Chemical Modification of the Human Ether-a-go-go-related gene (HERG) K⁺ Current by the Amino-Group Reagent Trinitrobenzene Sulfonic Acid

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We investigated the effects of trinitrobenzene sulfonic acid (TNBS), an amino-group reagent, on the human ether-a-go-go-related gene (HERG) K⁺ channels expressed in Xenopus oocytes. TNBS neutralizes the positively charged amino-groups of peptide N-terminal and lysine residues. External application of TNBS at 10 mM for 5 min irreversibly shifted the curves for currents at the end of the pulse and tail currents of HERG to a more negative potential and decreased the maximal amplitude of the I_{tail} curve ($I_{tail,max}$). TNBS had little effect on either the activated current-voltage relationship or the reversal potential of HERG current, indicating that TNBS did not change ion selectivity properties. TNBS shifted the time constant curves of both activation and deactivation of the HERG current to a more hyperpolarized potential; TNBS's effect was greater on channel opening than channel closing. External H⁺ is known to inhibit HERG current by shifting $V_{1/2}$ to the right and decreasing $I_{tail,max}$. TNBS enhanced the blockade of external H⁺ by exaggerating the effect of H⁺ on $I_{tail,max}$, not on $V_{1/2}$. Our data provide evidence for the presence of essential amino-groups that are associated with the normal functioning of the HERG channel and evidence that these groups modify the blocking effect of external H⁺ on the current.

Key words: H⁺, HERG channel, LQT, Rapidly activating delayed rectifier K⁺ current, Torsades de pointes, Trinitrobenzene sulfonic acid

INTRODUCTION

K⁺ channels encoded by the human ether-a-go-go-related gene (HERG) (Trudeau et al., 1995) play an important role in initiating cardiac repolarization by generating a rapid component of the delayed rectifier cardiac K⁺ current (I_{Kr}) (Sanguinetti et al., 1995). HERG is a major target in both acquired and inherited forms of long QT syndrome (LQT). Mutations in HERG have been shown to cause chromosome 7-linked inherited LQT (LQT2) (Curran et al., 1995), reflected by an increased ventricular action potential duration. Several drugs that block I_{Kr} and HERG cause acquired LQT and torsades de pointes (Snyders and Chaudhary, 1996; Suessbrich et al., 1996; Jo et al., 2000; Lee et al., 2004). Exploring mechanisms of mutation- or drug-induced HERG channel modulation requires molecular and chemical studies that can give us information on structure and function.

Several studies showed that chemical modifications of the specific group of amino acid residues, such as histidine residue (Spires and Begenisich, 1990) or amino-groups of peptide N-terminal and lysine residue (Cahalan and Pappone, 1983; Spires and Begenisich, 1992a) changed the properties of K⁺ currents of nerve cells. In contrast, modifying the sulfhydryl residue to a stable S-dinitrophenyl derivative had no effect on the K⁺ current (Spires and Begenisich, 1992a). Modification of histidine residue on the external surface of delayed rectifier K⁺ channel by diethylpyrocarbonate slowed channel opening

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in squid giant axon (Spires and Begenisich, 1990). Neutralization of positively charged peptide terminal amino and e-amino-groups of lysine residues of K^+ channel by trinitrobenzene sulfonic acid (TNBS) altered the size of ionic current and the kinetics of activation or deactivation (Cahalan and Pappone, 1983; Spires and Begenisich, 1992a). The groups also have shown to be involved in divalent cation such as Zn^{2+} binding to neuronal K^+ channel (Spires and Begenisich, 1992b).

In spite of the aforementioned investigations about the role of specific amino acids or reacting groups in regulation of K^+ channel, little is known about the effects of chemical reagents on HERG K^+ current and human cardiac I_{Kr}. As the primary structures for HERG channel has been determined (Warmke and Ganetzky, 1994), functional modification approach by specific reagents, together with available model of protein folding in the membrane, may become increasingly useful to map the location of groups relevant to channel function. In the present study, we have investigated the effects of TNBS, which neutralizes the positively charged amino-group of lysine residues on the outer membrane surface of HERG, on the properties of HERG channel expressed in *Xenopus* oocytes. Also, we examined the effect of TNBS on HERG current alteration by external pH (pH_o) to test the possible involvement of the residues in external H^+-induced channel blockade.

