 on I_{Kr} of rabbit ventricular myocytes.

Materials and Methods

Cell Culture. Chinese hamster ovary (CHO) cells were stably transfected to express hERG channels (BioCat ID number 97761; GlaxoSmithKline, Uxbridge, Middlesex, UK) and were used for patch-clamp recording. We chose CHO cells to stably express hERG channels because they have little endogenous voltage-dependent potassium channels and are therefore widely used to express voltage-gated potassium channels (Zeng et al., 2005). CHO cells expressing hERG channels were maintained at 37°C with 5% CO_2 in six-well culture dishes with Iscove's Dulbecco's modified Eagle's medium/F-12 nutrient mixture supplemented with 10% fetal bovine serum, 100 U/ml penicillin and streptomycin, and 0.5 mg/ml G-418 (Geneticin; Invitrogen, Carlsbad, CA).

Single Ventricular Myocyte Isolation. The studies were carried out in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the U.S. National Institutes of Health (Bethesda, MD) and approved by the GlaxoSmithKline Animal Care and Use Committee. Cardiac myocytes were isolated from the right ventricle of adult male New Zealand White rabbits using enzyme digestion described previously (Rials et al., 1997).

Patch Clamp. Whole-cell and inside-out patch currents were recorded from CHO cells stably expressing hERG channels and freshly isolated rabbit ventricular myocytes with an Axopatch 200B amplifier and Digidata 1322A digitizer (Molecular Devices, Sunnyvale, CA). Glass electrodes (2–4 MΩ resistance) were pulled from thin-wall glass (WPI, Sarasota, FL) using a P-97 horizontal puller (Sutter, Novato, CA) and fire-polished with MF-830 micro forge (Narishige, Long Island, NY). Cells were placed in a small chamber (volume = 0.7 ml) and continuously perfused with an external solution (3–4 ml/min). In whole-cell recording of CHO cells, bath solution contained 140 mM NaCl, 4 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM glucose, and 10 mM HEPES, pH 7.4. In the case of ventricular myocytes, bath solution contained 132 mM *N*-methyl-D-glucamine-Cl, 5 mM KCl, 1.2 mM $MgCl_2$, 10 mM glucose, 10 mM HEPES, and 0.1 mM $BaCl_2$, pH 7.4. Nifedipine (1 μM) was added to block L-type Ca^{2+} current. Pipette solution contained 119 mM potassium gluconate, 15 mM KCl, 5 mM EGTA, 5 mM K_2ATP, 3.2 mM $MgCl_2$, and 5 mM HEPES, pH 7.2. In inside-out patch recording, both bath and pipette solution contained 140 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, and 5 mM HEPES, pH 7.2. Currents were elicited by different voltage protocols (described in text and the figure legends) and acquired with pCLAMP 8 software (Molecular Devices) at room temperature. Whole-cell capacity transient was compensated near completely. Only cells with gigaohm seal were used for hERG current and I_{Kr} recording. No leak correction was applied.

Chemicals. PD-307243 (prepared by the chemists at GlaxoSmithKline, chemical structure shown in the inset of Fig. 1B), nifedipine (MP Biomedicals, Solon, OH), and dofetilide (APIN chemicals Ltd., Abingdon, UK) were made as 10 mM dimethyl sulfoxide stock solutions and diluted in the bath solutions to desired concentrations.

Molecular Modeling. The initial hERG channel model was based on the rat open state Kv1.2 crystal structure (Long et al., 2005) and has been described previously (Micheli et al., 2007). The loop region between the S5 and pore helices was then subsequently modified to ensure consistency with the biophysical data and molecular model published recently (Tseng et al., 2007). The Ser5-proline linker was modeled using the software package MOE with the two predicted α-helical domains, His576 to Ile583 and Trp585 to Gly594, constructed with the default parameters used in the program. The modified linker was then inserted between the Ser5 and pore helices by appropriate manual adjustment of the backbone torsion angles to ensure optimal alignment of the terminal residues. The process was repeated for the remaining three repeat domains and the resulting structure was geometry optimized using the OPLS 2005 force field as implemented in MacroModel (Schrodinger LLC, New York, NY). Molecular docking of the hERG channel activator, PD-370243, was performed with Glide (Schrodinger LLC) using the default SP parameters.

Data Analysis. All data were analyzed and illustrated using pCLAMP 8, Origin 7 (OriginLab Corp, Northampton, MA) and Excel (Microsoft, Redmond, WA) and presented as mean ± S.E. (n). Zero current level was indicated using broken lines in all figures. Statistic significance ($P < 0.05$) was determined using one-way analysis of variance or paired t test.

Results

PD-307243 Activated hERG Channel. When hERG currents were induced with a 2-s pulse to +20 mV followed by a 2-s tail step at −50 mV delivered every 10 s (Fig. 1 A, inset), bath perfusion of PD-307243 (10 μM) resulted in a gradual increase in both step and tail currents (Fig. 1A). We were surprised to find that continuous perfusion with 10 μM PD-307243 and constant stimulus resulted in an I_{to}-like current that is highlighted in Fig. 1A, top. Compared with the tail peak current, the onset of this I_{to}-like current was slower and took a much longer time to reach steady state (Fig. 1A,

bottom). It is interesting that the activation of the I_{to}-like current is seemingly dependent on channel opening. Continuous perfusion with 10 μM PD-307243 in the absence of stimulating pulses for 4.5 min did not result in the generation of the I_{to}-like current at the first stimulus pulse (Fig. 1B, top; trace labeled 1st), whereas increases in both step and tail currents were observed.

The activating effects of PD-307243 on hERG channels at various depolarization voltages were further investigated by a voltage protocol shown in Fig. 2A (inset). After recording control hERG current traces (left), 10 μM PD-307243 was perfused into the recording chamber, and the cells were continuously stimulated with the pulse shown in Fig. 1A (inset) at 10-s intervals until the drug effect reached steady state. The hERG current traces at various test potentials were recorded (middle; note the different current amplitude scale). The third group of hERG traces was recorded after extensive washout (~30 min) with control solution (right). The corresponding current-voltage relationships (I-V) for the instantaneous current (*I*), the step current (*S*), and the tail peak current (*T*) are shown to highlight changes that can be attributed to the effects of the compound on hERG gating (Fig. 2B). From this initial set of experiments, we observed that 10 μM PD-307243 increased all three current amplitudes (*I*, *S*, and *T*). It caused persistent hERG current at membrane potential as low as −60 mV. In addition, the hERG channel lost its characteristic rectification. The deactivation at −50 mV was extremely slow.

To further characterize the effects of the compound, we recorded step currents using an expanded voltage range (−100 to +80 mV) and measured the tail currents at −120 mV to allow more complete deactivation (Fig. 3A, inset). As illustrated in Fig. 3A, 3 μM PD-307243 at steady state induced instantaneous currents with little decay at very negative step voltages (≤ −40 mV). When the step voltages became more positive, an inactivating phase appeared [Fig. 3A (2)]. Dofetilide (1 μM), a specific blocker of hERG currents (Jurkiewicz and Sanguinetti, 1993), was able to completely

