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Activation of Estrogen Receptor α Enhances Bradykinin Signaling in Peripheral Sensory Neurons of Female Rats

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ABSTRACT

Numerous studies have demonstrated that females have a higher risk of experiencing several pain disorders with either greater frequency or severity than males. Although the mechanisms that underlie this sex disparity remain unclear, several studies have shown an important role for sex steroids, such as estrogen, in the modulation of nociception. Receptors for estrogen are present in primary afferent neurons in the trigeminal and dorsal root ganglia, and brief exposure to estrogen increases responses to the inflammatory mediator bradykinin (BK). However, the mechanism for estrogen-mediated enhancement of BK signaling is not fully understood. The aim of the present study was to evaluate the relative contributions of estrogen receptor α (ERα), ERβ, and G protein–coupled estrogen receptor 1 (GPER) to the enhanced signaling of the inflammatory mediator BK by 17β-estradiol (17β-E_2) in primary sensory neurons from female rats in culture (ex vivo) and in behavioral assays of nociception in vivo. The effects of 17β-E_2 on BK responses were mimicked by ERα-selective agonists and blocked by ERα-selective antagonists and by small interfering RNA knockdown of ERα. The data indicate that ERα is required for 17β-E_2–mediated enhancement of BK signaling in peripheral sensory neurons in female rats.

Introduction

Many epidemiologic studies have shown that women are at an increased risk for several clinical pain disorders, and are often more sensitive to experimentally induced pain than men (for reviews, see Craft, 2007; Fillingim et al., 2009). Among the many possible reasons for the disparity in pain responsiveness between men and women, which include cognitive and sociocultural differences, several studies have shown the importance of the sex hormone estrogen in regulating nociception (Craft, 2007; Fillingim et al., 2009; Gintzler and Liu, 2012).

Estrogen effects on nociception are complex and often contradictory. In studies of experimental pain in humans, increases, decreases, and no change in pain responsiveness during the menstrual cycle or during hormone replacement therapy have been reported (Fillingim et al., 2009). The complexity in these human studies may reflect differences in the way in which the phases of the menstrual cycle were defined, differences in pain models (electrical, thermal, mechanical, etc.), the age of the women studied (pre- versus postmenopausal), and the area of the body tested. However, even in animal studies, there are many contradictory reports on the effects of estrogen on nociception (for reviews, see Craft, 2007; Gintzler and Liu, 2012). For example, when estrogen is administered to ovariectomized (OVX) rats, nocifensive behavior in response to formalin injection into the rat hind paw is reduced (Hunter et al., 2011). However, when administered to the intrathecal space of intact or OVX female rats, estrogen produced mechanical allodynia and thermal hyperalgesia (Zhang et al., 2012). The nociceptive behavioral response to intra-articular injection of Freund's complete adjuvant to the temporomandibular joint can be enhanced (Kramer and Bellinger, 2009) or reduced (Kou et al., 2011) by estrogen. It is likely that such complex and contradictory actions of estrogen on pain responsiveness reflect differential actions of estrogen on different receptor subtypes and cell populations in the pain transmission/perception pathways within the central and peripheral nervous systems, along

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ABBREVIATIONS: ANOVA, analysis of variance; BK, bradykinin; BSA, bovine serum albumin; DMSO, dimethylsulfoxide; DPN, 2,3-bis(4-hydroxyphenyl)propionitrile; 17β-E_2, 17β-estradiol; E_2–BSA, 17β-estradiol-6-(*O*-carboxymethyl)oxime–bovine serum albumin; ER, estrogen receptor; G-1, (±)-1-[(3a*R**,4*S**,9b*S**)-4-(6-bromo-1,3-benzodioxol-5-yl)-3a,4,5,9*b*-tetrahydro-3*H*-cyclopenta[*c*]quinolin-8-yl]-ethanone; G-15, (3a*S**,4*R**,9b*R**)-4-(6-bromo-1,3-benzodioxol-5-yl)-3a,4,5,9*b*-3*H*-cyclopenta[*c*]quinoline; GPER, G protein–coupled estrogen receptor 1; HBSS, Hanks' balanced salt solution; ICI 182780, (7*R*,8*R*,9*S*,13*S*,14*S*,17*S*)-13-methyl-7-[9-(4,4,5,5,5-pentafluoropentylsulfinyl)nonyl]-6,7,8,9,11,12,14,15,16,17-decahydrocyclopenta[a]phenanthrene-3,17-diol; IP, inositol phosphate; i.pl., intraplantar; MPP, 1,3-bis(4-hydroxyphenyl)-4-methyl-5-[4-(2-piperidinylethoxy)phenol]-1*H*-pyrazole dihydrochloride; OVX, ovariectomized; PLC, phospholipase C; PPT, 1,3,5-tris(4-hydroxyphenyl)-4-propyl-1*H*-pyrazole; PWL, paw withdrawal latency; siRNA, small interfering RNA.

with time-dependent effects (including genomic versus nongenomic signaling) and differences in the type of pain studied.

Of the many possible targets for estrogen within the pain neurotransmission system, studies have suggested that estrogen can alter the function of the primary sensory neurons that respond to noxious stimuli (nociceptors). Receptors for estrogen are expressed by primary sensory neurons (Papka et al., 2001; Bereiter et al., 2005; Chaban and Micevych, 2005; Dun et al., 2009; Liverman et al., 2009b), and treatment of dorsal root ganglia neurons in culture with estrogen enhances capsaicin-induced currents (Chen et al., 2004), reduces translocation of protein kinase C subtype ε (Hucho et al., 2006), and attenuates ATP-induced calcium currents (Chaban and Micevych, 2005). We have shown that estrogen treatment of sensory neurons in culture from the adult rat trigeminal ganglion enhances signaling by the inflammatory mediator bradykinin (BK) (Rowan et al., 2010). Interestingly, each of these estrogen effects in sensory neurons occurs rapidly, within minutes, suggesting a rapid-onset, nongenomic mechanism mediated by membrane-associated estrogen receptors.