MATERIALS AND METHODS

Expression of HERG in oocytes

Complementary RNA of HERG was synthesized by in vitro transcription from 1 mg of linearized cDNA using T7 message machine kits (Ambion, Austin, TX, U.S.A.) and stored in 10 mM Tris-HCl (pH 7.4) at -80°C. Stage V-VI oocytes were surgically removed from female *Xenopus laevis* (Nasco, Modesto, CA, U.S.A.) anaesthetized with 0.17% tricane methanesulphonate (Sigma). Theca and follicle layers were manually removed from the oocytes by using fine forceps. Oocytes were then injected with 40 nl of cRNA (0.1-0.5 mg·mL⁻¹). After injection, oocytes were maintained in modified Barth's solution containing (mM): 88 NaCl, 1 KCl, 0.4 $CaCl_2$, 0.33 $Ca(NO_3)_2$, 1 $MgSO_4$, 2.4 $NaHCO_3$, 10 HEPES (pH 7.4), supplemented with 50 mg·mL⁻¹ gentamicin sulphonate. Currents were studied 2-7 days after injection.

Solutions and voltage clamp recording from oocytes

Normal Ringer solution contained (mM): 96 NaCl, 2 KCl, 1.8 $CaCl_2$, 1 $MgCl_2$, 10 HEPES (pH adjusted to 7.4 with NaOH). We used an amino-group reagent, TNBS, to neutralize the peptide terminal amino-group and the e-amino-group of lysine groups. Because TNBS react with

the neutral form of amino-groups, TNBS treatments were done with solutions of elevated pH 9.0 buffered with 10 mM CHES (2-[N-cyclohexylamino]ethane-sulfonic acid) (Means and Feeny, 1971; Cahalan and Pappone, 1983; Spires and Begenisich, 1992a; Spires and Begenisich, 1992b). Solution of TNBS was made up directly in the external solutions, shortly before the experiment. Solutions were applied to the oocyte by continuous perfusion of the chamber and solution exchanges were completed within 3 min. All the effects of TNBS were irreversible (our observations showed that the effects of TNBS on HERG current were not reversible even after 1.5 h-washout) due to the covalent conversion of amino-groups to a neutral trinitrobenzene derivative (Means and Feeny, 1971). Also, solutions of pH 9.0 (10 mM CHES) in the absence of TNBS produced no irreversible effects; the average changes of the potential required for half-maximal activation ($V_{1/2}$) and the maximal amplitude of tail currents (I_{tail}) curve were 1.8 ± 1.2 mV ($P > 0.05$, n = 3) and 2.7 ± 1.5% ($P > 0.05$, n = 4), respectively, after 5 - 10 min exposure to solutions of pH 9.0. Therefore, we examined the effects of TNBS by recording HERG current before the reagent treatment, during treatment with TNBS for 5 min, and 10 min after washout with normal Ringer solution. Experimental solutions of different pH values (pH 6.2 and 8.0) were made using HEPES-buffered solutions titrated with NaOH and decreasing the concentration of $CaCl_2$ (0.5 mM). Currents were recorded at room temperature (21-23°C) with a two-microelectrode voltage clamp amplifier (Warner Instruments, Hamden, CT, U.S.A.). Electrodes were filled with 3 M KCl and had a resistance of 2-4 MW for voltage-recording electrodes and 0.6-1 MW for current-passing electrodes. Stimulation and data acquisition were controlled with Digidata and pCLAMP software (Axon Instruments). All chemicals were purchased from Sigma (St. Louis, MO).

Analysis

All quantitative data are expressed as mean values ± S.E.M. The results were analyzed using ANOVA. Differences were considered significant when values were $P < 0.05$.