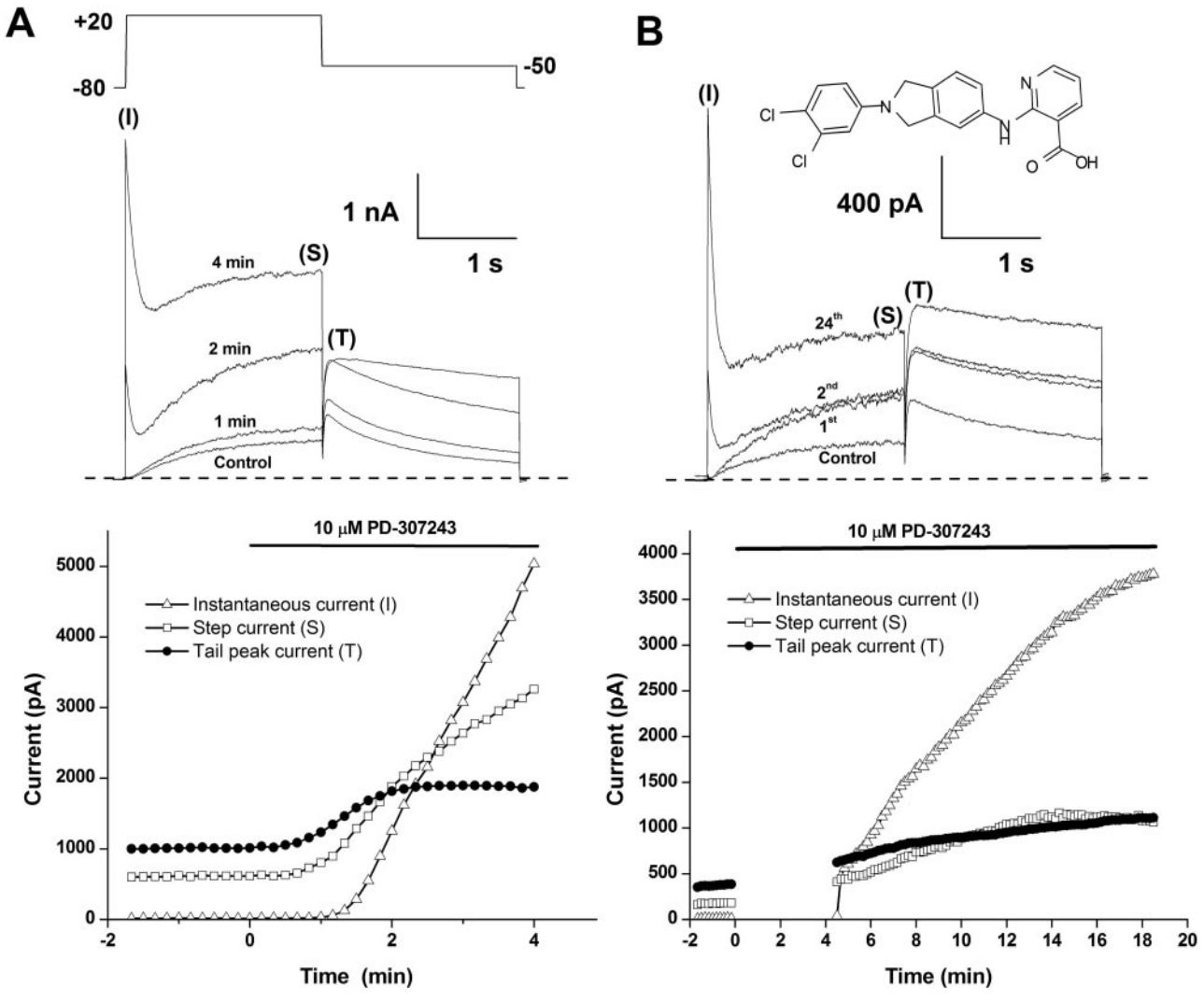

Fig. 1. Time course of hERG channel activation by PD-307243 in CHO cells. Whole-cell currents were generated from a holding voltage of −80 mV with a single step to +20 mV for 2 s followed by a tail pulse at −50 mV for 2 s. The protocol (inset) was repeated every 10 s. A, top, exemplar current traces of control and after 1-, 2-, and 4-min external perfusion with PD-307243 (10 μM) generated with constant pulsing. Bottom, time course of current amplitude measured at the beginning of +20 mV step (*I*), the last 100 ms of the +20-mV step (*S*), peak current at returning to −50 mV (*T*). Extracellular perfusion with PD-307243 was indicated by a horizontal bar. B, top, exemplar current traces of control and the 1st, 2nd, and 24th trace induced by constant pulsing after 4 min 30 s of external perfusion with PD-307243 (10 μM) without pulsing. Bottom, time course of *I*, *S*, and *T*. Inset, chemical structure of PD-307243.

block currents activated by 3 μM PD-307243 [Fig. 3A (3)], confirming that the instantaneous current is through hERG channels and is not due to the up-regulation of other channels endogenous to CHO cells (Lalik et al., 1993; Yu and Kerchner, 1998). The fact that PD-307243 (10 μM) did not activate any current in untransfected CHO cells (n = 3; data not shown) further validated this point. The decay of the tail current at −120 mV after a +20-mV depolarization step was fitted with a two-exponential function to determine the deactivation time constants. There was a statistically significant slowing of both the fast and slow deactivation time constants: τ_f was 17 ± 3 and 72 ± 16 ms (n = 8) and τ_s was 55 ± 8 and 199 ± 26 ms (n = 8) for the control and 3 μM PD-307243-perfused cells, respectively.

The I-V relationship shown in Fig. 3B revealed the concentration-dependent effects of PD-307243. Under control conditions, very little instantaneous current was detected. PD-307243 induced instantaneous current at voltage steps ≥ +20 mV with 1 μM and at all voltage steps with 3 and 10 μM (left). Before drug treatment, the I-V relationship of hERG step current had characteristic rectification with the maximal step current being observed at +30 mV (578 ± 144 pA, n = 8). The maximal step current was increased, and the voltage at which the maximum was reached shifted to +40 mV (1716 ± 538 pA, n = 6) and +60 mV (3180 ± 676 pA, n = 8) in the presence of 1 and 3 μM PD-307243. No reduction of step current was seen up to +80 mV with a concentration of 10 μM PD-307243 (middle). Under control conditions, no hERG tail current was recorded at step voltages ≤ −40 mV. PD-307243 (1 μM) slightly increased the tail current. Concentrations of 3 and 10 μM PD-307243 induced large tail currents even at step voltages from −100 to −40 mV (right), and the reduction of tail peak current within this voltage range reflected the minor voltage-dependent hERG channel inactivation. The increase in tail peak currents at voltages ≥ −30 mV was due to the voltage-dependent activation of hERG channels.

The appearance of persistent hERG current at negative voltages in the presence of PD-307243 required the opening

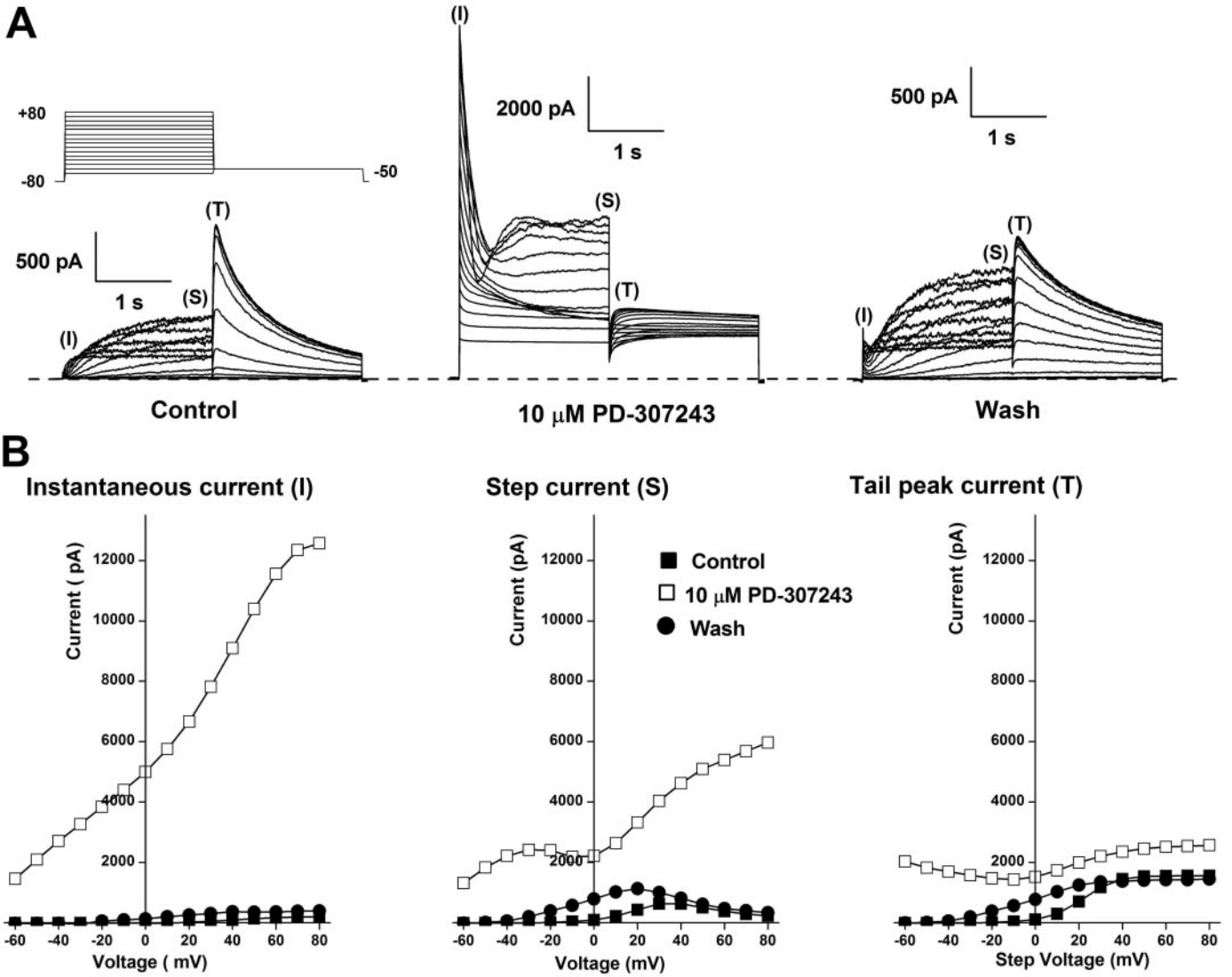

Fig. 2. Effects of PD-307243 on the I-V of hERG channels in CHO cells. Whole-cell currents were generated from a holding voltage of −80 mV and stepped to a range of voltages between −60 and +80 mV in 10-mV increments for 2 s followed by a tail pulse at −50 mV for 2 s. Interpulse interval was 10 s. A, exemplar traces showing the effect of PD-307243 (10 μM) on a family of currents. Left, control traces; middle, current traces after drug effects reached steady state; right, current traces after drug washout. B, left to right, I-V for I, S, and T in control, 10 μM PD-307243, and after washout.

of the hERG channels. Using the same protocol from the previous experiment (Fig. 3A) but limiting the maximal pulse step to −40 mV to avoid activating hERG channels, we assessed the effect of 6 min of PD-307243 (3 μM) perfusion without stimulus and observed no current activation in the voltage range tested (Fig. 4A, middle versus left). After stimulating the cell with the protocol shown in Fig. 1A (inset) for several minutes, we observed persistent hERG current at voltage steps from −100 to −40 mV (Fig. 4A, right). The corresponding I-V values were shown in Fig. 4B.

