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ORIGINAL ARTICLES

Effects of Estradiol on Voltage-Gated Potassium Channels in Mouse Dorsal Root Ganglion Neurons

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Abstract Voltage-gated potassium channels are regulators of membrane potentials, action potential shape, firing adaptation, and neuronal excitability in excitable tissues including in the primary sensory neurons of dorsal root ganglion (DRG). In this study, using the whole-cell patch-clamp technique, the effect of estradiol (E2) on voltage-gated total outward potassium currents, the component currents transient "A-type" current (I_A) currents, and "delayed rectifier type" (I_{KDR}) currents in isolated mouse DRG neurons was examined. We found that the extracellularly applied 17β-E2 inhibited voltage-gated total outward potassium currents; the effects were rapid, reversible, and concentration-dependent. Moreover, the membrane impermeable E2-BSA was as efficacious as 17β-E2, whereas 17α-E2 had no effect. 17β-E2-stimulated decrease in the potassium current was unaffected by treatment with ICI 182780 (classic estrogen receptor antagonist), actinomycin D (RNA synthesis inhibitor), or cycloheximide (protein synthesis inhibitor). We also found that I_A and I_{KDR} were decreased after 17β-E2 application. 17β-E2 significantly shifted the activation curve for I_A and I_{KDR} channels in the hyperpolarizing direction. In conclusion, our results demonstrate that E2 inhibited voltage-gated K^+ channels in mouse DRG neurons through a membrane ER-activated non-genomic pathway.

Keywords Estradiol · Patch-clamp recording · Voltage-gated potassium channels · Dorsal root ganglion neurons

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Introduction

The hormone estrogen regulates a remarkably large spectrum of neural functions, including learning, memory, emotions, and affective state, as well as motor coordination and pain sensitivity (Gintzler and Liu 2012). The mechanisms by which estrogen regulates nociception are complex, including direct action on the central and peripheral nervous systems, as well as indirect actions via modulation of the skeletal and immune systems. Estrogen has also been found to exert short-term actions on the electrical properties of neurons and alter the neuronal excitability (Vasudevan and Pfaff 2008). A wide variety of ion channels, playing important roles in the regulation of cell excitability, have been shown to be modulated by estrogen in dorsal root ganglion (DRG) neurons. For example, estradiol (E2) inhibits transient receptor potential vanilloid receptor 1 current activation by capsaicin (Xu et al. 2008), α,β-meATP-mediated currents (Lu et al. 2013), and high-voltage-activated Ca^{2+} channel currents (Lee et al. 2002)

in DRG neurons. E2 increased *N*-methyl-D-aspartic acid receptors currents in adult male and female DRG neurons (McRoberts et al. 2007). We also found that 17β-E2 inhibited voltage-gated Na^+ channels in mouse DRG neurons (Wang et al. 2013a).

Voltage-gated potassium (Kv) channels are important physiological regulators of membrane potentials, action potential shape, firing adaptation, and neuronal excitability in excitable tissues including nociceptive sensory neurons (Takeda et al. 2011). It follows that inhibition of Kv would increase excitability. The Kv channel current recorded from DRG neurons consists of two major biophysically distinct types: a transient "A-type" current (I_A) channels and "delayed rectifier type" (I_{KDR}) channels (Stewart et al. 2003). However, it is unknown whether E2 affects the Kv channels in mouse DRG neurons.

It is well known that estrogen may activate intracellular signals by two pathways: genomic or non-genomic activation (Watson et al. 2007). In genomic pathways, they activate nuclear estrogen receptor α and β (ERα and ERβ) (Beckett et al. 2006), resulting in the control of gene expression. In the case of non-genomic activation, estrogen activates cytoplasmic signaling events at or near the plasma membrane through either membrane-localized classical ERs or novel ERs (Micevych and Dewing 2011; Zhang et al. 2012). ERs are known to be expressed in various parts of the nociceptive pathway, including in the primary sensory neurons of the DRG (Takanami et al. 2010; Taleghany et al. 1999). Previous studies have demonstrated that the effects of estrogen on ion channel function and neuronal excitability may be governed by the non-genomic mode of estrogen action (Vasudevan and Pfaff 2008).

The present study investigated the effects of E2 specifically on dynamics of voltage-gated total outward potassium channels, transient I_A channels and I_{KDR} channels in isolated mouse DRG neurons using whole-cell patch-clamp recording.

Materials and Methods

All experiments were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals, and the study was approved by the local IACUC.

