Inactivation gating determines nicotine blockade of human HERG channels

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1Research Center, Montreal Heart Institute, Montreal H1T 1C8; 2Department of Medicine, University of Montreal, Montreal, Quebec, Canada H3C 3J7; and 3Department of Pharmacology, Zhelimuneng Medical School, Tongliao, Inner Mongolia 028000, China

Wang, Hui-Zhen, Hong Shi, Shu-Jie Liao, and Zhiguo Wang. Inactivation gating determines nicotine blockade of human HERG channels. Am. J. Physiol. 277 (Heart Circ. Physiol. 46): H1081–H1088, 1999.—We have previously found that nicotine blocked multiple K+ currents, including the rapid component of delayed rectifier K+ currents (IKr), by interacting directly with the channels. To shed some light on the mechanisms of interaction between nicotine and channels, we performed detailed analysis on the human ether-a-go-go-related gene (HERG) channels, which are believed to be equivalent to the native IKr, when expressed in Xenopus oocytes. Nicotine suppressed the HERG channels in a concentration-dependent manner with greater potency with voltage protocols, which favor channel inactivation. Nicotine caused dramatic shifts of the voltage-dependent inactivation curve to more negative potentials and accelerated the inactivation process. Conversely, maneuvers that weakened the channel inactivation gating considerably relieved the blockade. Elevating the extracellular K+ concentration from 5 to 20 mM increased the nicotine concentration (by ~100-fold) needed to achieve the same degree of inhibition. Moreover, nicotine lost its ability to block the HERG channels when a single mutation was introduced to a residue located after transmembrane domain 6 (S631A) to remove the rapid channel inactivation. Our data suggest that the inactivation gating determines nicotine blockade of the HERG channels.

Xenopus oocyte; voltage-clamp techniques

Nicotine, a cyclic alkaloid, is the main constituent of tobacco smoke responsible for the elevated risk of the cardiovascular disease and sudden coronary death associated with smoking (13). It is thought that nicotine causes its acute adverse effects by provoking cardiac arrhythmias (3, 8, 9, 15, 24, 31). The mechanisms by which nicotine favors arrhythmias have been attributed to its ability to enhance catecholamine release as a result of stimulation of nicotinic acetylcholine receptors (nAChRs) (2). However, we have recently found that nicotine can also block a number of K+ currents by interacting directly with these channels without the involvement of nAChRs and catecholamine release (26). The currents sensitive to nicotine include inward rectifier K+ current (Ito), transient outward K+ current (Ito), and the rapid component of the classic delayed rectifier K+ currents (IKr) in dog ventricular myocytes (26). Blockade of K+ currents may provide an explanation, complementary to the “catecholamine release” theory (2), for previously observed membrane depolarization and action potential duration (APD) prolongation caused by nicotine (7, 11, 16).

It is of interest and importance to understand in depth the mechanisms by which nicotine interacts with cardiac K+ channels to understand the pathophysiological implications of nicotine’s action. Yet, a potential problem of studying endogenous currents is the difficulty of accurately dissecting each of the multiple currents from others that coexist in a single cell. Studies using cloned channels equivalent to the native channels would help us better understand the problem and the characteristics of drug-channel interactions. In particular, the ability of nicotine to block Ikr may account to some extent for its ability to lengthen APD and to alter the propensity of arrhythmias. We therefore assessed in the present study the effects of nicotine on the human HERG channels that generate currents, when expressed in Xenopus oocytes, equivalent to the endogenous current Ikr (20).

METHODS

Functional expression in Xenopus laevis oocytes. Procedures for in vitro transcription and oocyte injection have been previously described in detail (30). HERG (a kind gift from Dr. Mark Keating) was subcloned into pSP64 vector. The S631A mutant of HERG is a kind gift from Dr. Terence E. Hébert. cRNAs were prepared with the mMESSAGE mMACHINE kit (Ambion) using SP6 RNA polymerase according to manufacturer’s protocols. cRNAs were dissolved in diethyl pyrocarbonate-treated sterile water, stored at −80°C, and diluted immediately prior to injection. Stage V–VI Xenopus oocytes were injected with 46 nl of cRNA.