The two primary receptors for estrogen, termed estrogen receptor (ER) α and ERβ, share greater than 60% sequence homology and are best known for their roles as nuclear receptors that regulate gene transcription and protein synthesis (Gibson and Saunders, 2012). ERα and ERβ are differentially expressed throughout the central and peripheral nervous systems (Perez et al., 2003) and are differentially activated by ligands in a tissue-specific manner, a characteristic exploited therapeutically by the selective estrogen receptor modulators (Hall et al., 2001; Nilsson and Koehler, 2005; Nelson et al., 2013). In addition to regulating gene transcription, both ERα and ERβ are found to be associated with the plasma membrane where they can rapidly regulate neuronal excitability (Woolley, 2007; Roepke et al., 2011; Srivastava et al., 2011) and can mediate rapid-onset, nongenomic signaling to many second messenger systems involved in nociceptive transmission, such as cAMP, calcium, and various kinases (Hammes and Levin, 2007; Levin, 2009). In addition, an estrogen-sensitive, G protein–coupled receptor, GPR30 or G protein–coupled estrogen receptor 1 (GPER), has recently been identified that is also capable of rapid-onset, nongenomic signaling (Barton, 2012).

We have recently found that 17β-estradiol (17β-E_2) rapidly (within minutes) enhances signaling by the inflammatory mediator BK in primary sensory neurons in culture and in an animal model of nociception (Rowan et al., 2010). The effect of 17β-E_2 was mimicked by a membrane-impermeable estrogen [17β-E_2 conjugated with bovine serum albumin (BSA)] and not blocked by the protein translation inhibitor anisomycin, indicating mediation by a membrane-associated ER. In the present work, we evaluated the relative contributions of ERα, ERβ, and GPER to the enhanced signaling of the inflammatory mediator BK by 17β-E_2 in adult female rats.

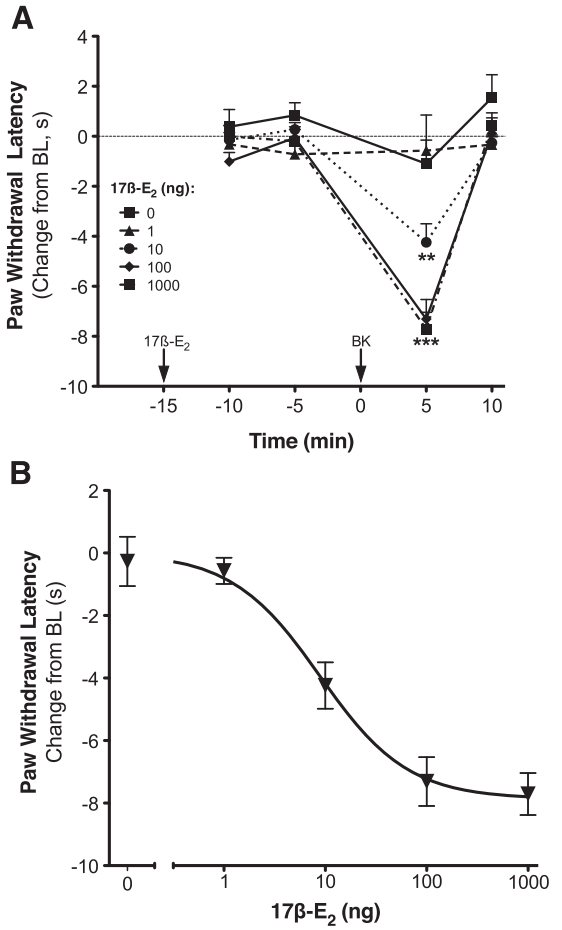

Fig. 1. Local application of 17β-E_2 rapidly and dose-dependently enhances bradykinin-induced thermal allodynia in OVX rats. (A) OVX rats received intraplantar injections (50 μl) of 17β-E_2 (doses indicated) or vehicle 15 minutes before injection with a subthreshold dose of BK (1 μg). PWL in response to a radiant heat stimulus applied to the ventral surface of the hind paw was measured in duplicate at 5-minute intervals before (baseline) and following each injection. Data are expressed as the change (seconds) from individual preinjection baselines (10 ± 2 seconds) and represent the mean ± S.E.M. of four to six animals per group. $^{**}P < 0.01$, $^{***}P < 0.001$ versus vehicle by two-way analysis of variance (ANOVA) with Bonferroni post-hoc analysis. (B) Dose-response curve for 17β-E_2 enhancement of BK-induced thermal allodynia. Data points are from (A) at the time of peak BK response (5 minutes post injection) as a function of 17β-E_2 dose. ED_{50} = 8.85 ng. BL, baseline.