DRG Neurons

DRG neurons were prepared as described previously (Wang et al. 2013a, 2011). Male C57 mice (10–12 week) were killed by decapitating under ethyl ether anesthesia. The vertebral columns were quickly removed and dissected in ice-cold Dulbecco's modified Eagle's medium (DMEM). DRGs were rapidly removed and enzymatically digested at 37 °C for 23–30 min in DMEM containing 2 mg/ml collagenase (type I) and 1 mg/ml trypsin (type I). After washing three times with fresh, enzyme-free external solution, single neuron was obtained by gentle agitation through a Pasteur pipette. The dissociated DRG neurons were plated into 3.5-cm culture dishes and incubated at 37 °C for at least 2 h before electrophysiological recording.

Whole-Cell Patch-Clamp Recording

Voltage-gated potassium channels currents in DRG neurons were recorded with whole-cell patch-clamp techniques by a patch-clamp amplifier (HUST-IBB, PC-2B) and then stored in a computer by software of IBBClamp as previously described (Wang et al. 2011). Patch pipettes were pulled from glass capillaries with an outer diameter of 1.5 mm on a two-stage puller (PP-830, Narishige, Tokyo). The resistance between the recording electrode filled with pipette solution and the reference electrode was 2–6 MΩ. Capacity transients were canceled as much as possible, and voltage errors were minimized with series resistance compensation at 80–90 %. Leakage current was digitally subtracted, while liquid junction potential was corrected throughout all experiments.

Drugs and Solutions

The external solution (in mM): choline chloride 150, KCl 5, $CaCl_2$ 2, $MgCl_2$ 2, HEPES 10, $CdCl_2$ 1, glucose 10. The pH was adjusted to 7.4 with KOH, and the osmolality was adjusted to 330 mosM. The pipette solution contained (in mM) potassium gluconate 120, KCl 20, $MgCl_2$ 2, EGTA 10, HEPES 10, Na_2-ATP 5, $CaCl_2$ 1, and the pH was adjusted to 7.3 with KOH, and the osmolality was adjusted to 300 mosM (Vydyanathan et al. 2005). 17β-E2, 17α-E2, 17β-estradiol-bovine serum albumin conjugate (E2-BSA), ICI 182780, actinomycin D, cycloheximide, Collagenase (type I) and trypsin (type I), dimethylsulfoxide (DMSO), choline chloride, HEPES, EGTA, TEA-Cl, Na_2-ATP, KCl were all purchased from Sigma (Sigma Chemical Co., St. Louis, MO, USA). DMEM was purchased from GIBCO. All other chemical reagents used were of analytic grade. 17β-E2, 17α-E2, E2-BSA, ICI 182780 were prepared as 100 mM stock solutions in DMSO and diluted in the bath solution for each experiment to give the desired final concentration just before use.

Data Analysis

Data were analyzed and fitted using Clampfit (Axon Instruments, Foster City, CA) and SigmaPlot (SPSS Inc., Chicago IL) software. Data were expressed as mean ± S.E.M. for all