RESULTS

Effects of TNBS on the HERG current were studied using *Xenopus* oocyte expression system. Throughout the experiments, holding potential was adjusted -70 mV and the repolarization potential was held constant at -60 mV for I_{tail}. Fig. 1A shows an example of voltage-clamp recording from the *Xenopus* oocyte cell with the representative current traces from a cell in control and after exposure to 10 mM TNBS. The amplitude of outward currents measured at the end of the depolarizing pulse
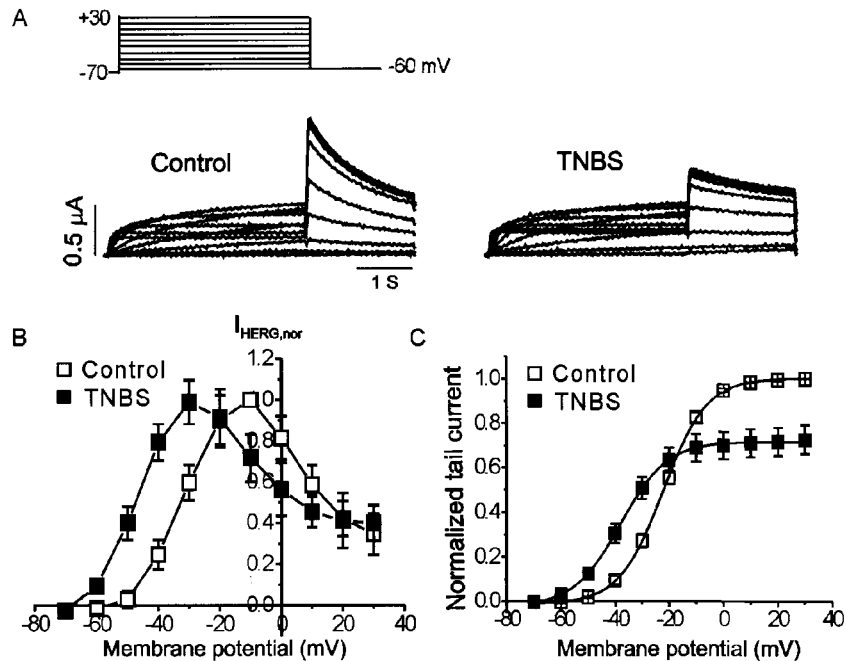
Fig. 1. Effect of trinitrobenzene sulfonic acid (TNBS) on the HERG currents expressed in *Xenopus* oocytes. Oocytes were treated with TNBS (10 mM) for 5 min and washed out for 10 min. All currents were recorded in normal Ringer solution. **A**: Superimposed current traces elicited by depolarizing voltage pulses (4 s) in 10 mV steps (upper panel) from a holding potential of -70 mV before treatment of TNBS (control) and after the treatment of TNBS. **B**: Plot of the normalized HERG current measured at the end of depolarizing pulses ($I_{HERG,nor}$) against the pulse potential in the control and TNBS conditions. The maximal amplitude of the I_{HERG} in the control was given a value of 1. **C**: Plot of the normalized tail current measured at its peak just after repolarization. The amplitude of the tail current in control was taken as 1. *Squares* with error bars represent mean ± S.E.M. (n = 5). Control and TNBS data were fitted to the Boltzmann equation, $y = 1/\{1 + exp[(-V + V_{1/2})/dx]\}$, with $V_{1/2}$ of -22.2 and -38.1 mV, respectively.
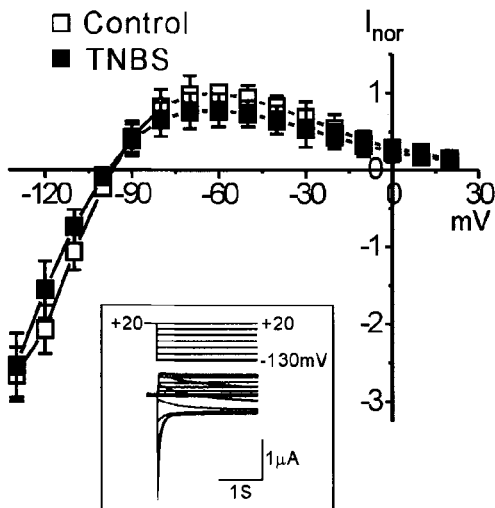
Fig. 2. Effects of TNBS on the fully activated current-voltage relationship. Oocytes were treated with TNBS (10 mM) for 5 min and washed out for 10 min. All currents were recorded in normal Ringer solution. **Inset** Superimposed current traces (lower traces) elicited by various level of test pulses ranging from -130 to +20 mV following the prepulse to +20 mV for 4 s (upper traces). Peak currents measured in the beginning of test pulses were plotted against the test pulse potential in the control and TNBS conditions and the maximal amplitude of the current for the control was assigned a value of 1. *Squares* with error bars represent mean ± S.E.M. (n = 6).