Materials and Methods

Fetal bovine serum was purchased from Gemini Bioproducts (Calabasas, CA). All other tissue culture reagents were from Invitrogen (Carlsbad, CA). (±)-1-[(3a*R**,4*S**,9b*S**)-4-(6-Bromo-1,3-benzodioxol-5-yl)-3*a*,4,5,9*b*-tetrahydro-3*H*-cyclopenta[*c*]quinolin-8-yl]-ethanone (G-1) and (3a*S**,4*R**,9b*R**)-4-(6-bromo-1,3-benzodioxol-5-yl)-3a,4,5,9b-3*H*-cyclopenta[*c*]quinoline (G-15) were purchased from Tocris Bioscience (Ellisville, MO). 17β-E_2, 1,3,5-tris(4-hydroxyphenyl)-4-propyl-1*H*-pyrazole (PPT), 2,3-bis(4-hydroxyphenyl)propionitrile (DPN), 1,3-bis (4-hydroxyphenyl)-4-methyl-5-[4-(2-piperidinylethoxy)phenol]-1*H*-pyrazole dihydrochloride (MPP), 4-(cyclohexylidenemethylene)-bis-phenol-1,1′-diacetate (cyclofenil), 17β-estradiol-6-(*O*-carboxymethyl)oxime–BSA (E_2–BSA), and all other drugs and chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

Animals. The animal study protocol was approved by the Institutional Animal Care and Use Committee of the University of Texas Health Science Center at San Antonio, and conformed to International Association for the Study of Pain and federal guidelines. OVX, adult female Sprague-Dawley rats, 200–250 g, were purchased from Charles River (Wilmington, MA). Experiments with OVX rats were performed at least 2 weeks following surgery. Animals were housed with food and water available ad libitum before experiments.

Rat Trigeminal Ganglion Culture. Primary cultures of rat primary sensory neurons were derived from adult OVX female rat trigeminal ganglion and prepared as described previously (Patwardhan et al., 2006; Berg et al., 2007a,b; Rowan et al., 2010). In brief, rats

were killed by decapitation, and ganglia were rapidly removed and chilled in Hanks' balanced salt solution (HBSS; Ca^{2+}, Mg^{2+} free) on ice. Ganglia were washed with HBSS, digested with 3 mg/ml collagenase for 30 minute at 37°C, and centrifuged. The pellet was further digested with 0.1% trypsin for 15 minutes at 37°C, pelleted by centrifugation (5000*g* for 5 minutes), and resuspended in Dulbecco's modified Eagle's medium (high glucose) containing 100 ng/ml nerve growth factor (Harlan, Indianapolis, IN), 10% fetal bovine serum, 1× penicillin/streptomycin, 1× L-glutamine, and the mitotic inhibitors uridine (7.5 μg/ml) and 5-fluoro-2′-deoxyuridine (17.5 mg/ml). After trituration to disrupt tissue clumps, the cell suspension was seeded on polylysine-coated 6- (mRNA), 24-, or 48-well [inositol phosphate (IP) accumulation] plates. The medium was changed 24 and 48 hours after plating and every 48 hours thereafter. Nerve growth factor and serum were removed 24 hours prior to experiments. Cells were used on the 5th or 6th day of culture.

Measurement of Inositol Phosphate Accumulation. BK-stimulated IP accumulation in primary sensory neuronal cultures was measured as described previously (Patwardhan et al., 2006; Berg et al., 2007a; Rowan et al., 2010). Cells grown in 24- or 48-well plates were labeled with 2 μCi/ml [^{3}H]myoinositol for 24 hours before experiments. After labeling, cells were rinsed three times with 1 ml of HBSS that contained 20 mM HEPES, and were preincubated in HBSS containing 20 mM HEPES and 20 mM LiCl for 15 minutes at 37°C in room air. Where indicated, antagonists were added 15 minutes before agonists, which were added 15 minutes before BK stimulation (1 nM, 25 minutes, 37°C). The final volume was 250 μl (48 wells) or 500 μl (24 wells). The incubation was terminated by the addition of 500 μl (48 wells) or 1 ml (24 wells) of ice-cold formic acid, and total [^{3}H]IPs were separated with ion-exchange chromatography and measured with liquid scintillation spectrometry. Data are expressed as accumulation of total IPs (disintegrations per minute) or as a percentage of basal IP accumulation.

Small Interfering RNA. ON-TARGETplus SMARTpool small interfering RNA (siRNA) for ERα and ERβ, siGLO Transfection Indicator, and DharmaFECT 3 transfection reagent were purchased from Dharmacon (Lafayette, CO). siRNA sequences for rat ERα were as follows: 5′-GAAUCAAGGUAAAUGUGUA, 5′-UCAAGUCGAUUCCG-CAUGA-3′, AACCAAUGCACCAUCGAUA-3′, and 5′-GCACAAGCGU-CAGAGAGAU-3′. siRNA sequences for rat ERβ were as follows: 5′-UCGCAAGUGUUAUGAAGUA-3′, 5′-GUAAACAGAGAGACACUGA-3′, 5′-AAUCAUCGCUCCUCUAUGC-3′, and 5′-GCACAAGGAGUAU-CUCUGU-3′. Forty-eight hours prior to experiments, cultures were transfected with 50 nM siRNA using 1:200 DharmaFECT. Twenty-four hours after transfection, the medium was removed and replaced with serum-free medium as in other experiments.