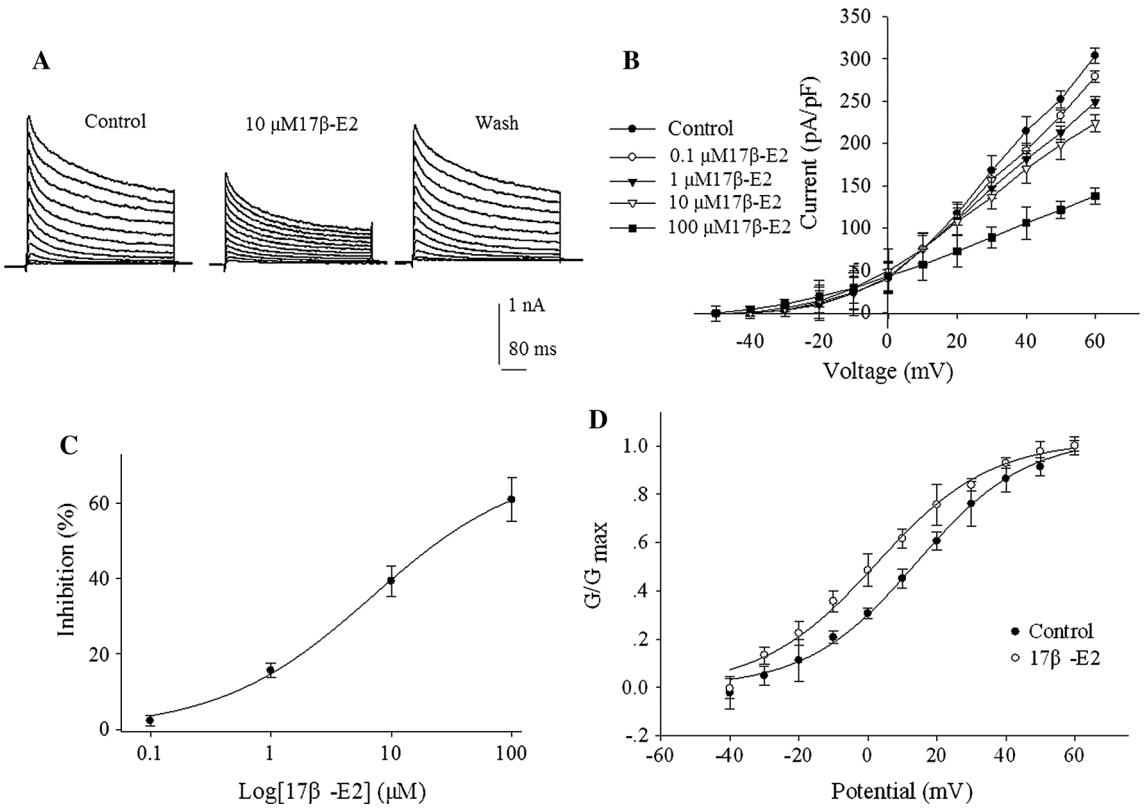

Fig. 1 17β-E2 inhibited voltage-gated total outward potassium currents recorded in DRG neurons. **a** Voltage-gated total outward K^+ currents before and after 17β-E2 perfusion. **b** Current–voltage (*I–V*) relationship of K^+ currents in control and 17β-E2 treatment groups. Each point represented mean ± S.E.M from peak K^+ current normalized by cell capacitance at different test potentials ($n = 8$). **c** The dose–response curve for 17β-E2 (0.1, 1, 10, and 100 μM) was fitted to the Hill equation and gave the values: $IC_{50} = 7.1 \pm 1.0$ μM, and $h = 0.7 \pm 1.0$ ($n = 10$). **d** Effects of 17β-E2 on the activation of voltage-gated total outward potassium channels. The activation curves were fitted with a Boltzmann equation with the parameters (control: $V_{1/2} = 14.1 \pm 1.5$ mV, $k = 15.8 \pm 1.1$; 10 μM 17β-E2: $V_{1/2} = 2.3 \pm 1.7$ mV, $k = 16.6 \pm 1.3$ $n = 8$, $P < 0.05$)

the experiments. Paired and/or unpaired Student's *t* test were used as appropriate to evaluate the statistical significance of differences between two group means, and ANOVA was used for multiple groups. Statistical significance is defined as $P < 0.05$. The concentration-dependent curve was fitted with the Hill equation: $I/I_{control} = 1-[\max/1 + (IC_{50}/C)^h]$, where max was the maximum inhibition attainable, IC_{50} was the half-inhibiting concentration, and h was the slope factor (Hill coefficient). To study the steady-state activation or inactivation kinetics, peak current values (I) were transformed into conductance (G) using the equation $G = I/(V_m - V)$, where V was the equilibrium potential and V_m was the membrane potential at which I was recorded. Normalized peak conductance (G/G_{max}) and the data describing the fractional decrease in the peak current during the steady-state inactivation (I/I_{max}) were fitted with a Boltzmann equation I/I_{max} or, $G/G_{max} = 1/\{1 + \exp[(V_m - V_{1/2})/k]\}$ where I_{max} was the maximal current amplitude; G_{max} was the maximal conductance; V_m was the command or conditioning voltage; $V_{1/2}$ was the potential of half-maximal activation or inactivation, and k was the slope factor.