In the presence of 3 μM PD-307243, a large component of the hERG tail current remained at the end of the 2-s step of −120 mV [Fig. 2A (2)]. To investigate whether the hERG channels would eventually close, we increased the interval between depolarizing pulses to 2 min and decreased the membrane potential to −130 mV (Fig. 5A, top). After recording a control trace, the cell was perfused for 2 min with 3 μM PD-307243, and subsequent recordings were made with continuous perfusion of the compound. In the presence of PD-307243, there was incomplete deactivation even with the very long interval and very negative voltage between depolarizing pulses (Fig. 5A, middle). The I_{to}-like current was observed at the second pulse in the presence of the compound (Fig. 5A, bottom), highlighting that it was a use-dependent phenomenon.

To investigate whether the compound had any effect on the hERG channel selectivity filter, we depolarized the channels with a step to +40 mV for 2 s and then recorded tail currents in a range of voltages between −120 to −40 mV for 2 s (Fig. 5B, inset). The reversal potential was not altered in the presence of 3 μM PD-307243 (Fig. 5B), the average values were −79.5 ± 1.0 and −78.4 ± 0.7 mV (n = 3) for control and 3 μM PD-307243, respectively ($P > 0.05$). This result implies that PD-307243 did not disrupt the selectivity filter.

PD-307243 Impaired hERG Channel Inactivation. The removal of rectification of hERG step I-V shown earlier suggested that there was modification in hERG channel inactivation. We investigated the effect of PD-307243 (3 μM) on channel inactivation by using a protocol that has been used (Zou et al., 1998) to determine the voltage-dependence of recovery from inactivation (Fig. 6A, inset). After control current traces were recorded (Fig. 6A, left), the cells were per-

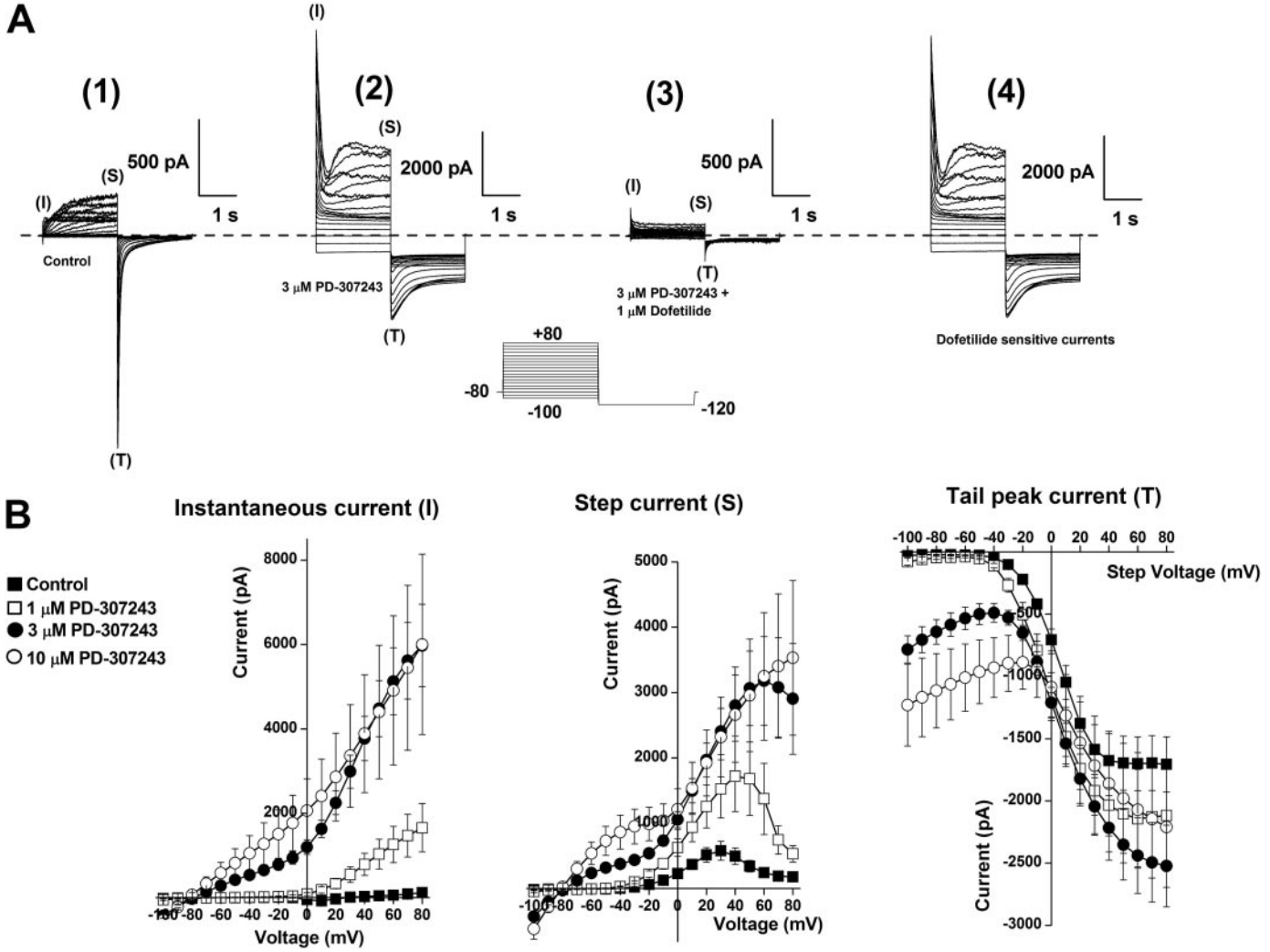

Fig. 3. Concentration-dependent effects of PD-307243 on hERG channels in CHO cells. A, exemplar traces showing the effect of PD-307243 (3 μM) on a family of currents. Whole-cell currents were generated from a holding voltage of −80 mV and stepped to a range of voltages between −100 and +80 mV in 10-mV increments for 2 s followed by a tail pulse at −120 mV for 2 s. Interpulse interval was 10 s: control traces (1), current traces after drug effects reached steady state (2), current traces in the presence of 3 μM PD-307243 + 1 μM dofetilide (3), and dofetilide-sensitive currents (4). B, mean I-V (n = 5–8 cells) for I, S, and T in control, 1, 3, and 10 μM PD-307243.

fused with 3 μM PD-307243 and stimulated with the protocol shown in Fig. 1A (inset) until the drug effect reached steady state; subsequently, a second group of current traces was recorded (Fig. 6A, right). PD-307243 shifted the voltage-dependent recovery from inactivation of hERG channels; the difference was significant at membrane potentials $\geq$0 mV. Fitting the data using the Boltzmann equation found $V_{1/2}$ to be -43 and -27 mV, and the slope factor (k) was 23 and 39 mV for the control and the treated group, respectively (Fig. 6B).

We next examined the effect of 3 μM PD-307243 on the onset rate of hERG current inactivation using a three-step protocol described previously (Fig. 7A, inset) (Smith et al., 1996; Spector et al., 1996). The results indicated a significant slowing of the rate of channel inactivation; the inactivation time constant was obtained using a single exponential fit (Fig. 7B). As the membrane potential increased, although there was the usual acceleration of inactivation in the control group, surprisingly, the drug group showed a significant slowing of inactivation.

PD-307243 Acted on hERG Channels in the Inside-Out Patch Configuration. The results from the whole-cell experiments indicated that there was a significant slowing of inactivation and a slowing of the deactivation of hERG channels in the presence of PD-307243, giving rise to the possibility that the compound could have multiple sites of action. To test this hypothesis, recordings were made in the inside-out patch configuration using a voltage protocol (Fig. 8A, inset) that was applied to the patch repeatedly at 10-s intervals. Representative current traces in control, 3, and 10 μM drug at steady state and after prolonged washout were illustrated in Fig. 8A. We observed that there was an increase in tail current amplitude and slowing of deactivation in the presence of PD-307243 (3 and 10 μM). The peak tail was increased 2.1 $\pm$ 0.6- (n = 7) and 3.4 $\pm$ 0.3-fold (n = 4), respectively, in the presence of 3 and 10 μM PD-307243. The fast deactivation time constant of the tail current (τ_f) was 17 $\pm$ 5 and 50 $\pm$ 12 ms (n = 7) for control and 3 μM PD-307243, and the slow deactivation time constant (τ_s) was 42 $\pm$ 12 ms in control compared with 118 $\pm$ 26 ms (n = 7) obtained after perfusion with 3 μM PD-307243 ($P < 0.05$). The activation effect of PD-307243 could also be largely reversed by washout of the compound (Fig. 8A) or completely blocked by 1 μM dofetilide (data not shown).