Behavioral Testing. Paw withdrawal latency (PWL) to a radiant thermal stimulus was measured with a plantar test apparatus (Hargreaves et al., 1988; Rowan et al., 2009, 2010) by observers blinded to the treatment allocation. In brief, rats were placed in plastic boxes with a glass floor maintained at 30°C. After a 30-minute habituation period, the plantar surface of the hind paw was exposed to a beam of radiant heat through the glass floor. The rate of increase in temperature was adjusted so that baseline PWL values were 10 ± 2 seconds; cut-off time was 25 seconds. PWL measurements were taken in duplicate (separated by 30 seconds) at 5-minute intervals. The average of the duplicate measurements was used for statistical analysis. 17β-E_2 stock solution was diluted in peanut oil. Stock solutions of all other drugs were diluted in saline with or without 2% Tween-20 as indicated. All drugs were administered via intraplantar (i.pl.) injection at a final volume of 50 μl. Where indicated, antagonists were administered 15 minutes before agonists, which were administered 15 minutes before BK injection. Doses of agonists and antagonists were chosen to produce maximal receptor occupancy based upon maximal concentrations (100 × K_i) reported in the literature (Stauffer et al., 2000; Meyers et al., 2001; Sun et al., 2002; Muthyala et al., 2003; Bologa et al., 2006; Zhao and Brinton, 2007; Dennis et al., 2009) and resulting concentrations following intraplantar injection into the paw assuming a volume of distribution of 1 ml (Rowan et al., 2010).

Data Analysis. For cell culture experiments, concentration-response data were fit to a logistic equation (eq. 1) using nonlinear regression analysis to provide estimates of maximal response (R_{max}), potency (EC_{50}) and slope factor (n):

$$R = \frac{R_{max}}{1 + \left(EC_{50}/[A]\right)^n} \quad (1)$$

where R = the measured response at a given agonist concentration (A), R_{max} = maximal response, EC_{50} = the concentration of agonist that produces half-maximal response, and n = slope index. Statistical differences in concentration-response curve parameters between groups were analyzed with Student's paired t test. For behavioral experiments, when only a single concentration was used, statistical significance was assessed using one-way analysis of variance followed by Dunnett's post-hoc or Student's t test (paired) using Prism software (GraphPad Software, Inc., San Diego, CA). P values less than 0.05 was considered statistically significant.

For behavioral experiments, time-course data were analyzed with two-way analysis of variance, followed by Bonferroni's post-hoc test. Data are presented as the mean ± S.E.M., and P values less than 0.05 were considered statistically significant.

Results

17β-E_2 Dose-Dependently Enhanced BK Responses. Intraplantar injection of 17β-E_2 to OVX rats at doses up to 1 μg had no effect on basal PWL but dose-dependently enhanced the response of a subthreshold dose of BK (1 μg; Fig. 1, A and B). As we have shown before (Rowan et al., 2010), BK-induced thermal allodynia was rapid and transient, with peak responses occurring 5 minutes after BK injection. Peak responses were analyzed to yield an ED_{50} for 17β-E_2 of 8.85 ng (Fig. 1B). In sensory neuronal cultures, a threshold concentration of BK (1 nM) increased IP accumulation by approximately 15% (Fig. 2). Although 17β-E_2 by itself, at concentrations up to 100 nM, did not alter basal IP accumulation, pretreatment (15 minutes) of cells with 17β-E_2 produced a concentration-dependent

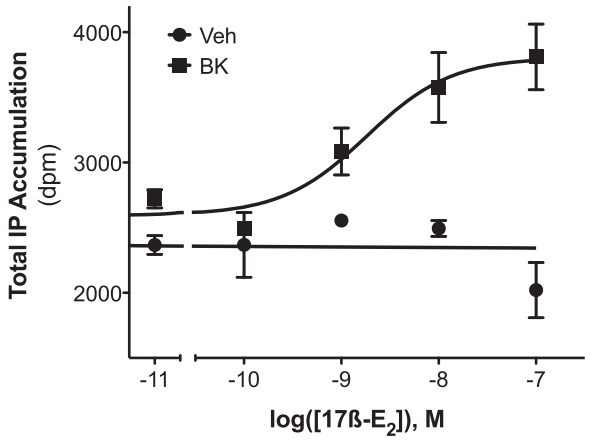

Fig. 2. Local application of 17β-estradiol rapidly and dose-dependently enhances BK-stimulated PLC activity in cultures of peripheral sensory neurons from female rats. Peripheral sensory neuron cultures, prepared from OVX rats, were treated with vehicle [Veh; 0.1% dimethylsulfoxide (DMSO)] or 17β-E_2 (concentrations indicated) for 15 minutes (37°C) before addition of BK (1 nM) or vehicle (HBSS). Total IP accumulation after 25 minutes was measured as described under *Materials and Methods*. Data are expressed as the total counts (dpm) and represent the mean ± S.E.M. of three experiments.

enhancement of BK-stimulated IP accumulation (Fig. 2; EC_{50} = 4.07 nM; E_{max} = 60% above basal).

ERα Agonists Mimicked the Effect of 17β-E_2 to Enhance BK Responses. OVX rats were injected (intraplantarly) with the ER subtype-selective agonists PPT (ERα; Stauffer et al., 2000; Sun et al., 2002), DPN (ERβ; Meyers et al., 2001; Muthyala et al., 2003; Zhao and Brinton, 2007), G-1 (GPER; Bologa et al., 2006), or vehicle 15 minutes prior to injection with a subthreshold dose of BK (1 μg i.pl.). None of the agonists alone affected basal PWL; however, PPT, but not DPN or G-1, significantly enhanced the allodynic response to BK (Fig. 3). Likewise, 17β-E_2, PPT, DPN, or G-1 did not alter basal phospholipase C (PLC) responses in sensory neuron cultures from OVX rats (not shown). However, 15-minute pretreatment with 17β-E_2 or PPT significantly enhanced BK (1 μM)-stimulated IP accumulation by 2-fold versus 4.5-fold, respectively (Fig. 4).