Results

Effects of 17β-E2 on Voltage-Gated Total Outward Potassium Channels

The voltage-gated total outward potassium currents in DRG neurons were elicited by stepping to a conditioning voltage of −110 from a holding potential of −50 mV; the membrane then was depolarized to −50 mV and on upto +60 mV in increments of 10 mV (Everill and Kocsis 1999; Nie et al. 2007). As shown in Fig. 1a, total outward potassium currents were decreased after 10 μM 17β-E2 application, and the inhibitory effect was partially reversed by washout. The effects of 17β-E2 on current–voltage (*I–V*) relationships of potassium currents density were studied

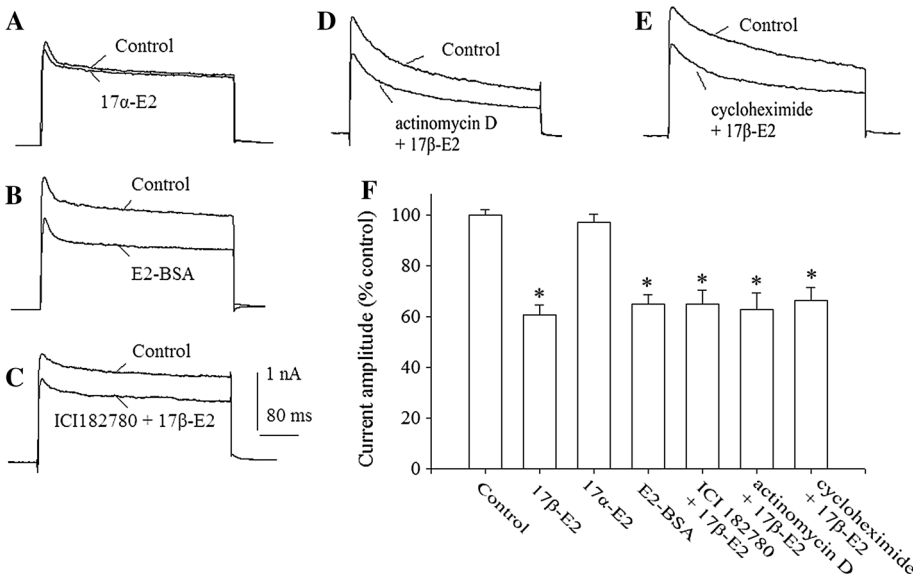

Fig. 2 Effects of 17α-E2, E2-BSA on the voltage-gated total outward potassium currents, and ICI 182780, actinomycin D, cycloheximide on the 17β-E2-induced inhibition of the voltage-gated K^+ currents recorded in DRG neurons. **a** Current traces taken from a representative cell exposed to 17α-E2 (10 μM). **b** The membrane impermeable E2-BSA (10 μM) reduces the voltage-gated total outward potassium currents. 17β-E2 decreases voltage-gated K^+ currents in the presence of 10 μM ICI 182780 (**c**), 10 μM actinomycin D (**d**) and 10 μM cycloheximide (**e**). **f** Summary of the effects of 17β-E2, 17α-E2, E2-BSA on the voltage-gated total outward potassium currents, and ICI 182780, actinomycin D, cycloheximide on the 17β-E2-induced inhibition of the voltage-gated K^+ currents in DRG neurons ($n = 10$, $^*P < 0.05$ vs Control)

at concentrations of 0.1, 1, 10, and 100 μM (Fig. 1b). 17β-E2 inhibited total outward potassium currents in a concentration-dependent manner. The dose–response curve for 17β-E2 was fitted to the Hill equation and gave the values: $IC_{50} = 7.1 \pm 1.0$ μM, and $h = 0.7 \pm 1.0$ (Fig. 1c). In all further studies, 10 μM 17β-E2 was used. The activation of K^+ channels was fitted with a Boltzmann function. 10 μM 17β-E2 significantly shifted the activation curve in the hyperpolarizing direction (control: $V_{1/2} = 14.1 \pm 1.5$ mV, $k = 15.8 \pm 1.1$; 10 μM 17β-E2: $V_{1/2} = 2.3 \pm 1.7$ mV, $k = 16.6 \pm 1.3$ $n = 8$, $P < 0.05$, Fig. 1d).

The effects of 17α-E2, the stereoisomer of the endogenous 17β-E2, on the voltage-gated total outward potassium currents in DRG neurons were also tested. Figure 2a shows that 17α-E2 (10 μM) had no significant effect on the outward potassium currents, indicating that the effect of estrogen observed in the present study is stereospecific. To determine if the effects of 17β-E2 were limited to the extracellular receptors, we performed experiments using a membrane-impermeable E2 analog, E2-BSA. Figure 2b shows 10 μM E2-BSA produced an inhibition of 34.9 ± 3.5 % ($n = 10$), an effect that was similar in magnitude to that produced by 17β-E2 (39.3 ± 4.1 %, $n = 10$, $P > 0.05$, Fig. 2d). To test whether the 17β-E2-induced inhibition of voltage-gated total outward K^+ currents was mediated by estrogen receptors, we treated the cells with 10 μM ICI 182780, a classical estrogen receptor antagonist. Figure 2c shows that the incubation with 10 μM ICI 182780 did not affect the inhibitory effect of 10 μM 17β-E2 on the voltage-gated K^+ currents. Besides, the decreased K^+ current was unaffected by treatment with 10 μM actinomycin D (inhibitor of RNA synthesis) or 10 μM cycloheximide (inhibitor of protein synthesis) (Fig. 2d, e), thus indicating that the effect of 17β-E2 was non-genomic effects and may not involve the synthesis of RNA and protein.

