Rapid Signaling of Estrogen in Hypothalamic Neurons Involves a Novel G-Protein-Coupled Estrogen Receptor that Activates Protein Kinase C

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Classically, 17β-estradiol (E2) is thought to control homeostatic functions such as reproduction, stress responses, feeding, sleep cycles, temperature regulation, and motivated behaviors through transcriptional events. Although it is increasingly evident that E2 can also rapidly activate kinase pathways to have multiple downstream actions in CNS neurons, the receptor(s) and the signal transduction pathways involved have not been identified. We discovered that E2 can alter μ-opioid and GABA neurotransmission rapidly through nontranscriptional events in hypothalamic GABA, proopiomelanocortin (POMC), and dopamine neurons. Therefore, we examined the effects of E2 in these neurons using whole-cell recording techniques in ovariectomized female guinea pigs. E2 reduced rapidly the potency of the GABA_B receptor agonist baclofen to activate G-protein-coupled, inwardly rectifying K⁺ channels in hypothalamic neurons. These effects were mimicked by the membrane impermeant E2–BSA and selective estrogen receptor modulators, including a new diphenylacrylamide compound, STX, that does not bind to intracellular estrogen receptors. We characterized the coupling of this estrogen receptor to a Gα_q-mediated activation of phospholipase C, leading to the upregulation of protein kinase Cδ and protein kinase A activity in these neurons. Moreover, using single-cell reverse transcription-PCR, we identified the critical transcripts, PKCδ and its downstream target adenyl cyclase VII, for rapid, novel signaling of E2 in GABA, POMC, and dopamine neurons. Therefore, this unique Gα_q-coupled estrogen receptor may be involved in rapid signaling in hypothalamic neurons that are critical for normal homeostatic functions.

Key words: GABA_B receptor; GIRK; dopamine; GAD; POMC; SERMs

Introduction

It is becoming increasingly evident that the gonadal steroid hormone 17β-estradiol (E2) imparts a multifaceted influence over synaptic transmission in the mammalian CNS. Not only can estrogen alter synaptic responses via estrogen response element-driven target gene transcription, but E2 can also rapidly modulate cell-to-cell communication via membrane-initiated, rapid signaling events (for review, see Kelly and Wagner, 1999). These synaptic alterations are brought about via changes in the cellular responsiveness to the activation of various receptor systems (both G-protein-coupled and ionotropic) to their respective first messengers. E2 can alter the linkage of G-protein-coupled receptor (GPCR) systems such as opioid (both μ and κ), GABAA, and dopamine D1 receptors to their respective effector systems (Demotes-Mainard et al., 1990; Kelly et al., 1992; Takano et al., 1994; Wagner et al., 1994; Lagrange et al., 1996). In addition, E2 can act as an allosteric modulator of ionotropic receptors, such as 5-HT3 and nicotinic receptors (Wetzel et al., 1998; Paradiso et al., 2001), or by direct binding to subunits of ion channels, such as the β1 subunit of the maxi-K⁺ channel (Valverde et al., 1999). These fundamentally distinct signaling pathways give rise to a coordinated regulation by estrogen of complex physiological processes to maintain homeostasis in the mammal (McEwen, 2001).

The quintessential role of estrogen in the CNS is to transmit feedback information to gonadotropin-releasing hormone (GnRH) neurons that control the female reproductive cycle. Estrogen can alter GnRH neuronal activity directly (Kelly et al., 1984; Lagrange et al., 1995) or it can act upstream to alter synaptic input to GnRH neurons (Watson et al., 1992; Herbison et al., 1995, 2001; Sullivan et al., 1995; Simonian et al., 1999). Two of the major presynaptic target neurons of estrogen are the proopiomelanocortin (POMC) and GABA neurons, both of which provide a prominent synaptic input onto GnRH neurons (Morrell et al., 1985; Leranth et al., 1992; Herbison, 1997). Both opioid peptides and GABA inhibit GnRH output (Ferin et al., 1984; Mitsushima et al., 1996) and luteinizing hormone release (Ferin et al., 1984; Akema et al., 1990; Seltzer and Donoso, 1992; Jarry et al., 1994).
1995) from the anterior pituitary. Another target of the actions of estrogen is the arcuate dopamine (tubero-infundibular) neurons that are located in the arcuate nucleus and project to the median eminence, in which they release dopamine into the portal circulation, which directly inhibits prolactin secretion from anterior pituitary lactotrophs (Neill, 1980; Björklund and Lindvall, 1984; Hökfelt et al., 1984).