ERα Antagonists Blocked the Effects of 17β-E_2, PPT, and 17β-E_2–BSA to Enhance BK Responses. The nonselective ER antagonist ICI 182780 [(7*R*,8*R*,9*S*,13*S*,14*S*,17*S*)-13-methyl-7-[9-(4,4,5,5,5-pentafluoropentylsulfinyl)nonyl]-6,7,8,9,11,12,14,15,16,17-decahydrocyclopenta[a]phenanthrene-3,17-diol] (1 μg i.pl.) did not affect basal PWL in OVX rats, but when injected 15 minutes prior to 17β-E_2 (100 ng), PPT (2 ng), or 17β-E_2–BSA (30 μg), ICI 182780 completely blocked the enhancement of BK-induced (1 μg) thermal allodynia (Fig. 5). Likewise, the ERα-selective antagonist MPP (1 μg) also blocked the 17β-E_2– and 17β-E_2–BSA-mediated enhancement of BK-induced thermal allodynia, without altering PWL on its own (Fig. 6). By contrast, the 17β-E_2–mediated enhancement of BK-induced thermal allodynia was unaffected by the ERβ antagonist cyclofenil or the GPER antagonist G-15 at doses chosen to produce maximal receptor occupancy (100 × K_i).

When tested in sensory neuron cultures from OVX female rats at concentrations (100 × K_i) chosen to produce maximal receptor occupancy (Stauffer et al., 2000; Meyers et al., 2001; Sun et al., 2002; Muthyala et al., 2003; Bologa et al., 2006; Zhao and Brinton, 2007; Dennis et al., 2009), only ICI 182780 (ERα and ERβ, 30 nM) and MPP (ERα, 300 nM) blocked 17β-E_2 (50 nM)–mediated enhancement of BK (1 nM)-stimulated IP accumulation (Fig. 7). Cyclofenil (ERβ, 10 nM) and G-15 (GPER, 2 μM) were without effect. None of the antagonists alone altered basal IP accumulation.

Expression of ERα Is Required for 17β-E_2–Mediated Enhancement of BK Signaling. Sensory neuron cultures were treated for 48 hours with siRNA (50 nM) selective for ERα, ERβ, or nontargeting control siRNA (siGLO). Reverse-transcription polymerase chain reaction analysis of total

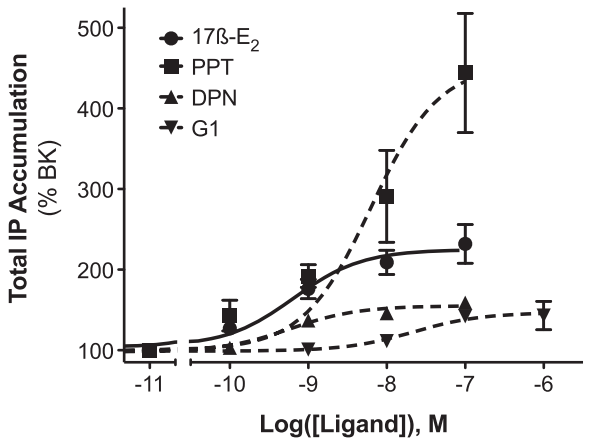

Fig. 4. Activation of ERα enhances BK-stimulated PLC activity in female peripheral sensory neuron cultures. Peripheral sensory neuron cultures from OVX rats were treated with vehicle (0.1% DMSO), 17β-E_2, PPT, DPN, or G-1 (concentrations indicated) for 15 minutes (37°C) before addition of BK (1 nM) and further incubation for 25 minutes (37°C). Total IP accumulation was determined as described under *Materials and Methods*. Data are expressed as the percentage of the BK response and represent the mean ± S.E.M. of six to eight experiments. Basal IP accumulation = 310 ± 19 dpm; BK-stimulated IP accumulation = 408 ± 25 dpm (31% over basal).

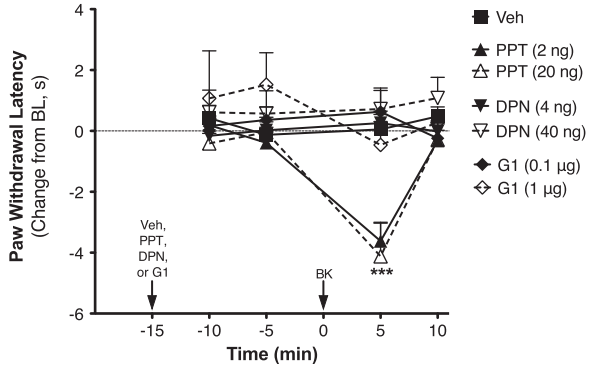

Fig. 3. BK-induced thermal allodynia is enhanced by the ERα-selective agonist PPT but not by the ERβ-selective agonist DPN or the GPER-selective agonist G-1, in OVX rats. Separate groups of OVX animals received intraplantar injections of PPT (2 or 20 ng), DPN (4 or 40 ng), G-1 (0.1 or 1 μg), or vehicle (Veh; 0.1% DMSO, 2% Tween-20) 15 minutes before injection with a subthreshold dose of BK (1 μg). PWL was measured in duplicate at 5-minute intervals before (baseline) and after each injection. Data are expressed as the change (seconds) from individual preinjection baseline (10 ± 2 seconds) and represent the mean ± S.E.M. of four to six animals per group. ***$P < 0.001$ versus Veh by two-way ANOVA with Bonferroni post-hoc analysis. BL, baseline.