(steady-state current, I_{ss}) was increased with more positive voltage steps, and reached a maximum value at -10 mV. Depolarizing steps to even more positive caused a decrease of the current, resulting in a negative slope of the IV curve (Fig. 1B). Current-voltage relationships for I_{ss} obtained at TNBS are plotted in Fig. 1B. The curve of I_{ss} was shifted 15.6 ± 1.9 mV (n = 5, $P < 0.05$) in the hyperpolarizing direction following TNBS treatment.

After the depolarizing steps, repolarization to -60 mV induced outward I_{tail} whose amplitude was even larger than the amplitude of I_{ss} observed during depolarization. This is a characteristic property of HERG current, and it is known to be due to the rapid inactivation mechanism (Sanguinetti *et al.*, 1995). The amplitude of I_{tail} was increased with depolarizing steps from -60 to +10 mV and was then superimposed on further depolarizing steps to +30 mV. After the application with TNBS, I_{tail} was changed, as shown in Fig. 1A. Amplitude of I_{tail} was normalized to the peak amplitude obtained in the control condition at a maximum depolarization, and was plotted against the potential of the step depolarization (Fig. 1C). The normalized I_{tail} reflects voltage dependence of activation of the HERG channels. Data obtained in control conditions were well fitted by the Boltzmann equation with $V_{1/2}$ at -22.2 ± 1.2 mV (n = 5). After TNBS treatment, the maximal amplitude

of I_{tail} curve was reduced to 72 ± 6% of control (n = 5, P < 0.05) indicating that the decrease in maximum conductance of HERG channels by TNBS. Also, TNBS shifted $V_{1/2}$ of the activation curve to the left; $V_{1/2}$ after exposure to the reagent was -38.1 ± 2.7 mV (n = 5, P < 0.05). The average of the $V_{1/2}$ shift by TNBS was -15.9 ± 2.1 mV (n = 5, P < 0.05) showing effects of TNBS on the voltage dependence of HERG channel activation.

In Fig. 2, the effect of TNBS on activated currents was tested. The activated current- voltage relationship was obtained by using a double pulse protocol shown in the inset of Fig 2. Depolarizing pulse to +20 mV, which