Effects of 17β-E2 on I_A

The outward A-type (I_A) K^+ currents were delineated using 25 mM TEA, a blocker of native I_{KDR} K^+ currents in DRG neurons (Liu and Simon 2003; Vydyanathan et al. 2005). The protocol to measure I_A activation was performed at a holding potential of −80 mV and consisted of 300 ms depolarization pulses from −80 to +60 mV in 10 mV steps. Figure 3a shows that 17β-E2 reversibly inhibits I_A currents. The current–voltage relationship was examined, and we found that 10 μM 17β-E2 significantly shifted the *I–V* curve downwards stepwise (Fig. 3b). We also found that 10 μM 17β-E2 significantly shifted the activation curve in the hyperpolarizing direction (control: $V_{1/2} = 15.8 \pm 2.1$ mV, $k = 15.4 \pm 1.4$; 10 μM 17β-E2: $V_{1/2} = -9.7 \pm 2.6$ mV, $k = 13.3 \pm 2.2$ $n = 9$, $P < 0.05$, Fig. 3c).

The steady-state inactivation-voltage protocol consisted of 500 ms preconditioned pulses ranging from −120 to +10 mV followed by 500 ms test pulse depolarizing to

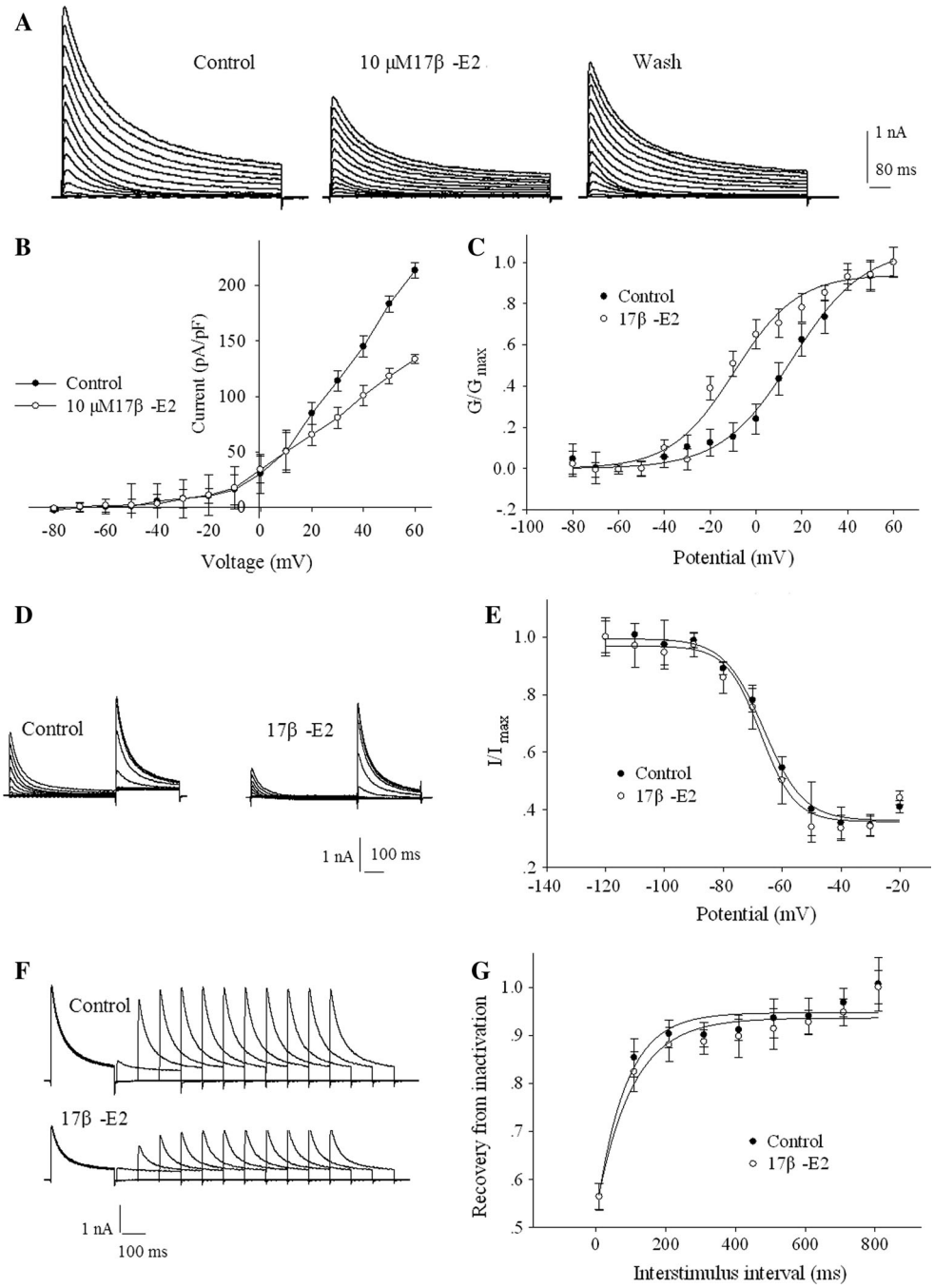

Fig. 3 Effects of 17β-E2 on I_A currents in DRG neurons. **a** A representative voltage-clamp experiment showing that 10 μM 17β-E2 decreased (reversibly) the I_A currents. **b** A *I–V* relationship of I_A currents in control and 10 μM 17β-E2 treatment groups. Each point represented mean ± S.E.M from peak I_A current normalized by cell capacitance at different test potentials ($n = 9$). **c** Comparison of activation of I_A before and after 10 μM 