Two-electrode voltage-clamp recording. Approximately 48 h after cRNA injection, two-electrode voltage clamp was performed on individual oocytes as previously described (30). Electrodes filled with 3 M KCl containing 10 mM HEPES had a resistance of ~0.6–2 MΩ when measured in the bath solution containing the following (in mM): 100 NaCl, 5 KCl, 0.3 CaCl2, 2 MgCl2, and 10 HEPES (pH 7.4). The electrodes were connected to a GeneClamp-500 amplifier (Axon Instrument, Burlington, CA). The pCLAMP suite of programs was employed for data acquisition and analysis. Records were digitized at 5 kHz and filtered at 2 kHz. Experiments were conducted at room temperature (22–23°C).

Data analysis. Group data are means ± SE. Statistical comparisons among groups were performed by ANOVA. If significant effects were indicated by ANOVA, a t-test with Bonferroni correction or a Dunnett’s test was used to evaluate the significance of differences between individual means. Otherwise, baseline and drug data were compared by Student’s t-test. A two-tailed P < 0.05 was taken to indicate a statistically significant difference. A nonlinear least-square curve-fitting program (CLAMPFIT in pCLAMP 6.0 or Graphpad Prism) was used to perform curve-fitting procedures.
Expression of HERG resulted in the induction of a K\textsuperscript{+} conductance with characteristic activation and rectification properties of the endogenous I\textsubscript{Kr}. Because of the rapid C-type inactivation of the HERG channels compared with their activation, outward currents at potentials positive to 0 mV became smaller (21, 23, 27). The inactivation is rapidly weakened on hyperpolarization to negative potentials, as indicated by the rising phase of the tail currents (Fig. 1). Nicotine applied to the superfusion solution produced concentration-dependent suppression of the HERG channels, as illustrated in Fig. 1.

Voltage dependence. The voltage dependence of HERG blockade by nicotine was assessed with three different I-V protocols, i.e., the standard current-voltage (I-V) protocols, the fully activated I-V protocols, and the instantaneous I-V protocols. With the standard I-V protocols, activating currents were elicited by 2-s depolarizing pulses ranging from -60 to +50 mV and tail currents by 1-s repolarizing pulses to -50 mV. The voltage steps were delivered from a holding potential (HP) of -70 mV at an interpulse interval of 10 s. The standard I-V relationship demonstrated strong inward rectification from potentials positive to -10 mV. Nicotine diminished the currents at various concentrations from 1 to 1,000 µM (Fig. 2A, top). The degree of blockade appeared enhanced with stronger depolarization and stronger apparent inward rectification or rapid inactivation from -30 and +30 mV (Fig. 2A, bottom). To determine the fully activated I-V relationships, a 1-s prepulse to +40 mV was applied before each of the repolarizing pulses to the test potentials ranging from -140 mV to +20 mV. Note that the prepulse
potential to +40 mV was positive enough to induce full conductance of the channels but also rendered a large amount of channels into the inactivated state (see Fig. 4). Thus the fully activated I-V curve also demonstrated striking inward rectification with a negative slope from potentials more positive than −20 mV. Nicotine produced a block of the currents at all test potentials with more pronounced inhibition at potentials between −60 to 0 mV (Fig. 2B). Similar to the standard I-V data, the inhibitory effects of nicotine were more pronounced as the channel rectification (inactivation) manifested at positive potentials. The reduced potency at potentials positive to +30 mV with the standard I-V protocols (Fig. 2A, bottom) could be explained by the relatively slow association rate of nicotine to the HERG channels compared with the rate of endogenous channel inactivation at such positive potentials. In other words, the HERG channel inactivation occurred largely before nicotine could bind to the channels and produce the inhibitory effects. If it is true that nicotine blockade depends on channel inactivation, then removal of rectification should eliminate the correlation between the blockade and the voltage. Hence, the instantaneous I-V protocols were used to test this possibility. The channels were first inactivated by clamping the membrane at +40 mV for 1 s followed by a prepulse to −100 mV for 20 ms. This prepulse was long enough for rapid recovery of the HERG channels from inactivation but was short enough to prevent significant channel deactivation. After the recovery prepulse, a family of test pulses were delivered to potentials ranging from −140 to +20 mV. The currents recorded at the test pulses were fitted by the single exponential function with extrapolation to the initial point of the test pulse, and the amplitude was plotted against test potentials (TPs) to construct the instantaneous I-V curves. The I-V relationships from such protocols were linear (Fig. 2C, top) because minimal inactivation occurred during the prepulse. This protocol resulted in significantly less HERG-channel blockade (Fig. 2C, bottom) when compared with the standard and the fully activated I-V protocols.