induced a full activation, was followed by various level of test pulse. The amplitude of the current was measured at its peak before deactivation occurred, and plotted against the test potential (Fig. 2). The fully activated current-voltage relationship showed a typical inward rectification of the HERG channel due to the rapid inactivation. The reversal potential and the fully activated current-voltage relationship were little changed by TNBS; the reversal potentials before and after treatment of TNBS were -97.1 ± 2.3 mV and -98.7 ± 1.9 mV, respectively (P > 0.05, n = 6). These results indicate that neutralization of lysine residue by TNBS did not affect either inactivation pro-
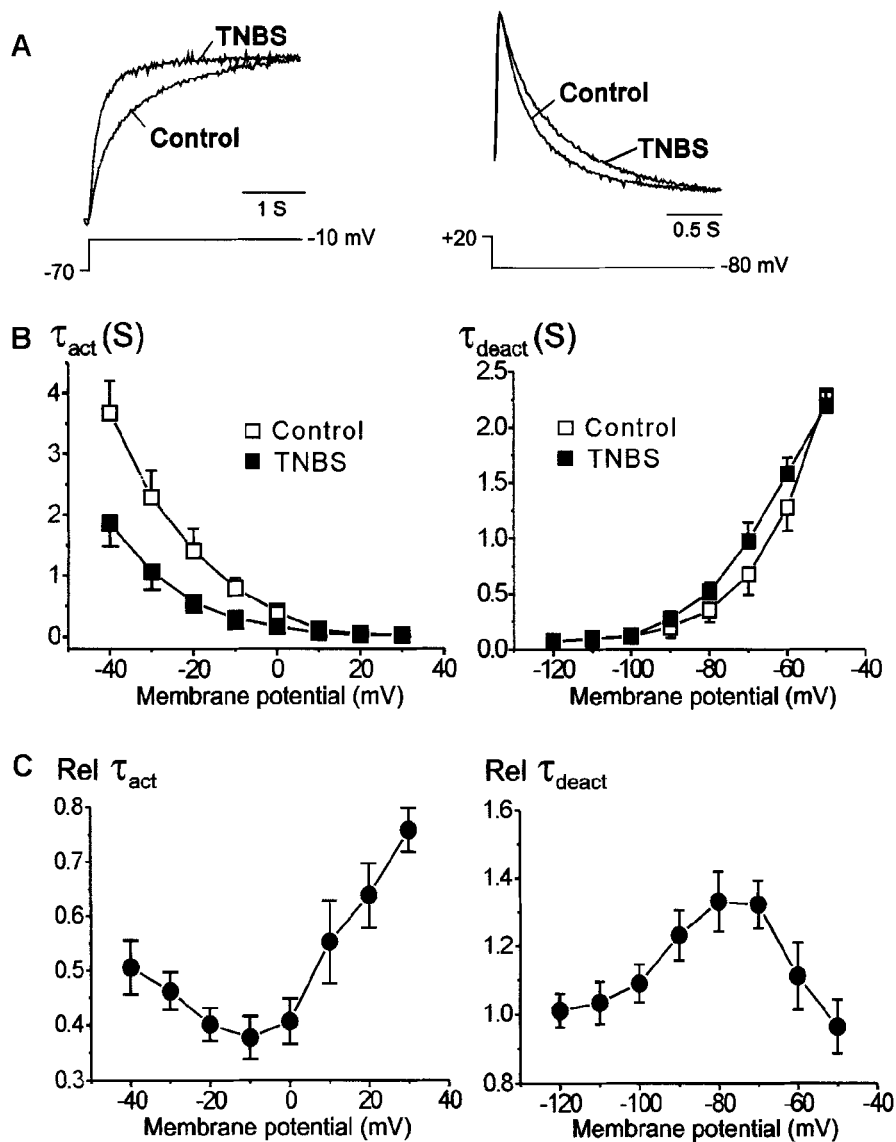
Fig. 3. Effects of TNBS on the kinetics of current onset and current decay. Oocytes were treated with TNBS (10 mM) for 5 min and washed out for 10 min. All currents were recorded in normal Ringer solution. A: Superimposed current traces recorded before and after TNBS treatment showing activation time course at -10 mV (left panel) and deactivation time course at -80 mV (right panel). The TNBS and control records were normalized. B: Effect of TNBS on the activation (left panel) and deactivation (right panel) time constants. C: Effect of TNBS on relative time constants. Activation (left panel) and deactivation (right panel) time constants after TNBS treatment relative to control values are shown at different voltages. Symbols with error bars in B and C represent mean ± S.E.M. (n = 4).