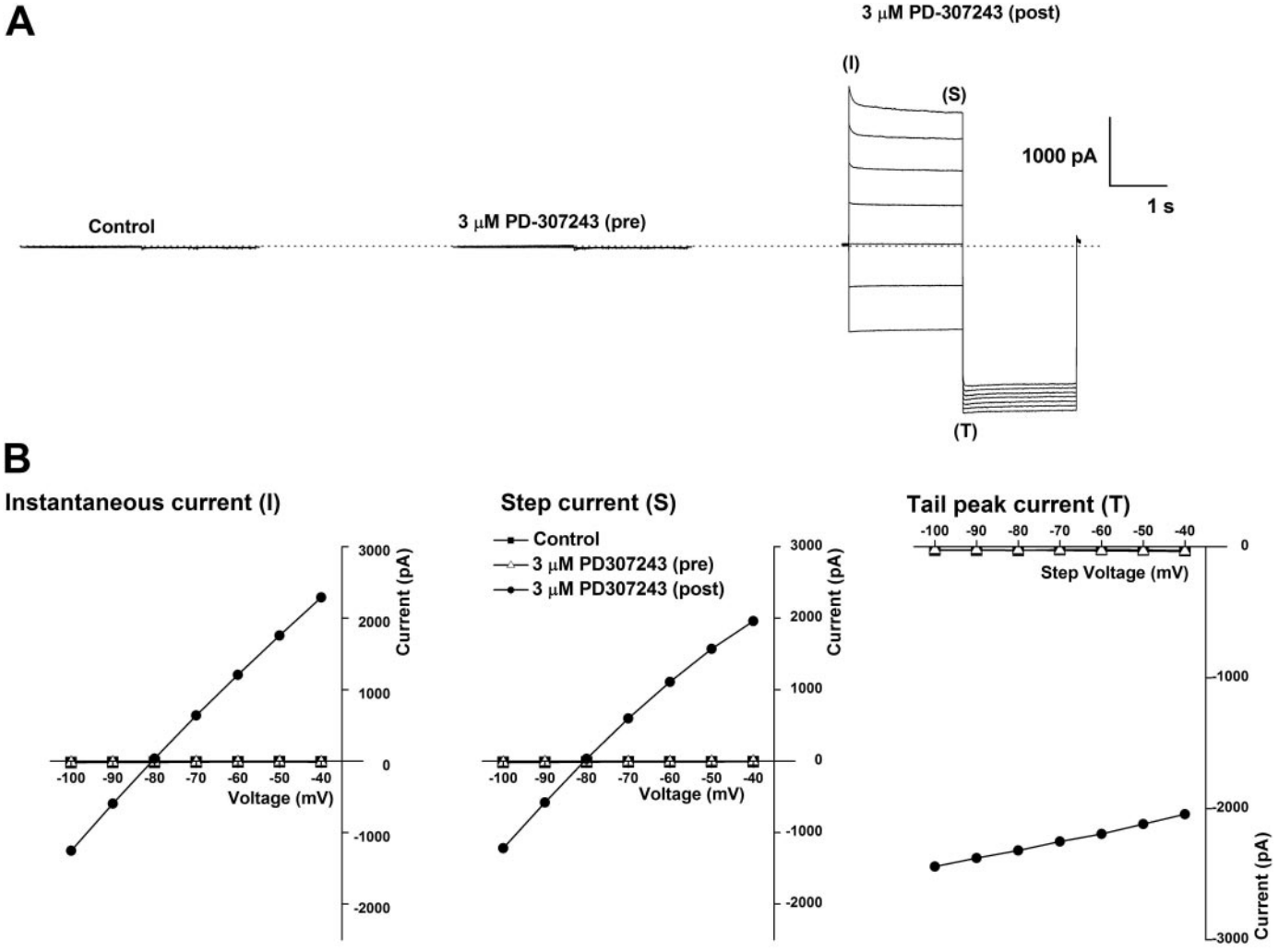

Fig. 4. Appearance of persistent hERG current in the presence of PD-307243 requires channel opening. A, exemplar traces showing the effect of PD-307243 (3 μM) on a family of currents. Whole-cell currents were generated using the voltage protocol from Fig. 3A, inset, with testing steps from -100 to -40 mV. Left, control traces; middle, first group of traces induced after 6-min drug perfusion (prepulsing); right, a group of traces induced after constant pulsing using the voltage protocol shown in Fig. 1A, inset (postpulsing). B, I-V for I, S, and T in control, 3 μM PD-307243, before and after constant pulsing.

We further examined how PD-307243 affected the voltage-dependent activation of hERG current in inside-out patches. The voltage protocol (Fig. 8B, inset) was identical with what used in the whole-cell experiment (Fig. 3A, inset). The major effect of PD-307243 was a concentration-dependent increase of the tail current amplitude and slowing of deactivation (Fig. 8B). All of the tail currents were normalized by the amplitude of the control tail current after +80 mV depolarization and plotted against step membrane potentials for control, 3, and 10 μM PD-307243 (Fig. 8C). The maximal tail current after the +80-mV step was increased 2.5- and 3.8-fold compared with control and with concentrations of 3 and 10 μM PD-307243, respectively. However, the half-maximal activation voltage ($V_{1/2}$) obtained by fitting the data with the Boltzmann equation was not changed: $V_{1/2}$ was 9.7 ± 7.9 mV (n = 3), 13.5 ± 1.7 mV (n = 3), and 10.7 ± 2.5 mV (n = 2) for control, 3, and 10 μM PD-307243 ($P > 0.05$). When applied on the intracellular side of the membrane, PD-307243 did not induce I_{to}-like hERG current at depolarizing voltage steps, and only very small persistent current was seen at step voltages ≤ −80 mV in the presence of the drug (Fig. 8C).

PD-307243 Activated I_{Kr} in Rabbit Ventricular Myocytes. Next, we wanted to confirm that the unique activation effects of PD-307243 on hERG channels expressed in CHO cells could be reproduced in native cells expressing the rapid component of I_{Kr} channels. For this purpose, we recorded currents from ventricular myocytes freshly isolated from the rabbit heart. Membrane current (Fig. 9A, left) was elicited by the voltage-clamp protocol shown in the inset when Na^+, Ca^{2+}, and inward rectifier K^+ current were eliminated by ion substitution and blocking agents in the external solution. The cell was then perfused with 3 μM PD-307243 and repeatedly stimulated by the same protocol until drug effects reached steady state. The second group of current traces were then recorded that clearly demonstrated the activation effects of PD-307243 (Fig. 9A, middle). The currents acti-