17β-E2 treatment. The activation curves were fitted to a Boltzmann equation with the parameters (control: $V_{1/2} =$ 15.8 ± 2.1 mV, $k = 15.4 \pm 1.4$; 10 μM 17β-E2: $V_{1/2} = -9.7 \pm 2.6$ mV, $k = 13.3 \pm 2.2$ $n = 5$, $P < 0.05$). **d** Effects of 10 μM 17β-E2 on the steady-state inactivation of I_A channels. **e** The inactivation curves were fitted to the Boltzmann equation with the parameters (control: $V_{1/2} = -66.2 \pm 1.1$ mV, $k = -6.7 \pm 0.9$, 10 μM 17β-E2: $V_{1/2} = -67.3 \pm 1.7$ mV, $k = -5.8 \pm 1.5$ $n = 5$, $P > 0.05$). **f** Representative current traces of I_A inactivation recovery in the absence (*top traces*) and presence (*bottom traces*) of 10 μM 17β-E2. **g** Time courses of recovery were fitted by single-exponential functions with time constants of 82 ± 6.5 ms during control, and 101 ± 12.3 ms after 10 μM 17β-E2 treatment ($n = 10$, $P < 0.05$)

+30 mV. Figure 3e shows 10 μM 17β-E2 did not significantly affect the steady-state inactivation curve for I_A channels (control: $V_{1/2} = -66.2 \pm 1.1$ mV, $k = -6.7 \pm 0.9$, 10 μM 17β-E2: $V_{1/2} = -67.3 \pm 1.7$ mV, $k = -5.8 \pm 1.5$ $n = 11$, $P > 0.05$). The recovery from inactivation of I_A was studied using a double-pulse protocol as shown in the Fig. 3f. The time courses of recovery can be fitted by single-exponential functions with time constants of 82.0 ± 6.5 ms in control, these increased considerably to 101.0 ± 12.3 ms in the presence of 10 μM 17β-E2 ($n = 10$, $P < 0.05$, Fig. 3g).