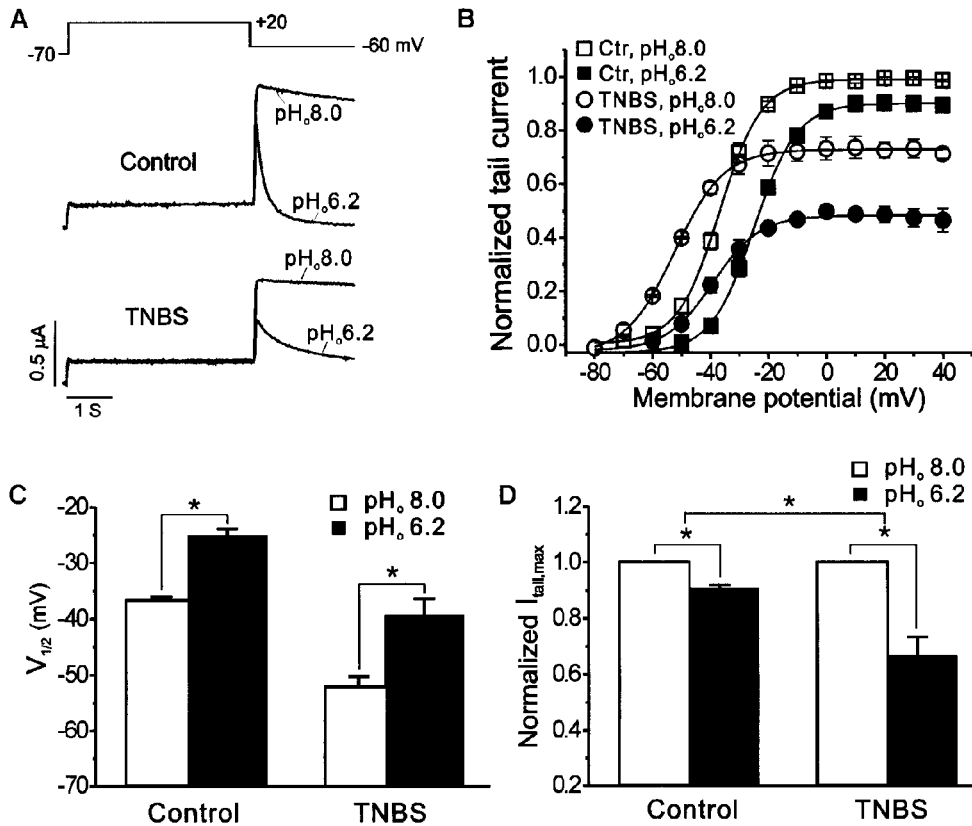
Fig. 4. Effects of TNBS on H$^+$-induced HERG channel blockade. Oocytes were treated with TNBS (10 mM) for 5 min and washed out for 10 min. All currents were recorded in normal Ringer solution. **A**: Superimposed current traces recorded in the presence of solutions of different external pH (pH$_o$ 8.0 and 6.2) before and after TNBS treatment. Note that the H$^+$- induced decrease of tail current amplitude measured at its peak just after repolarization was enhanced after TNBS treatment. **B**: Plot of the normalized tail current measured at its peak just after repolarization. The amplitude of the tail current in control, pH$_o$ 8.0 was taken as 1. *Symbols* with *error bars* represent mean ± S.E.M. (n = 5). The lines are the fits to the Boltzmann equation, $y = 1/\{1 + \exp[(-V + V_{1/2})/dx]\}$, where $V_{1/2}$ is the potential required for half-maximal activation ($V_{1/2}$ for Ctr, pH$_o$8.0, Ctr, pH$_o$6.2, TNBS, pH$_o$8.0, and TNBS, pH$_o$6.2 were -36.8, -24.9, -51.9 and -39.2 mV, respectively). **C**: The average data of $V_{1/2}$ values obtained from 5 cells. **D**: Bar graph shows the average decrease of the maximal amplitude of I$_{tail}$ curve (I$_{tail,max}$) from the pH$_o$ drop from 8.0 to 6.2 before and after TNBS treatment. I$_{tail,max}$ in pH$_o$ 6.2 solution was normalized to the I$_{tail,max}$ obtained in a solution of pH$_o$ 8.0. Data were from **B** (n = 5). *Bars* with error bars in **C** and **D** represent mean ± S.E.M and *P < 0.05.