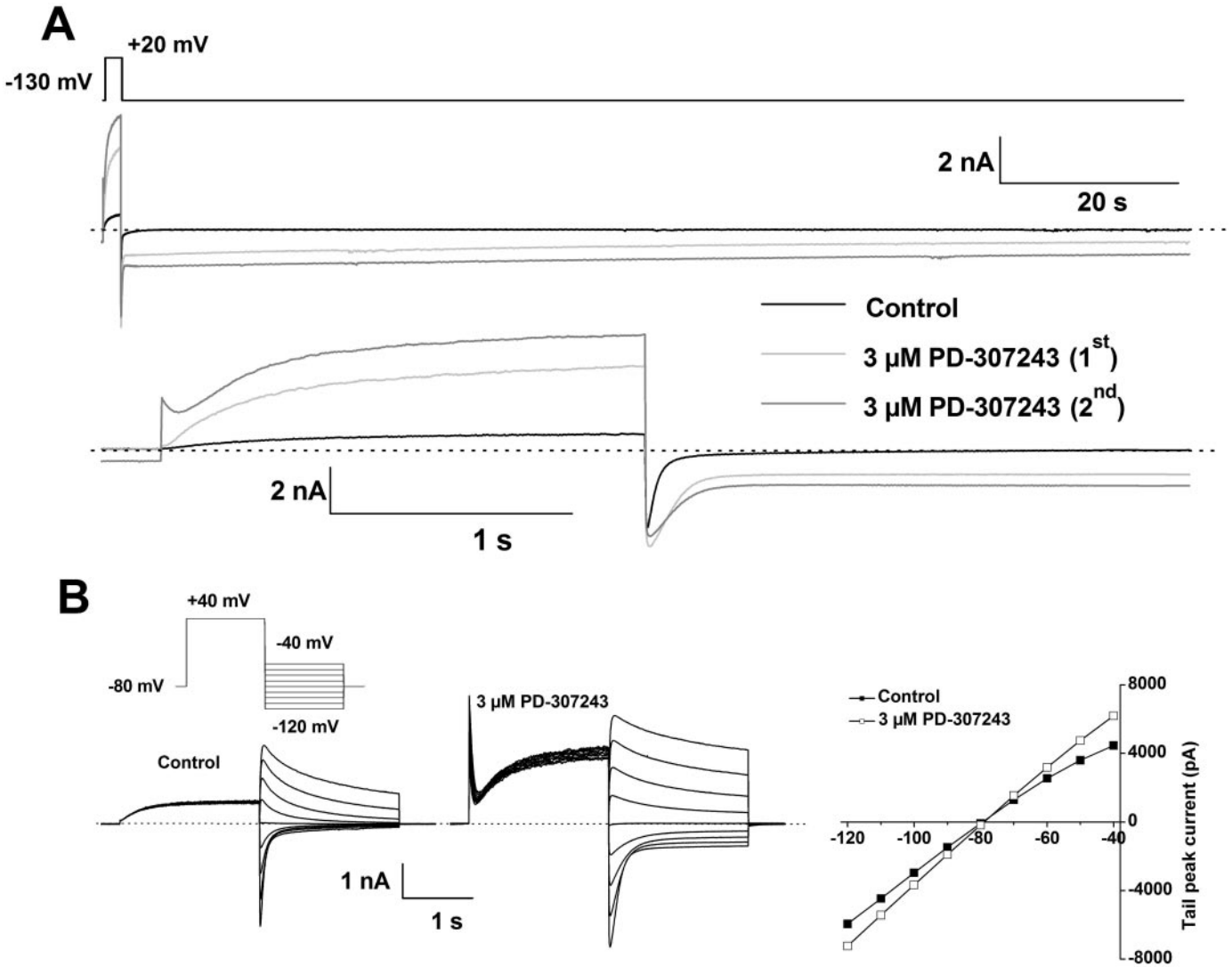

Fig. 5. Influence of PD-307243 on deactivation and the reversal potential of hERG channels. A, whole-cell currents were generated from a holding voltage of −130 mV with a single step to +20 mV for 2 s, followed by a tail pulse at −130 mV for 2 min (top). Middle, exemplar current traces of control and the first two traces after 2-min perfusion with PD-307243 (3 μM) generated with constant pulsing. Bottom, the same traces showing the first 4.5-s to highlight the development of the I_{to}-like current in the second trace after perfusion with the compound. B, whole-cell currents were generated from a holding potential of −80 mV with a single step to +40 mV for 2 s followed by tail currents recorded at voltages in the range of −120 to −40 mV for 2 s (inset). Exemplar traces for control (left) and after perfusion with 3 μM PD-307243 (middle). Right, I-V of tail peak current recorded at various voltages for control (■) and after perfusion with 3 μM PD-307243 (□).

vated by PD-307243 at −30, 0, and +30 mV were obtained by subtracting the control current traces from the corresponding recordings in the presence of the drug (Fig. 9A, right). The PD-307243 activated an I_{to}-like current, which resembled the drug effect observed in CHO cells expressing hERG channels. Similar results were obtained in two additional ventricular myocytes. We further confirmed that the PD-307243 activated current was I_{Kr} by demonstrating its inhibition by 1 μM dofetilide (Fig. 9B).

Intergraded Effect of PD-307243 on hERG Current during an Action Potential. Because PD-307243 affected multiple properties of hERG channels, we wanted to evaluate the overall effect of PD-307243 on hERG currents during an action potential. For this purpose, we used a voltage-clamp protocol modeled on an action potential prerecorded from a rabbit ventricular myocyte (Fig. 10A). The current trace was recorded in CHO cells expressing hERG channels before (control in Fig. 10B) and after 3 μM PD-307243 perfusion with repetitive stimuli at 5-s intervals. The trace labeled as 3 μM PD-307243 was recorded when the drug response reached steady state (Fig. 10B). The shape of the hERG current resembled the action potential. Measurement of the area under the hERG current trace in control and in the presence of 3 μM PD-307243 revealed that there was an 8.8 ± 1.0-fold (n = 5) increase in the total potassium ions conducted by hERG channels during the action potential in the presence of the compound.

Action of PD-307243 on Other Cardiac Ion Channels. We next wanted to examine the specificity of action of PD-307243 and tested it on a range of known cardiac ion channels. We tested the action of PD-307243 on the KCNQ1 + minK complex heterologously expressed in CHO cells, the molecular correlate of I_{Ks} (Sanguinetti et al., 1996). At a concentration of 3 μM, PD-307243 significantly increased KCNQ1 + minK current 64 ± 12% (n = 5) at +50 mV (Supplemental Fig. S1). PD-307243 was also found to acti-

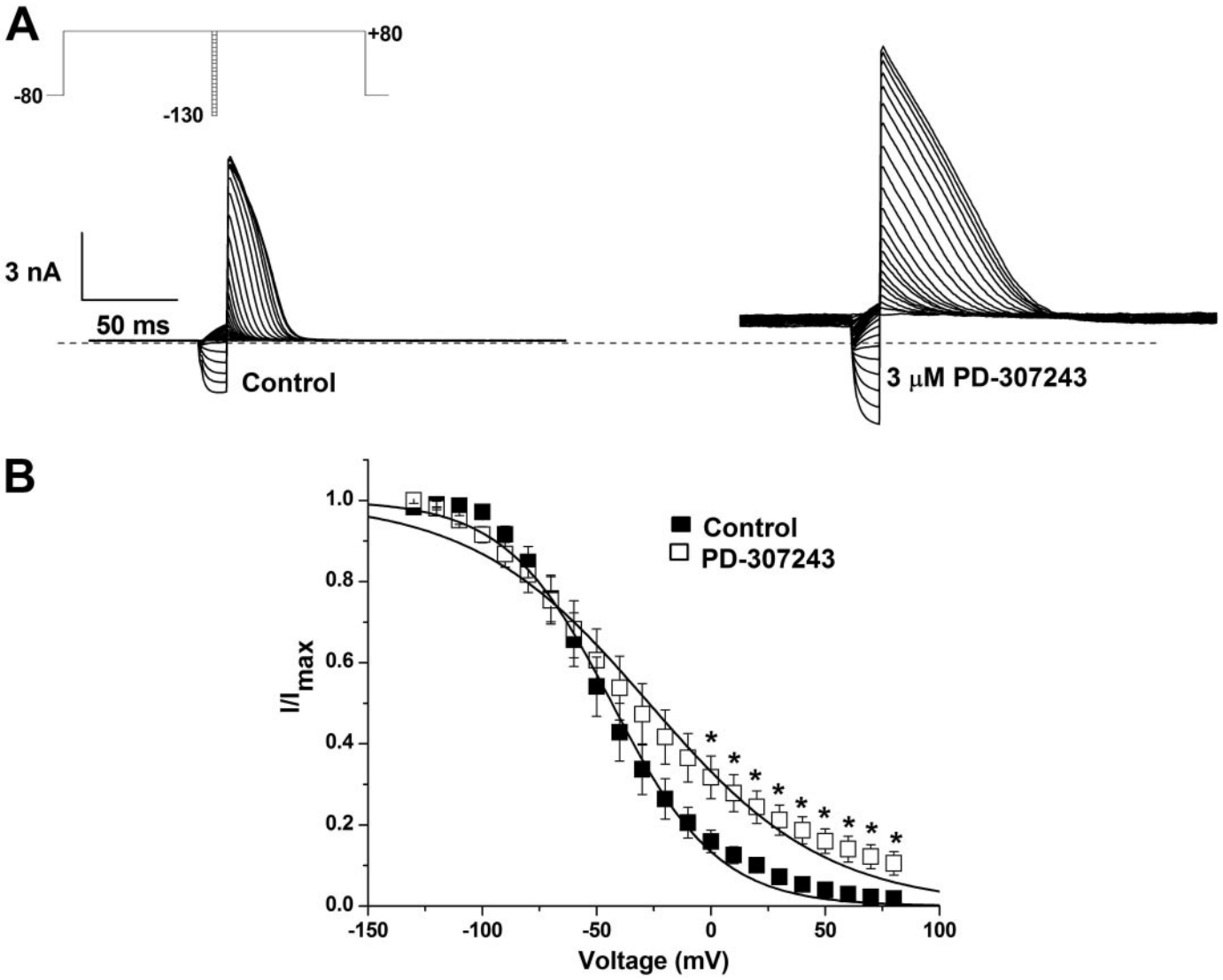

Fig. 6. Effect of PD-307243 on voltage-dependent recovery from inactivation of hERG channels in CHO cells. Whole-cell currents were generated by a three-step protocol (inset). From a holding potential of −80 mV, channels were allowed to inactivate at +80 mV for 500 ms; this was followed by stepping the membrane to a range of voltages between −130 and +80 mV in 10-mV increments for 15 ms to allow recovery from inactivation, and this was followed by a tail pulse to +80 mV for 500 ms. A, exemplar traces recorded under control conditions and in the presence of PD-307243 (3 μM). B, peak tail currents were normalized to the maximal peak tail current at −130 mV and plotted against voltages for the control and PD-307243 (n = 5 cells). The curves were fitted with the Boltzmann function: $I/I_{max} = 1/(1 + \exp[(V - V_{1/2})/k])$.*, $P < 0.05$.

vate L-type Ca^{2+} current recorded in ventricular myocytes isolated from rabbit hearts. At concentrations of 3 and 10 μM, PD-307243 increased peak Ca^{2+} current recorded at +10 mV by 29 ± 4% (n = 6) and 52 ± 11% (n = 5), respectively (Supplemental Fig. S2); the increases were statistically significant. PD-307243 (3 μM) had no effect on I_{to} recorded from ventricular myocytes (Supplemental Fig. S3) or human $Na_v1.5$ channels expressed heterologously in human embryonic kidney 293 cells (Supplemental Fig. S4).