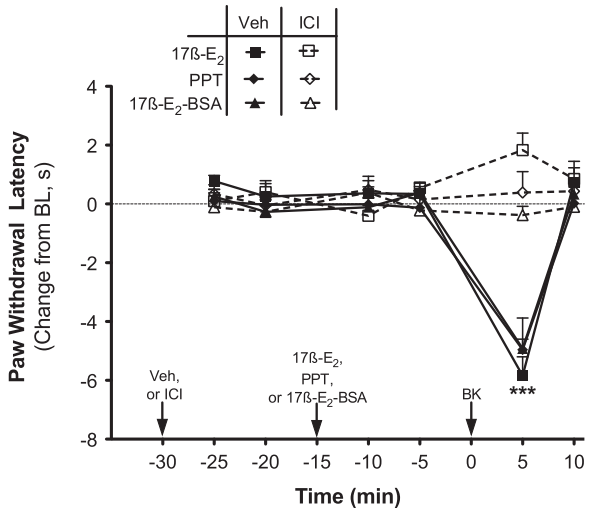

Fig. 5. 17β-Estradiol, PPT, and 17β-E_2–BSA enhancement of BK-induced thermal allodynia is blocked by the ER antagonist ICI 182780. Separate groups of OVX animals received intraplantar injections of the nonselective ER antagonist ICI 182780 (ICI; 1 μg) or vehicle (Veh; 0.1% EtOH) 15 minutes prior to injection with 17β-E_2 (100 ng), PPT (2 ng), or 17β-E_2–BSA (30 μg). Fifteen minutes later, animals received intraplantar injections of BK (1 μg). PWL was measured in duplicate at 5-minute intervals before (baseline) and after each injection. Data are expressed as the change from individual preinjection baselines and represent the mean ± S.E.M. of four to six animals per group. ***$P < 0.001$ respective Veh versus ICI by two-way ANOVA with Bonferroni post-hoc analysis. BL, baseline.

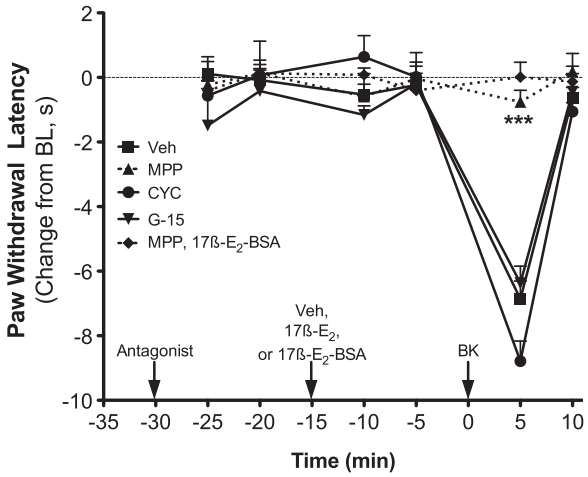

Fig. 6. 17β-E_2 and 17β-E_2–BSA-mediated enhancement of BK-induced thermal allodynia is blocked by the ERα antagonist MPP, but not by antagonists of ERβ, cyclofenil, or GPER, G-15. Separate groups of OVX animals received intraplantar injections of MPP (1 μg), cyclofenil (1 ng), G-15 (1 μg), or vehicle (Veh; 0.1% EtOH) 15 minutes prior to injection with 17β-E_2 (100 ng) or 17β-E_2–BSA (30 μg). Fifteen minutes later, animals were injected with BK (1 μg). PWL was measured in duplicate at 5-minute intervals before (baseline) and after each injection. Data are expressed as the change (seconds) from individual preinjection baselines and represent the mean ± S.E.M. of four to six animals per group. ***$P < 0.001$ versus all other groups by two-way ANOVA with Bonferroni post-hoc analysis. BL, baseline.

mRNA showed that treatment with siRNA for ERα or ERβ selectively reduced their respective mRNA by 47% and 38%, respectively (Fig. 8A). Treatment with ERα or ERβ siRNA did not alter threshold BK (1 nM)-stimulated IP accumulation (BK stimulation, percentage above basal: siGLO, 25 ± 4; ERα siRNA, 31 ± 5; ERβ siRNA, 30 ± 4). In cells transfected with control siRNA, 17β-E_2 enhanced BK-stimulated IP accumulation as shown previously. Consistent with experiments using subtype-selective agonists and antagonists, the 17β-E_2–mediated enhancement of BK-stimulated IP accumulation was blocked by siRNA directed toward ERα but not ERβ (Fig. 8B).

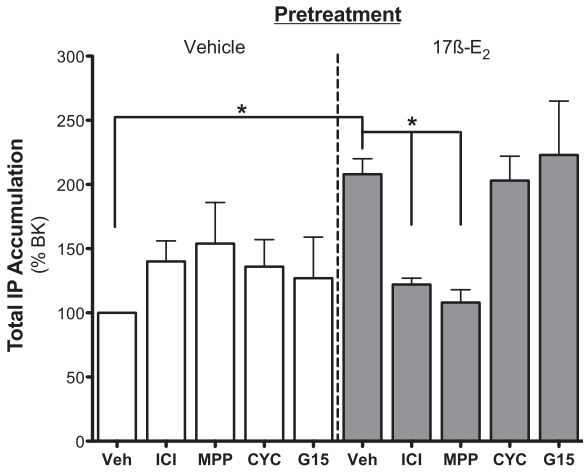

Fig. 7. 17β-E_2–mediated enhancement of BK-stimulated PLC signaling is blocked by the nonselective antagonist ICI 182780 and by the ERα-selective antagonist MPP but not by the ERβ- or the GPER-selective antagonists cyclofenil and G-15, respectively. Cultures of peripheral sensory neurons from OVX rats were treated with vehicle (Veh; 0.1% DMSO) or antagonist [ICI 182780 (ICI), 30 nM; MPP, 300 nM; cyclofenil (CYC), 10 nM; G-15, 2 μM] 15 minutes before treatment with vehicle (0.1% DMSO) or 17β-E_2 (50 nM). Fifteen minutes after 17β-E_2 addition, cells were treated with BK (1 nM) for 25 minutes, and total IP accumulation was determined as described under *Materials and Methods*. Data are expressed as a percentage of BK stimulation and represent the mean ± S.E.M. of five to six experiments. *$P < 0.05$ by one-way ANOVA with Bonferroni post-hoc analysis. Basal dpm 470 ± 23.1, BK dpm 578 ± 34.4 (23% stimulation over basal).