The percent reduction at a given TP was smaller with instantaneous I-V protocols compared with the other two protocols. For example, at 0 mV, the reduction of HERG currents by 5 µM nicotine was 23.1 ± 6.0% with the standard I-V protocols, 43.7 ± 6.7% with the fully activated I-V protocols, and only 12.6 ± 4.5% with the instantaneous I-V protocols (Fig. 2B). The inhibitory potency of nicotine was evaluated with the three different voltage protocols, as illustrated in Fig. 3C. The dose-response curves were constructed by plotting the percent block of the step currents at a TP of 0 mV as a function of drug concentrations. The IC₅₀ values calculated from the Hill equation were 16.8 ± 2.2 µM (Hill coefficient = 0.74) for the standard I-V protocols and 1.8 ± 0.3 µM (Hill coefficient = 0.51) for the fully activated I-V protocols. The values for the instantaneous I-V protocols could not be calculated because the concentrations of nicotine used were not high enough to reach maximal effects. Apparently, nicotine is ~10 times and at least 100 times more potent with the fully activated I-V protocols than with the standard and the instantaneous I-V protocols, respectively. It appears that the conditioning pulses that render the channels inactivated can facilitate the drug binding and blockade.

Effects on the activation and inactivation properties. The fact that nicotine blocks the HERG channel more strongly at potentials with apparent rectification (or inactivation, the standard I-V and the fully activated I-V vs. the instantaneous I-V) and particularly with inactivating prepulses (the fully activated I-V vs. the standard I-V) suggests that nicotine preferentially blocks the HERG channels when they are in the inactivated state. If this is true, then it is expected that nicotine should be able to alter the inactivation parameters but leave the activation gating unchanged. Therefore, we performed analysis on the data regarding the voltage-dependent
activation and inactivation gating properties and the drug effects. The activation curves were constructed by normalizing the tail currents recorded with the standard I-V protocols. The normalized data (or conductance) were plotted against the prepulse potentials and fitted to the Boltzmann distribution. Nicotine at concentrations lower than 1,000 µM did not alter the activation parameters (Fig. 4): the half-maximum activation voltages (activation V½ values) were −36.6 ± 4.3 mV under control conditions and −30.6 ± 5.1 mV with 100 µM nicotine (P > 0.05, n = 5). Nicotine at 1,000 µM produced slight but statistically significant changes in V½ values. In contrast, the inactivation characteristics were markedly affected by nicotine, as illustrated in Fig. 4, C and D. Nicotine caused apparent shifts of the inactivation curves to the negative direction in a concentration-dependent fashion. The half-maximal inactivation voltage (inactivation V½ values) was changed from −49.9 ± 4.4 mV under control conditions to −55.4 ± 3.6, −61.3 ± 6.5, and −88.6 ± 8.7 mV with the drug concentrations of 1 (P < 0.05, n = 5), 5 (P < 0.01, n = 6), and 1,000 µM (P < 0.01, n = 6), respectively. The slope factor was not significantly altered (−26.7 ± 3.4 mV for control vs. −23.9 ± 2.9 mV for nicotine at 1 mM, P > 0.05, n = 6). The blockade of the currents elicited at the TP of 0 mV was more prominent with less negative prepulse potentials. For example, at a holding potential of −80 mV, nicotine produced a 22.4 ± 9.0, 34.1 ± 7.5, and 50.9 ± 11.1% reduction of the activating current amplitude at 1, 5, and 10 µM, respectively. In comparison, the respective percent block increased to 36.9 ± 7.5, 50.2 ± 6.0, and 71.5 ± 3.5 at a holding potential of −20 mV. Note that even at −140 mV, nicotine still caused certain degrees of blockade (15.4 ± 7.0, 23.5 ± 7.8, and 37.9 ± 14.9% by 1, 5, and 10 µM nicotine, respectively). The results indicated that the inactivation of the HERG channels facilitated nicotine binding to the channels and that the channel blockade was not merely a consequence of a steady-state inactivation shift.