PD-307243 Docked in the Extracellular Mouth of the Pore. A model of the hERG channel incorporating the extracellular S5, P, and S6 segments was constructed. This was based initially on its homology with a mammalian Shaker family potassium channel (Kv1.2), but the S5-P loop regions were subsequently modified in accordance with details described in a recent publication (Tseng et al., 2007). The important aspects of the biophysical studies that have been applied to the model are as follows: residues 576 to 583 and 585 to 594 are most likely to adopt an amphipathic α helical structure; the hydrophobic residues on the S5-P2 helix are in high-impact positions oriented toward the internal face of the hERG channel, with the hydrophilic residues facing the solvent front. After geometry optimization of the protein receptor model, the hERG channel activator PD-370243 was docked in the extracellular portion of the molecule, with the docking grid loosely defined around the center of the pore. The docking was performed using Glide under standard SP conditions, with the top 10 scoring poses being retained. All 10 poses were oriented in a similar manner, with the carboxylic acid moiety pointed toward the center of the pore and interacting with the side-chain hydroxyl group of serine 631, as shown in Fig. 11. The hydrophobic domains of the small molecule, primarily the dichlorophenyl moiety, were making positive interactions with the lipophilic residues leucine 589 and isoleucine 593 of the protein. This orientation was consistent with the limited structure-activity data for this com-

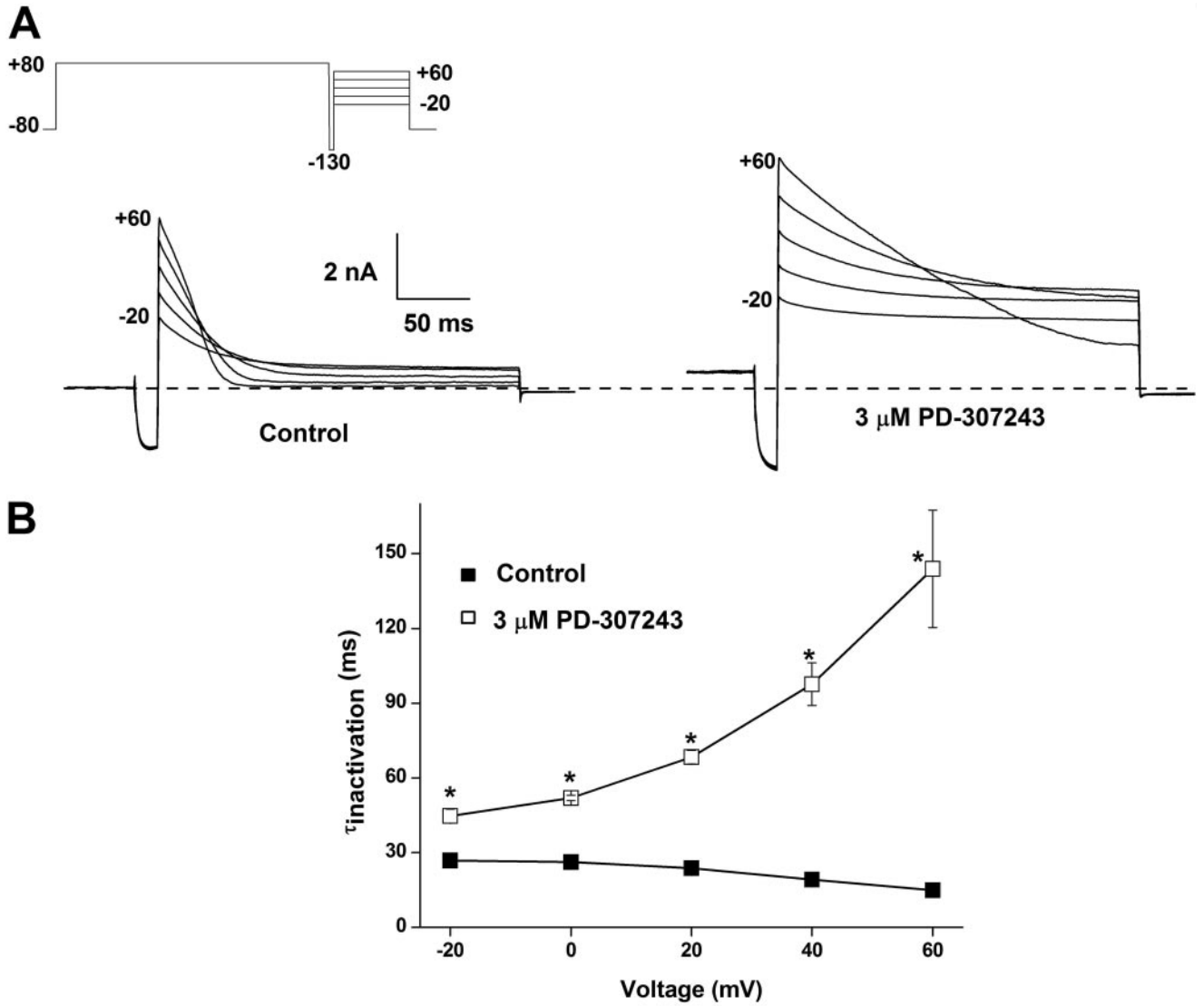

Fig. 7. Effect of PD-307243 on the inactivation time constant of hERG current in CHO cells. A, exemplar traces showing the measurement of hERG current inactivation under control conditions and in the presence of PD-307243 (3 μM). Whole-cell currents were generated by a three-step protocol. From a holding potential of −80 mV, channels were allowed to inactivate at +80 mV and then briefly were allowed to recover at −130 mV for 15 ms; the membrane was then stepped to a range of voltages between −20 and +60 mV, in 20-mV increments. B, the time constants obtained in the range of voltages −20 to +60 mV were plotted (n = 4 cells). *, $P < 0.05$.

pound (Zhou et al., 2005), highlighting the importance of the carboxylic acid moiety and hydrophobic domain to the activity of this molecule.

Discussion

In this study, we demonstrated that PD-307243 concentration-dependently increased hERG currents and elucidated its mode of action. Here we show for the first time that PD-307243 has a site of action close to the extracellular mouth of the channel pore.

PD-307243 induced persistent hERG current at very negative membrane potentials and I_{to}-like hERG current followed by a rising phase at positive voltages. The generation of the instantaneous current at all the voltages was attributable to dramatic slowing of channel deactivation, with some channels being perpetually held in an open state, even at very negative membrane potentials. Because hERG channel inactivation was quite limited at negative voltages (≤ -60 mV), the hERG current showed little decay. At more positive voltages, hERG channel inactivation became more apparent. Because PD-307243 dramatically slowed the onset of hERG current inactivation, a clearcut inactivating phase of hERG current was observed at depolarization steps, which was absent under normal circumstances because of very fast hERG channel inactivation (Zhou et al., 1998; Kiehn et al., 1999). The slowly rising phase of hERG current after initial decay reflected the opening of additional hERG channels in the close state in response to membrane depolarization.

The induction of the instantaneous hERG current by PD-307243 required channel opening; this is consistent with the observation made for RPR260243 (Kang et al., 2005), a hERG channel activator that mainly slows hERG channel deactivation without affecting other channel properties. The I_{to}-like current appears at the second pulse after RPR260243 treatment, and the amplitude of the instantaneous hERG current is unchanged with continuous pulsing. In contrast, the binding of PD-307243 to the hERG channel seemed to be use-dependent. With continuous stimulation, more and more hERG channels were accumulated in open state as a result of incomplete deactivation, resulting in increasing I_{to}-like current amplitude. This use-dependent action of PD-307243 strongly implied that the site of action of the compound may be within the channel pore and only became accessible on