perties or the ion selectivity of HERG channels.

To examine the effects of TNBS on HERG current kinetics, we measured the time courses of current activation and deactivation using a single-exponential function fit (Fig. 3A). Treatment with TNBS accelerated the activation time course (Fig. 3B, left panel; n = 4) and, in contrast, slowed deactivation time course (Fig. 3B, right panel; n = 4). To compare the effect of TNBS on activation and deactivation time course, ratio of the values after TNBS treatment to the control values was calculated. As shown in Fig. 3C, TNBS decreased relative activation time constant over a wide range of potentials and decreased the value maximally by 62 ± 4% at -10 mV. For the relative deactivation time constant, TNBS only increased the values over a narrow range of potentials and the maximum extent of the increase was 33 ± 9% at -80 mV (n = 4). The results show that TNBS produced a larger modification of activation than deactivation time course yet the modifica-

tions of activation and deactivation kinetics were both voltage dependent.

Next, we have examined the possible involvement of TNBS in H$^+$-induced HERG channel blockade. It has been known that external H$^+$ inhibited HERG current by two different mechanisms; voltage-dependent blockade (right shift of $V_{1/2}$) and the decreasing of g$_{max}$ in our previous study (Jo *et al.*, 1999). As shown in control of Fig. 4B, decrease of pH$_o$ from 8.0 to 6.2 shifted activation curve to the right by 11.9 ± 2.7 mV (n = 5, P < 0.05) and decreased g$_{max}$ by 9.0 ± 1.5% (n = 5, P < 0.05; see also Control of Fig. 4A). After TNBS treatment, the same change of pH$_o$ induced the right shift of the curve by 12.7 ± 2.1 mV (n = 5, P < 0.05) and the decrease of 33.3 ± 5.1% in g$_{max}$ (n = 5, P < 0.05; see also TNBS of Fig. 4A). The modification of amino-groups by TNBS did not significantly change voltage-dependent block induced by external H$^+$ (n = 5, P > 0.05, Fig. 4C), in contrast, it potentiated the ion's inhi-

bitory effect on g_{max} by ~3-folds (n = 5, P < 0.05, Fig. 4D). The results showed that TNBS enhanced the blockade of external H^+ via exaggerating the ion's effect on g_{max} not on $V_{1/2}$.

DISCUSSION

TNBS is a membrane impermeable agent reacting with amino-groups, resulting in production of a stable neutral trinitrobenzene derivative, but it can also modify sulfhydryl residues producing S-trinitrophenyl derivatives (Freedman and Radda, 1968; Means and Feeny, 1971; Bell and Bell, 1988). However, S-trinitrophenyl derivatives are unstable and the original sulfhydryl residue is spontaneously restored (Freedman and Radda, 1968; Means and Feeny, 1971). The apparent irreversibility of the TNBS effects reported here are consistent with amino but not sulfhydryl modification. Moreover, the information on HERG amino acid sequence indicates that its N-terminal group should be in the cytoplasm where externally treated TNBS could not access (Warmke and Ganetzky, 1994). Therefore, we conclude that an externally accessible charged amino-group of lysine residue plays important roles in activation and deactivation process and g_{max} of HERG channel.

Our results show that modification of HERG current by TNBS shifted the activation curve in a hyperpolarized direction and concomitantly decreased g_{max}. The both effects caused an increase of the tail current amplitudes at more negative than -20 mV, in contrast, the amplitudes were reduced by TNBS at more positive potentials than the voltage (Fig. 1C). Shifting of the voltage dependence of activation by neutralization of positively charged lysine could be considered to be a general phenomenon caused by a non-specific surface charge effect (Green and Andersen, 1991; Hille, 1992). This effect is thought to reflect the neutralization of lysine residue on the external surface of the channel, altering the voltage field that is sensed by the channel gating mechanism. According to this hypothesis, current activation and deactivation should be affected in a similar fashion and voltage-dependent curves for activation and deactivation kinetics shift to the left. Indeed, TNBS shifted the curves of activation and deactivation time constant to the left (Fig. 3B). However, there was a larger curve shift of activation time constant than that of deactivation. In addition, the g_{max} of HERG channels (current magnitude at highly depolarized potentials where there is already a high probability of opening) was significantly reduced by TNBS (Fig. 1C), which cannot be explained by the surface charge theory. Therefore, we propose that the neutralization with positively charged lysine on the external surface of HERG changed channel properties not only by increasing negative surface charge density but also by another mechanism which allows

HERG channel to decrease g_{max} and to open rapidly upon depolarization, possibly via conformational change. Also, the activation rates of the channel were not altered by TNBS in the voltage range from 0 to +30 mV (Fig. 3B), while the amplitudes of tail current were reduced by ~30% in the range (Fig. 1C). The results suggest that TNBS treatment could increase inactivation and/or deactivation rates of HERG channel.