Effects of 17β-E2 on I_{KDR}

The delayed rectifier type (I_{KDR}) channel currents were initiated via a −50 mV prepulse protocol, followed by a series test pulses rising from −50 to +60 mV in 10 mV steps of 400 ms. To record I_{KDR}, 5 mM 4-aminopyridine

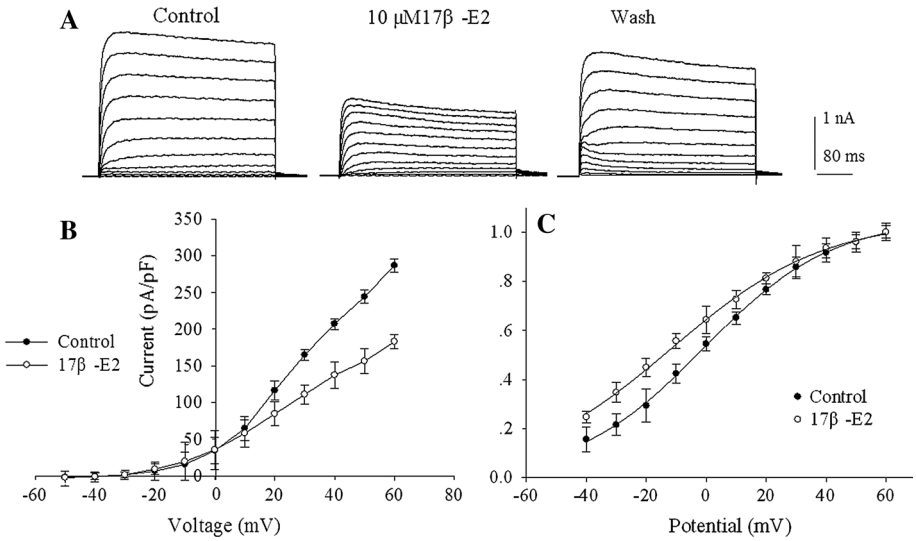

Fig. 4 Effects of 17β-E2 on I_{KDR}. **a** Voltage-dependent I_{KDR} traces recorded in a representative cell using the whole-cell patch-clamp technique. I_{KDR} was reversibly inhibited by 10 μM 17β-E2. **b** A *I–V* relationships of I_{KDR} showing the inhibitory effect of 17β-E2. **c** Comparison of activation of I_{KDR} before and after 10 μM 17β-E2 treatment. The activation curves were fitted to a Boltzmann equation with the parameters (control: $V_{1/2} = -0.9 \pm 0.6$ mV, $k = 21.5 \pm 0.5$; 10 μM 17β-E2: $V_{1/2} = -11.7 \pm 0.8$ mV, $k = 25.5 \pm 0.8$ $n = 8$, $P < 0.05$)

was added to the external solution to eliminate I_A. As shown in Fig. 4a, the I_{KDR} currents were decreased after 10 μM 17β-E2 application, and the inhibitory effect was partially reversed by washout. We also found that 10 μM 17β-E2 significantly shifted the *I–V* curve downwards stepwise (Fig. 4b). The activation of I_{KDR} K^+ channels was fitted with a Boltzmann function. We found that 10 μM 17β-E2 induced a statistically significant hyperpolarizing shift in the conductance–voltage relation (control: $V_{1/2} = -0.9 \pm 0.6$ mV, $k = 21.5 \pm 0.5$; 10 μM 17β-E2: $V_{1/2} = -11.7 \pm 0.8$ mV, $k = 25.5 \pm 0.8$ $n = 8$, $P < 0.05$, Fig. 4c).

Discussion

In the present study, we investigated the effects of E2 on Kv channels in mouse DRG neurons. We found that the extracellularly applied 17β-E2 inhibited voltage-gated total outward potassium currents; the effects were rapid, reversible, and concentration-dependent. Moreover, the membrane impermeable E2-BSA was as efficacious as 17β-E2, whereas 17α-E2 had no effect. The short time scales of application of 17β-E2 as well as the use of actinomycin D and cycloheximide point to non-genomic effects. We also found that I_A and I_{KDR} were decreased after 17β-E2 application.

A wide variety of ion channels, playing important roles in the regulation of cell excitability, have been shown to be modulated by estrogen in DRG neurons. Kv channels are regulators of neuronal excitability, opening in response to depolarizing membrane potentials allowing outward flux of K^+ to depolarize the neuron and play a key role in pain sensation by controlling afferent impulse discharge. I_A potassium channel can modulate action potential firing frequency and slow the rate of depolarization by altering the duration of the after hyperpolarization, I_{KDR} potassium channel function to limit the duration of action potentials by remaining open for as long as depolarization occurs, thereby promoting the onset of repolarization.