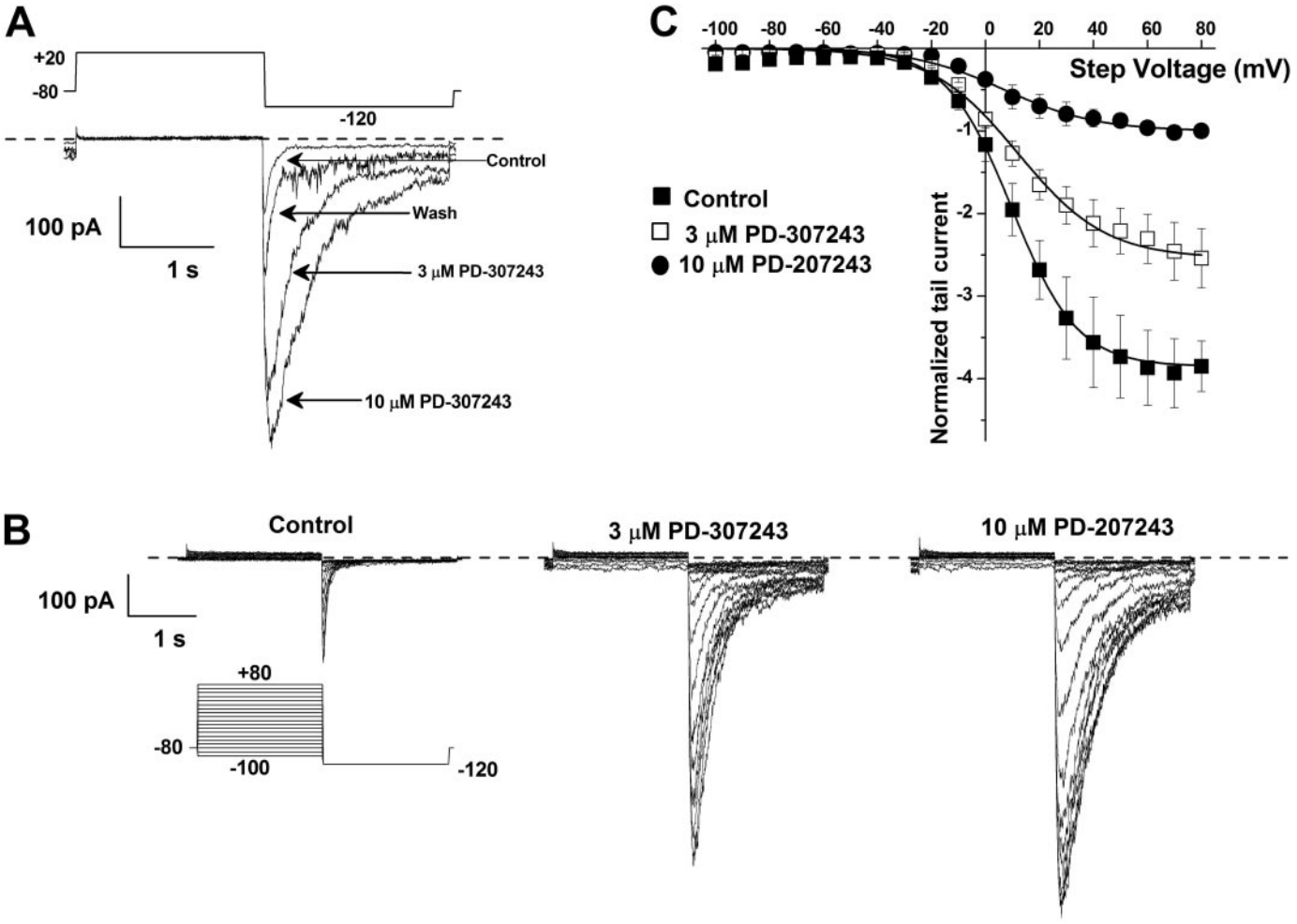

Fig. 8. Intracellular effect of PD-307243 on hERG channels expressed in CHO cells. A, exemplar traces showing the current recorded in an inside-out patch in control, 3, and 10 μM PD-307243, and after washout. Currents were evoked from a holding voltage of -80 mV, and stepped to $+20$ mV for 2 s followed by a tail current at -120 mV for 2 s. Interpulse interval was 10 s. B, exemplar traces showing the effect of PD-307243 at 3 and 10 μM in an inside-out patch on a family of currents. Currents were generated from a holding voltage of -80 mV and stepped to a range of voltages between -100 and $+80$ mV in 10-mV increments for 2 s, followed by a tail pulse at -120 mV for 2 s. Interpulse interval was 10 s. C, averaged I-V of tail peak current normalized by the tail current amplitude at $+80$ mV in control (n = 2–3 cells).

channel opening. The reversal potential of the hERG current was not altered in the presence of the drug, and this argues against it modulating the channel by interacting with the selectivity filter.

Extracellular application of PD-307243 gave a small rightward shift in $V_{1/2}$ for voltage-dependent recovery from inactivation. This property is consistent with other reported hERG channel activators, such as mallotoxin (Zeng et al., 2006), NS1643, and NS3623 (Hansen et al., 2006a,b). However, the small shift was not observed for NS1643 in another study (Casis et al., 2006). In addition, PD-307243 dramatically slowed the onset of hERG current inactivation. Although NS1643 (Casis et al., 2006) and NS3623 (Hansen et al., 2006b) also slow the rate of hERG current inactivation, these drugs do not change the characteristic acceleration of inactivation with more depolarized membrane potentials. In the presence of PD-307243, hERG current inactivates much more slowly at more positive voltages. The slowing of time-dependent inactivation coupled with the retarded channel closure generated the I_{to}-like hERG current at positive membrane potentials.

Our experimental findings suggested that PD-307243 acted on the outer regions of the channel pore leading to the modification of inactivation gating. The extracellular turret and the pore loop are important for channel inactivation (Witchel, 2007). In particular, amino acid residues 583 to 597 have been identified to play a critical role in the C-type inactivation of hERG channel (Liu et al., 2002; Torres et al., 2003; Jiang et al., 2005). In fact, a mutation resulting in an asparagine-to-lysine substitution at position 588 (N588K) is associated with an inherited form of SQTS, which is characterized by a significant slowing of channel inactivation (Cordeiro et al., 2005; McPate et al., 2005).

Leu589 and Ile593 have been reported previously to play important roles in hERG channel inactivation (Liu et al., 2002; Torres et al., 2003; Jiang et al., 2005); docking studies suggest that PD-307243 binds in the extracellular region of the channel pore and showed points of contact with these two residues. In addition, the carboxylic acid moiety of PD-307243 was shown to form a hydrogen bond with the serine residue at position 631. A mutation of this residue to an alanine impairs but does not abolish channel inactivation (Zou et al., 1998), which seems to be consistent with the action of PD-307243.

It is plausible that binding of the compound to the extracellular region of the channel pore leads to conformational changes in the channel, which ultimately results in the impaired deactivation that was observed. The slowing of channel deactivation that is observed in the inside-out configuration can therefore result from two scenarios. The first could involve the binding of PD-307243 to a low-affinity site on the intracellular side of the channel pore that attenuates channel deactivation. In the second instance, there could be a slow diffusion of the compound to the extracellular binding site, and the identified difference that we observed on channel

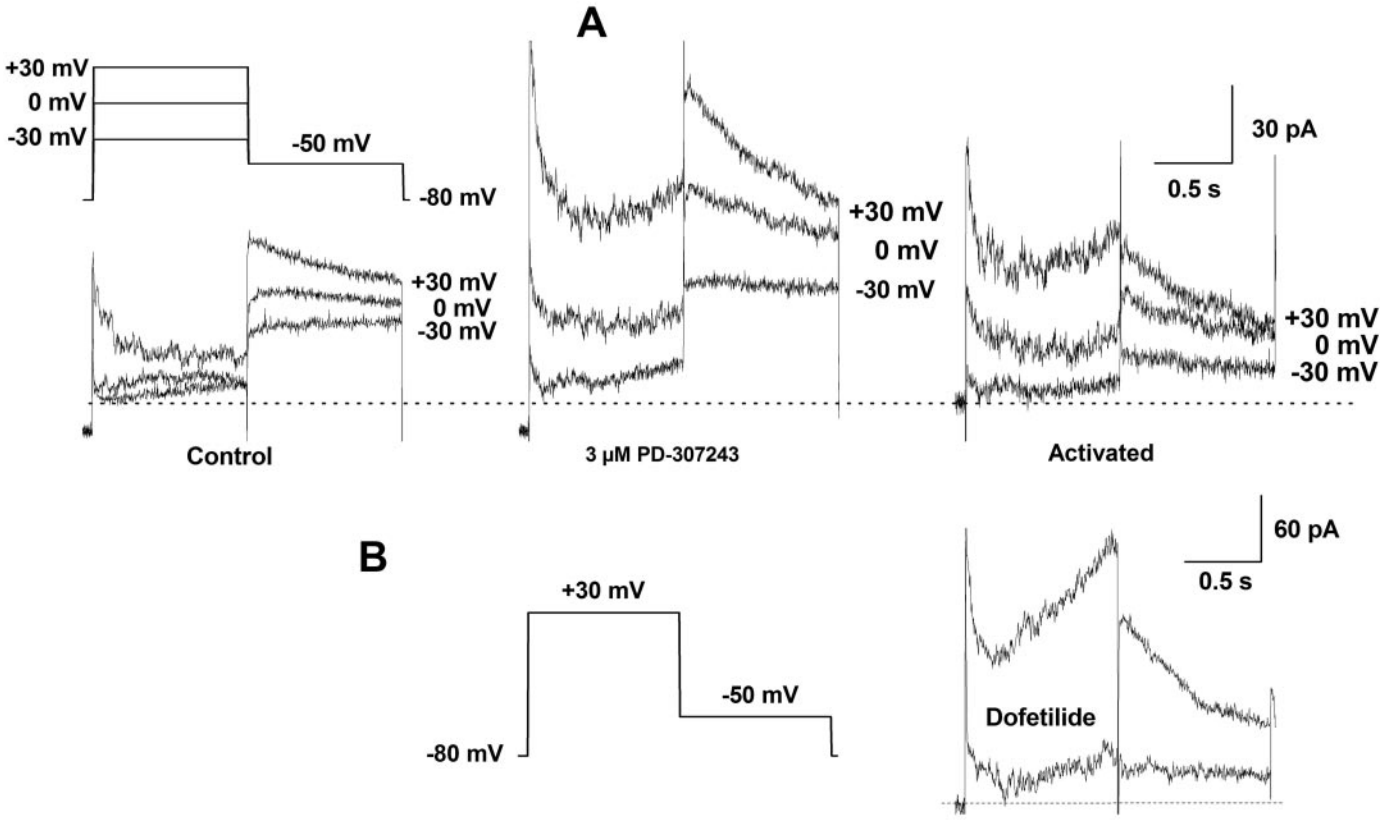

Fig. 9. PD-307243 activates I_{Kr} in rabbit ventricular myocytes. A, whole-cell currents were generated from a holding potential of −80 mV and stepped to −30, 0, and +30 mV for 1 s followed by a tail pulse at −50 mV for 1 s. Interpulse interval was 10 s. Left, control current traces; middle, current traces in 3 μM PD-307243 when drug response reached steady state; right, the PD-307243 activated current obtained by subtracting the current traces on the left from those in the middle. B, same voltage-clamp protocol as in A but only one step. Traces shown were the PD-307243 activated current before and after dofetilide (1 μM) blockade.

inactivation compared with that seen in the whole-cell mode could be explained by the accumulation of low concentrations at the site of action.