Effects on kinetics. Once activated, the HERG channels undergo complex process from closed states to open/inactivation, inactivation, deactivation, and finally to closed states again. Open channel blockers often cause apparent acceleration of activation time course and/or apparent deceleration of deactivation.

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Fig. 4. Effects of Nic on voltage-dependent activation and inactivation properties of HERG channels in Xenopus oocytes. A: analog data showing tail currents elicited on repolarization to −50 mV, after 2-s prepulses from −60 to +20 mV. B: average data showing effects of Nic on conductance curve of HERG channels. Tail currents (A) were normalized to maximum value obtained with a prepulse to +20 mV, and conductance (normalized tail currents) was plotted against prepulse voltage to construct activation conductance curve. Symbols are experimental data (n = 6), and lines represent best fits to Boltzmann distribution: 1/\(I_{\text{max}} = 1/(1 + \exp(V_{\text{V_{0}}} - V/k))\), I, current amplitude at a prepulse potential V; \(I_{\text{max}}\), maximal tail current amplitude after a prepulse to +20 mV; \(V_{\text{0}}\), voltage for half-maximal activation; k, slope factor. C: examples of currents elicited with protocols shown (inset) to evaluate inactivation properties of HERG channels before and after Nic. D: negative shift of inactivation curves by Nic. To construct inactivation curves, voltage protocols (inset, C) were employed: a 2-s depolarizing pulse to inactivate HERG channels followed by varying repolarizing pulses to potentials between −140 and +20 mV for a short period of 20 ms, followed by a test pulse to +20 mV. Current amplitude at TP was normalized and plotted against prepulse potentials. Symbols are experimental data and curves represent best fits to Boltzmann distribution.
time course because of reopening of the channels caused by drug unbinding. The activation time course was described by the single exponential function on the currents evoked at the TP of −20 mV from a HP of −70 mV. Nicotine did not significantly alter the activation time course, as indicated by the similar time constants before (403 ± 30 ms) and after 100 µM nicotine (384 ± 34 ms, P > 0.05, n = 7). The deactivation time constants were obtained by fitting, to the double exponential function, the tail currents elicited by a hyperpolarizing pulse to −120 mV after a 2-s prepulse at +40 mV. The descending phase represents recovery from inactivation, and the following rising phase represents the deactivation time course. The deactivation time constants were 75.6 ± 8.9 ms under control conditions and 78.4 ± 11.6 ms in the presence of 100 µM nicotine (P > 0.05, n = 6). Similarly, nicotine failed to change the recovery time constant (2.7 ± 0.4 ms for control and 2.8 ± 0.4 ms for nicotine at 100 µM, P > 0.005, n = 6), too.

In sharp contrast, nicotine markedly accelerated the inactivation time course of the HERG channels. The inactivation process was analyzed by fitting the currents elicited with the voltage protocols described in the inset of Fig. 5 to the single exponential function. A 20-ms hyperpolarizing pulse to −100 mV was sufficient for recovery from inactivation but too short to cause significant deactivation. The following depolarization-induced initial outward current reflects the open state of the HERG channels, which then again inactivate rapidly. Therefore, the decaying tail currents represent the HERG-channel inactivation but not deactivation. Examples are shown in Fig. 5A, and the averaged data on the concentration-dependent decreases in the inactivation time constant induced by nicotine are illustrated in Fig. 5B.