To obtain a better understanding of how estrogen may affect neuronal excitability, we measured how estrogen modulated voltage-gated total outward potassium channels, I_A and I_{KDR} potassium channels. We found that 17β-E2 inhibited voltage-gated total outward potassium currents in a concentration-dependent manner, after 17β-E2 application, the current density of the I_A and I_{KDR} K^+ channels was also significantly reduced. On the other hand, we found that 17β-E2 altered the kinetics properties of I_A and I_{KDR} K^+ channels. 17β-E2 significantly shifted the activation curve for I_A and I_{KDR} channels in the hyperpolarizing direction. Our results were consistent with previous reports that E2 may affect voltage-gated K^+ channels currents. Bath application of 17β-E2 (10–100 μM) reversibly reduced voltage-gated outward potassium currents in a concentration-dependent manner in rat parabrachial nucleus cells. This effect was mimicked by E2-BSA but not mimicked by 17α-E2 (Fatehi et al. 2005). The selective estrogen receptor modulator raloxifene remarkably suppressed transient outward and ultra-rapid delayed rectifier potassium

currents (I_A and I_{KDR}) in human atrial myocytes. 17β-E2 inhibited I_A (IC_{50} = 10.3 μM) without affecting I_{KDR}. The inhibitory effects of raloxifene and 17β-E2 on I_A and/or I_{KDR} were unaffected by the estrogen receptor antagonist ICI 182780 (Liu et al. 2007). E2 alters I_A and I_{KDR} in mouse gonadotropin-releasing hormone (GnRH) neurons, E2 affected the amplitude, decay time, and the voltage dependence of both inactivation and activation of I_A and altered a slowly inactivating current of I_{KDR} (DeFazio and Moenter 2002). In the mouse colon, estrogen suppresses I_A, which are important for regulating excitability (Beckett et al. 2006).

The broad spectrum of estrogen action is mediated by two types of estrogen receptors (ERs), ERα and ERβ, through genomic regulation by the modulation of gene transcription. In addition to these actions, there is increasing evidence of rapid non-genomic effects of estrogen via membrane-associated receptor (Takanami et al. 2010). In this study, to examine whether 17β-E2 modulates voltage-gated K^+ channels through a membrane ER, the membrane impermeable E2-BSA and the electrophysiological inactive stereoisomer 17α-E2 were tested. The rapid effect of 17β-E2-induced inhibition of the voltage-gated K^+ currents was mimicked by E2-BSA, whereas 17α-E2 had no effect. We also found that ICI 182780, which blocks classical ERs, failed to reverse the inhibitory effect of 17β-E2 on the voltage-gated K^+ currents, suggesting that the effect is not mediated by a classical ER. Besides, the effects of 17β-E2 were not blocked by pretreatment with actinomycin D (inhibitor of RNA synthesis) or cycloheximide (inhibitor of protein synthesis), suggesting non-genomic effects of estrogen via membrane-associated receptor in DRG neurons.

Previous studies have shown that E2 may affect ion channel function via a non-genomic mechanism. E2 inhibits transient receptor potential vanilloid receptor 1 current activation by capsaicin (Xu et al. 2008) and high-voltage-activated Ca^{2+} channel currents (Lee et al. 2002) in DRG neurons by a non-genomic estrogen signaling pathway. 17β-E2 significantly attenuated α, β-meATP-mediated currents by acting on ERα and GPR30 receptors, which probably involves the intracellular cAMP-PKA-ERK1/2 pathway (Lu et al. 2013). Both 17β-E2 and E2-BSA increased the Ca^{2+}-activated K^+ channels (BK_{Ca}) current in a concentration-dependent manner. 17β-E2 -stimulated increase in the BK_{Ca} current was unaffected by treatment with ICI 182780, actinomycin D or cycloheximide (Wong et al. 2008). 17β-E2 rapidly increases the ATP-sensitive potassium channel (K_{ATP}) channel activity in GnRH neurons through a membrane ER-activated PKC-PKA signaling pathway (Zhang et al. 2010). In the previous study, we found that 17β-E2 inhibited voltage-gated Na^+ channels in mouse DRG neurons through a membrane ER-activated PKC-PKA signaling pathway (Wang et al. 2013a). We also found 17β-E2 inhibited high-voltage-activated Ca^{2+} channels in cortical neurons via independent of ICI 182780-sensitive ER, PKC, and PKA-dependent signaling pathway (Wang et al. 2013b).

Conclusion

E2 inhibited voltage-gated K^+ (I_A and I_{KDR}) channels in mouse DRG neurons through non-genomic effects via membrane ER-activated pathway. This study focused on immediate effects of E2 on K^+ channel activity; however, further investigation will be required to determine any long-term effects.

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