Discussion

Although ERα and ERβ share a significant amount of sequence homology, especially in the DNA-binding domain, their differential tissue expression and differential sensitivity to ligands creates an exciting area of potential therapeutic intervention for a variety of disease states. Although both ERα and ERβ have been strongly implicated in regulating nociception (Craft, 2007; Coulombe et al., 2011; Gintzler and Liu, 2012), such regulation of pain responsiveness by estrogen is complex and often contradictory. Such complexity is probably attributed to differential effects mediated by ERα and ERβ that are expressed at multiple levels of the pain neurotransmission system (Coulombe et al., 2011). Furthermore, effects of estrogen may differ depending upon the type of pain studied (Craft, 2007). Thus, to begin to clarify estrogen's role in pain, it is important to study estrogen's effects in defined regions of the pain-processing system.

Nociceptors respond to intense, potentially tissue-damaging stimuli and send signals to the central nervous system that are interpreted as pain, and thus are generally the first stage in the pain-processing system. Nociceptors express three receptor subtypes that respond to estrogen—ERα, ERβ, and GPER (Papka et al., 2001; Bereiter et al., 2005; Chaban and Micevych, 2005; Dun et al., 2009; Liverman et al., 2009b)—and therefore, estrogen is in a position to regulate pain neurotransmission at this initial stage. We recently found that 17β-E_2 rapidly (within 15 minutes) enhances the responsiveness of nociceptors to activation by BK in vitro and in vivo (Rowan et al., 2010). The rapid-onset effect of 17β-E_2 is mediated by a nongenomic mechanism via a membrane-associated receptor (Rowan et al., 2010). Here, we provide evidence that the rapid-onset effect of 17β-E_2 to enhance nociceptor sensitivity to BK is mediated by the ERα receptor subtype.

17β-E_2 dose-dependently enhanced BK-induced thermal allodynia in the rat hind paw in vivo and PLC activity in sensory neurons in culture, consistent with our previous report (Rowan et al., 2010). Only the ERα-selective agonist PPT mimicked the enhancement of BK responses seen with 17β-E_2. The effects of 17β-E_2, PPT, and the membrane impermeable 17β-E_2–BSA were blocked by the ERα-selective antagonist MPP but not by the ERβ-selective antagonist cyclofenil or the GPER antagonist G-15. Neither the ERβ-selective agonist DPN nor the GPER-selective agonist G-1 enhanced BK-stimulated thermal allodynia when injected into the hind paw at doses chosen to produce maximal receptor occupancy. Collectively, these data suggest that the estrogen receptor that mediates rapid-onset enhancement of BK responses by estrogen in peripheral sensory neurons is ERα.

The utility of selective agonists and antagonists to identify receptors that mediate a response is limited by the degree of ligand selectivity for the target receptor and the dose/concentration of the ligands used. Although the affinities of