Effects of high external K+ concentration. The data discussed above point to one possibility: nicotine blocks the HERG channels by preferentially interacting with the inactivated channels. It is known that the unusually rapid inactivation of HERG channels is caused by C-type inactivation that is highly sensitive to external K+ concentrations ([K+]o) (28). Elevated [K+]o markedly slowed the kinetics and amplitude of the HERG-channel inactivation. Weakening of the HERG inactivation gating should relieve nicotine blockade. This was indeed confirmed by the following experiments. The HERG currents were recorded with 20 mM [K+]o, and the drug effects were evaluated with the three different I-V voltage protocols as already described above. The extent of channel blockade by nicotine also became smaller with higher [K+]o: 10 µM nicotine failed to affect the HERG channels regardless of I-V protocols used. In addition, to reach statistically significant blockade, nicotine concentration has to be raised to 100 µM (decreased by 35.9 ± 3% at 0 mV with the standard I-V protocols). Comparison of the percent inhibition of the HERG channels with different [K+]o is presented in Fig. 6.

Effects on inactivation-deficient mutant (S631A). To further investigate the possibility of inactivation block of the HERG channels by nicotine, we evaluated the effects of this compound on S631A mutant of the HERG channel, in which the rapid C-type inactivation gating is removed. The expression of S631A in oocytes gave rise to delayed-rectifier-like currents with rapid activation and only slight inactivation during the 2-s pulse. The inward rectification seen with the wild-type HERG channels was absent in the mutant (Fig. 7, A and B). For comparison, three different I-V protocols were used to assess the drug effects. The results are illustrated in Fig. 7. The potency of the drug effects was apparently diminished in the mutant. Nicotine at 5 µM, which suppressed the wild-type HERG channels by 12 to ~42% depending on different voltage protocols, did not at all affect the currents expressed by the mutated HERG channels. Elevation of the drug concentration by 2,000-fold to 10 mM still failed to exhibit any effects on the channels.

**DISCUSSION**

In the present study, we performed detailed analysis of nicotine-HERG channel interactions, in terms of the voltage dependence and concentration dependence as well as time dependence. We demonstrated that nicotine blocked the human HERG channels expressed in Xenopus oocytes, consistent with its ability to block Ikr...
The physiological counterpart of the HERG channels in our previous study (26). This study represents the first detailed characterization of direct interactions between nicotine and K₈ channels. A novel finding in this study is that the inactivation gating of the human HERG channels determines the potency of nicotine blockade.

The detailed analyses of our data revealed that blockade of the HERG channels by nicotine is mainly caused by the binding of nicotine to the inactivated channels. Our data provided several lines of evidence in support of this notion. First, nicotine produced more pronounced inhibition of the HERG channels with the voltage protocols that rendered the channels more in the inactivated state. For example, nicotine has higher potency when the test pulses are preceded by a conditioning pulse (2 s, to +40 mV), which favors channel inactivation. In addition, depolarized membrane potentials or less negative holding potentials facilitate nicotine binding to the HERG channels and promote the inhibitory effects. The second line of evidence is that nicotine caused marked negative shifts of the inactivation curves and alterations of inactivation parameters (the V₁/₂ values), although it left the voltage-dependent activation properties unchanged. The inactivation time course was also substantially accelerated by nicotine, whereas the activation time constant was not altered. Third, maneuvers that weakened or removed the channel inactivation such as the use of the instantaneous I-V protocols and elevation of [K⁺]o all substantially relieved the channels from blocking. Finally, more convincing data were obtained from the experiments demonstrating a failure of nicotine (up to 10 mM) to affect the inactivation-deficient mutant (S631A) of the HERG channels. The results from the mutant seem to suggest that the channel inactivation is absolutely required for nicotine block of the HERG channels. A similar mode of drug-HERG interactions has also been documented in several studies using antiarrhythmic (6, 22) and nonantiarrhythmic agents (17, 18, 25). For example, Suessbrich et al. (25) reported blockade of HERG by haloperidol (an antipsychotic drug) with similar potency to nicotine. The mechanism of haloperidol block involves binding to inactivated channels as