Although hERG activators RPR260243 (Kang et al., 2005), NS1643 (Hansen et al., 2006a), NS3623 (Hansen et al., 2006b), and PD-118057 (Zhou et al., 2005) have been shown

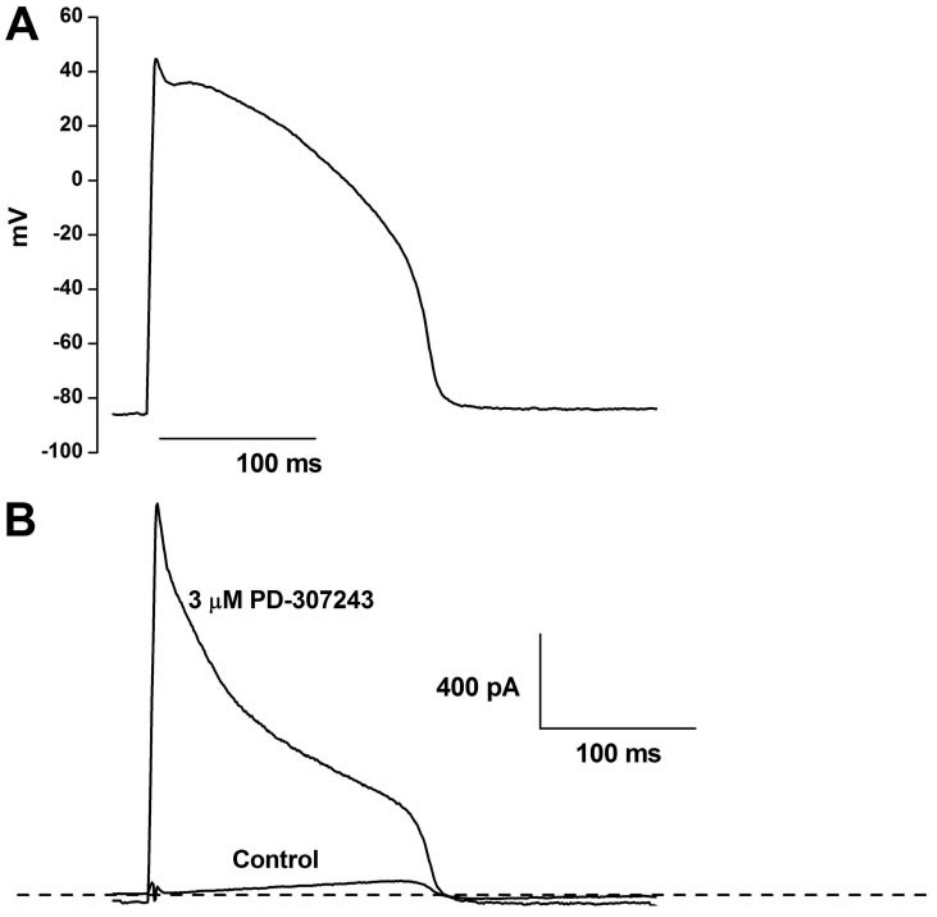

Fig. 10. PD-307243 increases total potassium ions conducted by hERG channels during an action potential stimulus. A, a prerecorded action potential trace was used as the voltage protocol to induce hERG currents. The protocol was repeated at 5-s interval. B, hERG current traces before and after PD-307243 (3 μM) application when drug effect reached steady state.

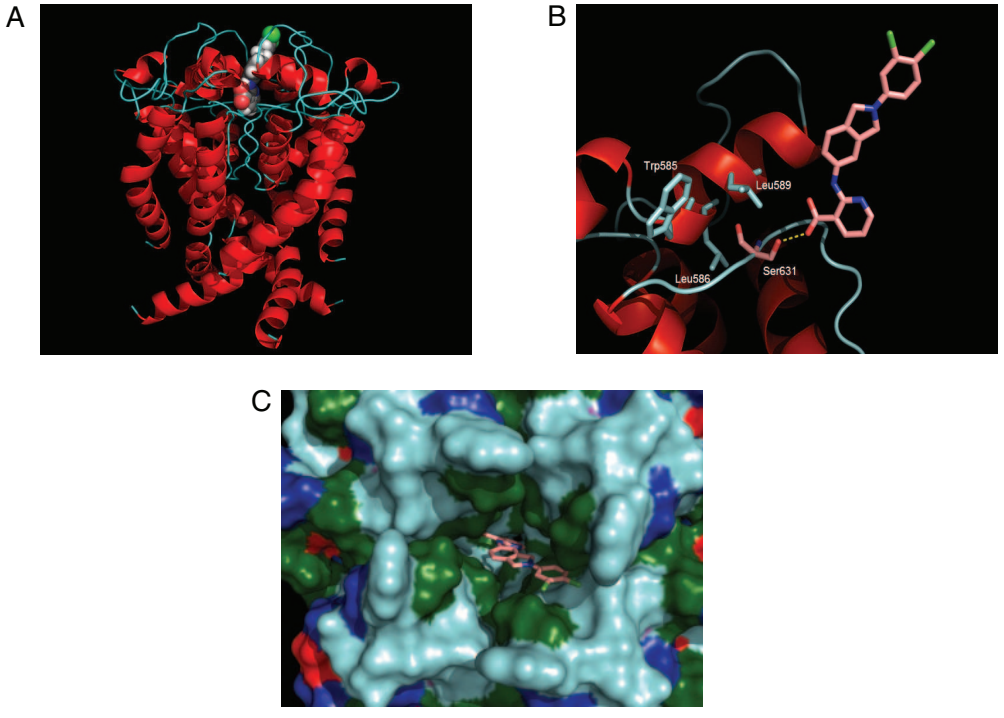

Fig. 11. PD-307243 docks in the extracellular regions of the channel pore. A, hERG channel model with backbone cartoon representation, where red domains are α-helical and cyan domains are turns. The channel activator, PD-370243, is shown as a space-filling model, colored by atom type. B, the carboxylic acid moiety of the hERG channel activator, PD-370243, is shown interacting with the side chain hydroxyl group of serine 631. The conserved hydrophobic residues, Trp585, Leu586, and Leu589 are shown with their side chains oriented toward the center of the pore channel. C, the hERG channel model surface is colored by chemical nature, with hydrophobic residues in green, hydrophilic residues in cyan, basic residues in blue, and acidic residues in red. The pore channel is surrounded by hydrophobic residues at the mouth, where the more lipophilic domains of the activator are interacting, notably the dichlorophenyl moiety.

to shorten cardiac action potential, only RPR260243 has been tested on I_{Kr} in native cells. As seen in the hERG channels expressed in CHO cells, RPR260243 slows I_{Kr} deactivation in guinea pig ventricular myocytes (Kang et al., 2005). We also clearly demonstrated that I_{Kr} activated by PD-307243 in rabbit ventricular myocytes had an upstroke followed by inactivation as seen in CHO cells expressing hERG channels, although less dramatic. This confirmed that the effects of PD-307243 on the deactivation and inactivation properties of hERG channels were not limited to the heterologous expression system but were reproducible in native channels passing I_{Kr} in ventricular myocytes.

The observations that hERG channel deactivation and inactivation were greatly altered in the presence of the compound could also explain the hERG current profile during an action potential. In the presence of the drug, hERG channels became constitutive and lose characteristic rectification. Therefore, the hERG current more or less passively followed the membrane potential. hERG channel gating is suited for the role it plays in driving phase III of the ventricular action potential, rapid inactivation rendering the channels unable to conduct potassium ions during phase II of the action potential (Vandenberg et al., 2004; Witchel, 2007). If this was altered, it could have consequences on the generation of other ion channel currents. We postulate that the instantaneous I_{Kr} observed in the presence of PD-307243 could prematurely terminate the action potential of ventricular myocytes and be potentially proarrhythmic, provided that PD-307243 is a selective hERG (I_{Kr}) activator. It should be noted that activators of hERG have been shown to shorten the QT interval (Kang et al., 2005; Zhou et al., 2005). Therefore, the mechanisms by which small molecule activation of hERG channels occurs need to be carefully studied in the search for activators that will optimally modify the action potential repolarization in patients with LQTS. The nonspecific nature of activities of PD-307243 on a number of cardiac ion channels that are known to play important roles in the genesis and maintenance of the ventricular action potential renders PD-307243 inappropriate to demonstrate the effect of I_{Kr} activation on action potential shortening. Activation of I_{Kr} and L-type Ca^{2+} currents would offset each other and make the action potential data difficult to interpret.

Taken together, the results of this study reveal that hERG channel activation by PD-307243 might be the result of its binding in the extracellular region of the pore. The compound modified channel inactivation and retarded deactivation holding the channel in a constitutively open state. Our findings provide the basis for further studies that will not only be useful in the rational design of hERG channel openers but could also be critical for the elimination of unwanted effects resulting from hERG channel activation.

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