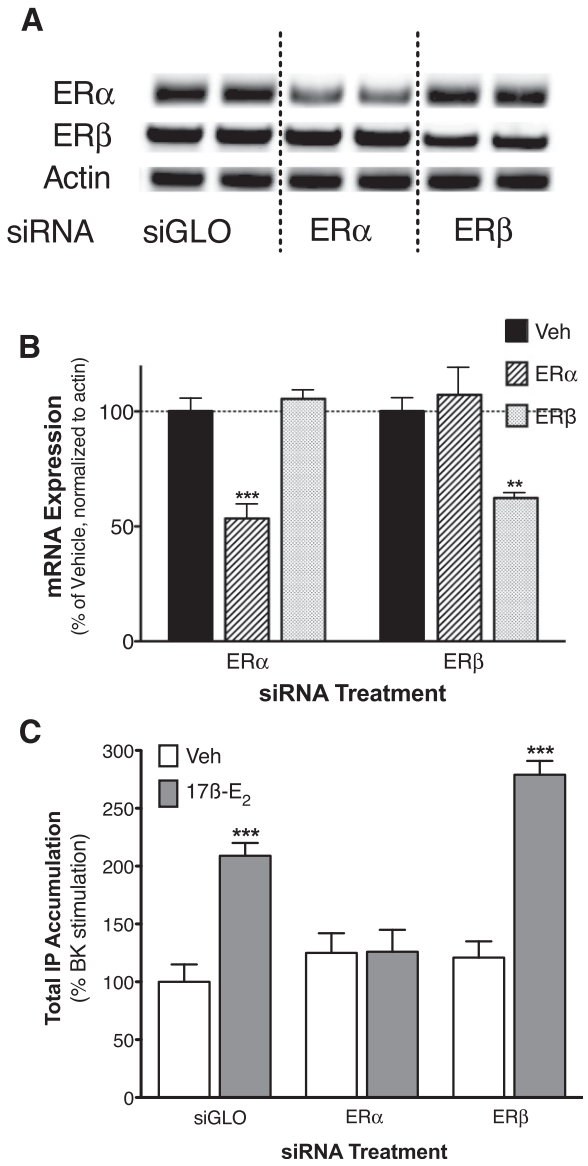

Fig. 8. ERα is required for 17β-E_2–mediated enhancement of BK-stimulated PLC activity in female peripheral sensory neuron cultures. Peripheral sensory neuron cultures from OVX rats were incubated with 50 nM siRNA or siGLO control for 48 hours prior to experiments as described under *Materials and Methods*. Total mRNA was isolated, amplified by reverse-transcription polymerase chain reaction, and separated by agarose gel electrophoresis as described under *Materials and Methods*. (A) Representative agarose gel bands acquired using primers specific to ERα (top row), ERβ (middle row), or β-actin (bottom row) following treatment with siGLO (left two columns), ERα siRNA (middle two columns), or ERβ siRNA (right two columns). (B) Quantification of band densities normalized to β-actin. Data represent the mean ± S.E.M. of three experiments. $^{**}P < 0.01$, $^{***}P < 0.001$ versus vehicle (Veh) by one-way ANOVA with Bonferroni. (C) Following siRNA treatment, peripheral sensory neuron cultures were treated with vehicle (0.1% DMSO) or 17β-E_2 (50 nM) for 15 minutes (37°C) before addition of BK (1 nM) and further incubation for 25 minutes. Total IP accumulation was determined as described under *Materials and Methods*. Data are expressed as a percentage of BK stimulation and represent the mean ± S.E.M. of eight experiments. $^{***}P < 0.001$ by one-way ANOVA with Bonferroni post-hoc analysis.

the drugs used in the present study provide the highest available level of selectivity for their respective targets, there is some concern because affinity/selectivity of ER ligands has generally been characterized with the nuclear ERs that regulate gene transcription responses. The affinity of ligands for a receptor can be influenced by the molecular partners of the receptor. For example, the influence of a G protein on the binding affinity of agonists for seven transmembrane–spanning receptors is well known. Thus, it is possible that the selectivity of ligands for ERs may differ depending upon the location (membrane versus nuclear) and molecular partners of the ERs. To address this issue, and to support the pharmacological results, we used SMARTpool siRNA to selectively reduce ERα or ERβ expression in sensory neurons in culture. Treatment with siRNA selectively reduced expression of ERα or ERβ mRNA. Whereas nontargeting, control siRNA, or treatment with siRNA directed at ERβ had no effect on either the baseline BK-stimulated IP accumulation or the 17β-E_2–mediated enhancement of BK responses, treatment with siRNA directed at ERα completely abolished the enhancing effect of 17β-E_2 on BK-stimulated PLC activity, without affecting the baseline BK response. These results support the conclusion that the ERα subtype mediates the enhancing effect of estrogen on BK responses in peripheral sensory neurons.

In addition to ERα, ERβ and GPER are expressed in sensory neurons, and both DPN and G-1 produced small enhancements of BK-stimulated PLC activity when applied to sensory neurons in culture. The lack of the ability of DPN and G-1 to enhance BK-stimulated thermal allodynia in vivo suggests that the magnitude of the effect on BK signaling may be below the threshold needed to produce behavioral effects on thermal allodynia. Alternatively, ERβ and GPER could be expressed by sensory neurons that are not responsive to thermal stimulation but could regulate responsiveness to noxious mechanical stimulation. In addition, differences in the responses to DPN and/or G-1 between the experiments with sensory neurons of the trigeminal ganglion and the behavioral assay could reflect differences between the trigeminal versus the dorsal root ganglia sensory neurons, which mediate the paw withdrawal response to drug administration in the hind paw.

The mechanism by which activation of membrane-associated ERα rapidly enhances BK signaling in peripheral sensory neurons is not known. A large number of signaling pathways have been shown to be regulated by membrane-associated ERs in a variety of tissues, including mitogen-activated protein kinases, phosphatidylinositol 3-kinase, endothelial nitric-oxide synthase activation, cAMP production, and intracellular calcium mobilization (Hammes and Levin, 2007; Levin, 2009). However, little is known about the contribution of these signaling pathways in mediating rapid effects of estrogen on nociception. In one study, rapid enhancement of inflammation-induced mechanical allodynia of the masseter muscle by estrogen was shown to be mediated by activation of extracellular signal–regulated kinase in sensory neurons (Liverman et al., 2009a). Additional experiments are needed to delineate the signaling pathway(s) regulated by membrane-associated ERα activation that mediates effects of estrogen to enhance BK-stimulated signaling in peripheral sensory neurons.

Many studies have now shown that women experience a disproportionate amount of pain, and that estrogen may be a contributory factor. Estrogen has been implicated in a variety of pain-causing conditions, including arthritis, migraine, and temporomandibular disorder. However, the effect of estrogen on pain processing is multifaceted and complex, with reports of

both pro- and antinociceptive actions. Adding to the complexity are the multiple ER subtypes, genomic (long-term) and non-genomic (rapid-onset), and developmental and activational effects of estrogen. Consequently, unraveling the complexity of estrogen's role in pain, as with many other estrogen-regulated physiologic processes, requires careful dissection of effects at multiple levels of the pain-processing system. Our results indicate that acute activation of membrane-associated ERα in peripheral sensory neurons by estrogen rapidly enhances thermal allodynia elicited by the inflammatory mediator BK. It is possible that targeting membrane–associated ERα with selective antagonists could be effective analgesics for inflammatory pain in women.

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Authorship Contributions

Participated in research design: Berg, Clarke, Hargreaves, Roberts, Rowan.

Conducted experiments: Berg, Rowan.

Performed data analysis: Berg, Clarke, Rowan.

Wrote or contributed to the writing of the manuscript: Berg, Clarke, Hargreaves, Roberts, Rowan.

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