![Fig. 6. Effects of Nic on HERG channels under elevated external K⁺ concentration ([K⁺]o). Currents were recorded with [K⁺]o of 20 mM. A: analog data obtained with standard I-V protocols as described in Fig. 1. B: percent blockade of HERG channels with [K⁺]o of 20 mM at 0 mV with 3 different protocols, compared with that with [K⁺]o of 5 mM. *P < 0.05 and **P < 0.01 vs. control.](#)

![Fig. 7. Effects of Nic on inactivation-deficient mutant of HERG channels (S631A). A: raw data from a representative oocyte. Currents were elicited with standard I-V protocols. B: superimposed I-V curves in absence and presence of 10 mM Nic, C: percent blockade of S631A mutant by 10 mM Nic with 3 different I-V protocols as described in Fig. 2.](#)
inactivation enhanced and removal of inactivation weakened the inhibition. A study utilizing inactivation-deficient mutant (S620T) by Ficker et al. (10) also elegantly demonstrated that the inhibitory potency of dofetilide on HERG was considerably reduced in the absence of channel inactivation gating, as in our study with S631A mutant. The authors concluded that a C-type inactivation process is crucial for high-affinity binding of dofetilide (10). Similarly, a study by Herzberg et al. (12) also clearly demonstrated that the blockade of HERG by E-4031 was largely diminished with the mutations disabling the rapid HERG channel inactivation.

Decrease in \( I_{Kr} \) has been implicated in a variety of diseased states of the heart, including myocardial infarction and ischemia, cardiac hypertrophy, and heart failure, etc. (4, 5, 14, 29, 30, 32). This decrease is often accompanied by generation of arrhythmias, such as ectopic beats, early afterdepolarization, and triggered activity (1, 32). Reduction of \( I_{Kr} \) could be antiarrhythmic (e.g., the reentrant types of arrhythmias) or proarrhythmic (e.g., early afterdepolarization, long Q-T syndrome). Nicotine has been associated with the elevated risk of sudden coronary death (13) by provoking arrhythmias (3, 8, 9, 15, 24, 31) through interfering with the electrical activity of the heart. The averaged blood levels of nicotine are about 200 nM in smokers (2) and the peak concentration can reach up to 440 nM 2 h after one cigarette and could be much higher in heavy smokers (19). Nicotine (0.1 to 1 \( \mu \)M) used in our experiments is therefore comparable with the blood levels in the smokers. Nicotine (0.1 \( \mu \)M) blocks the HERG channels by about 11% at the TP of 0 mV in the present study and blocked 17% \( I_{Kr} \) in our previous studies (26). The percent blockade by 0.1 \( \mu \)M nicotine (11%) is small but could be physiologically significant considering the high impedance of the plateau phase repolarization. It should be noted that the efficacy ofnicotine might have been underestimated in our study because the cytoplasm of oocytes is packed with lipophilic yolk granules that can absorb the drugs and also because the experiments were conducted at room temperature (22–23°C), which can often decrease drug-sensitivity because the cytoplasm of oocytes is packed with lipophilic yolk granules that can absorb the drugs and also because the experiments were conducted at room temperature (22–23°C), which can often decrease drug-sensitivity.

In conclusion, nicotine blocks the HERG channels expressed in Xenopus oocyte in a concentration-dependent manner, which is in agreement with our observations on the inhibition of \( I_{Kr} \) in canine ventricular cells. The effects of nicotine appear to be dependent on the inactivation gating of the HERG channels, with the blockade enhanced with predominant inactivation and relieved in the absence of inactivation. Our study represents the first detailed investigation of nicotine's direct interaction with the cloned channels, which reveals a novel aspect of nicotine's pharmacology.

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