There are several studies showing the effect of TNBS on K^+ channels in multicellular nerve tissues. Neutralization of positively charged peptide terminal amino and e-amino-groups of lysine residues by TNBS increased the size of K^+ current and slowed the activation in squid giant axon (Spires and Begenisich, 1992a). Also, the positively charged groups were involved in divalent cation binding to the channel in the same tissue (Spires and Begenisich, 1992b). In frog myelinated nerve fibers, TNBS slowed delayed rectifier K^+ channel closing without effect on channel opening time course and tail current amplitude (Cahalan and Pappone, 1983). Considering that TNBS accelerated channel opening rather than closing and decreased g_{max} of a molecular equivalent of I_{Kr} in the present study, it could be suggested the presence of tissue-, species-, and channel subtype-specific effects of TNBS on K^+ channel.

External H^+ is known to inhibit HERG current by two features, shifting $V_{1/2}$ to the right and decreasing g_{max}, via different binding sites (Jo et al., 1999). In the present study, we showed that TNBS did not change the blockade of external H^+ in respect to $V_{1/2}$ shift, while it enhanced the effect of the ion on g_{max} (Fig. 4). The reduction in conductance by H^+ was also observed in other K^+ current in squid giant axon and frog node of Ranvier (Hille, 1973; Schauf and Davis, 1976). From the quantitative analysis of this effect, it was suggested that one of the acidic groups within the pore is titrated by H^+, resulting in a decrease in cation permeability (Hille, 1973; Schauf and Davis, 1976). Therefore, it is possible that TNBS could induce conformational change of HERG channel, which would make external H^+ prone to access the acidic groups within the pore of the channel thereby facilitating reduction of conductance by external H^+.

Several mutagenesis studies have shown to be useful to our understanding the roles of specific amino acids in HERG function although it is not known the role of lysine residues. For example, an point mutations in HERG, T474I mutation, altered gating process of the channel (Zhou et al., 1998). A histidine located at the extracellular loop between S5 and the pore region of HERG has been reported to be involved in the C-type inactivation, K^+ selectivity, and voltage-dependence of activation (Dun et al., 1999). Furthermore, mutations of acidic residues in the S4-S5 linker of HERG to neutral or basic residue

accelerated the deactivation rate (Sanguinetti and Xu, 1999). Interestingly, a C-terminal splice variant of HERG (HERG$_{USO}$) has been identified in normal human heart, and this subunit coexpressed with HERG suppresses the HERG current amplitude, accelerates activation, and shifts the voltage-dependence by -8.8 mV, which are qualitatively similar to our findings with TNBS treatment in the present study (Kupershmidt et al., 1998). Therefore, neutralization of positively charged e-amino-groups of lysine residues by TNBS could change property of c-terminus of HERG subunit although direct biochemical evidence will be required to further test the postulate. There are four lysine residues in the extracellular region of HERG channel - one in between transmembrane segment S1 and S2 and three in between S5 and S6 (Warmke and Ganetzky, 1994). Given the large changes in g_{max} and activation kinetics we observed with amino-group reagent in the present study, these lysine residues may be interesting candidates for future site-directed mutagenesis investigation.

In summary, the present study shows that a modifier amino-group of lysine residues located on the external surface of HERG channel is involved in determining the channel properties including voltage dependences of channel activation and deactivation and g_{max}. Also, the group influences the blockade of external H^+ via expediting H^+-induced reduction of g_{max} when neutralized. The present functional modification study for lysine residues would be useful to map the location of groups relevant to channel function, which could shed light on the basic mechanism of HERG channel kinetics and open new pharmaceutical possibilities.

ACKNOWLEDGEMENTS

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