μ-Opioid and GABA_β receptors are linked to the same population of G-protein-coupled inwardly rectifying K^+ (GIRK) channels in hypothalamic POMC, dopamine, and GABA neurons (Loose et al., 1990, 1991; Wagner et al., 2000). Activation of either μ-opioid or GABA_β receptors elicits an outward K^+ current that robustly hyperpolarizes hypothalamic neurons. However, maximum activation of either receptor occludes the response of the other receptor (Loose et al., 1991; Wagner et al., 1999, 2001). Interestingly, short-term exposure to estrogen reduces the potency of both μ-opioid and GABA_β receptor agonists to activate GIRK channels in hypothalamic neurons (Lagrange et al., 1994, 1996, 1997). The underlying mechanism of this estrogen-induced decrease in the responsiveness of hypothalamic neurons to the μ-opioid and GABA_β agonists is not known. Recent experiments have shown that selective protein kinase A (PKA) inhibitors can block the effects of estrogen, and PKA activators mimic the effects of estrogen on the coupling of μ-opioid receptor to GIRK (Lagrange et al., 1997). Therefore, in the present study, we characterized the estrogen-mediated rapid signaling pathway in hypothalamic neurons by using novel estrogen receptor (ER) ligands. In addition, by using specific protein kinase inhibitors and single-cell reverse transcription (scRT)-PCR, we found that this novel estrogen receptor is coupled to Go_q and activates a phospholipase C (PLC)–protein kinase Cβ–protein kinase A pathway. We conclude that stimulation of this pathway by binding of natural estrogen hormone and certain selective estrogen receptor modulators (SERMs) to the novel G_q-coupled estrogen receptor mediates the rapid steroid response in hypothalamic neurons.

Materials and Methods

Animals and treatments. All animal procedures described in this study are in accordance with institutional guidelines based on National Institutes of Health standards. Female Topeka guinea pigs (400–600 gm), bred in our institutional breeding facility, and female multicolor guinea pigs (400–500 gm; Elm Hill Breeding Labs, Chelmsford, MA) were used in these experiments. The guinea pigs were maintained under constant temperature (25°C) and light (on between 6:30 A.M. and 8:30 P.M.). Animals were housed individually, with food and water provided ad libitum. They were ovariectomized under ketamine–xylazine anesthesia (33 and 6 mg/kg, respectively, s.c.) 5–7 d before experimentation, and they were given sesame oil vehicle (0.1 ml, s.c.) 24 hr before experimentation. Serum estrogen concentrations were determined by radioimmunoassay (Wagner et al., 2001) from trunk blood collected on the day of experimentation and were <10 pg/ml. An additional group of animals (n = 6) were ovariectomized and, after 1 week, were injected with oil vehicle, estradiol benzoate (25 μg in oil), or STX (25 μg in oil) 24 hr before they were killed. The uteri were collected, weighed, and fixed in 4% paraformaldehyde for later histological analysis, which is not being reported in this paper.

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The wild-type C57BL/6 mice in these studies were obtained from The Jackson Laboratory (Bar Harbor, ME). All animals were maintained under controlled temperature (25°C) and photoperiod conditions (14/10 hr light/dark cycle; lights on between 7:00 A.M. and 9:00 P.M.) with food and water ad libitum. Adult mice were ovariectomized under isoflurane anesthesia and allowed to recover for 1 week. At this time, the animals were injected daily for 2 d with oil vehicle, estradiol benzoate (1 μg), or STX (2 or 5 μg) and anesthetized and killed by decapitation after 24 hr.

The uteri were collected, weighed, and fixed in 4% paraformaldehyde for later histological analysis, which is not being reported in this paper.

Drugs. All drugs were purchased from Calbiochem (La Jolla, CA) unless otherwise specified. Tetrodotoxin (TTX) (Alomone Labs, Jerusalem, Israel) was dissolved in Milli-Q H2O and further diluted with 0.1% acetic acid (final concentration, 1 μM). pH 4–5. 17β-Estradiol was purchased from Steraloids (Wilton, NH), recrystallized to ensure purity, and dissolved in 100% ethanol to a stock concentration of 1 mM. 17α-Estradiol (17α-E2; 1 mM; Steraloids), anti-estrogen (ICI 182,780; 10 μM; Tocris Cookson, Ballwin, MO), and the selective estrogen receptor modulators 4-OH-tamoxifen (10 μM; Steraloids), raloxifene (10 μM; Eli Lilly and Company, Indianapolis, IN), and STX (10 μM) were also dissolved in 100% ethanol. 17β-Estradiol 17-hemisuccinate: BSA (E2–BSA) (1 μg; Steraloids) was dissolved in H2O. The protein kinase A inhibitor H-89 dihydrochloride (10 μM) (Stressgen Biotechnologies, Waltham, MA), the protein kinase A inhibitor forolin (50 μM), the protein kinase C inhibitors bisindolylmaleimide I hydrochloride (BIS) (100 μM), Go6976 (2 μM), and rottlerin (10 μM), the phospholipase C inhibitor U73122 (20 μM), and the MEK1 [mitogen-activated protein (MAP) kinase kinase-1] inhibitor PD98059 (50 μM) were dissolved in DMSO. Protein kinase A inhibitory peptide 6–22 amide (1 μM), the protein kinase A inhibitor Rp-cAMPS (50 μM), and cholera toxin (CTX) A subunit (1 μg/μl) were dissolved in H2O. The Gα_q-binding protein designed to mimic the C terminus of the Gα_q subunit and the Gα_q-binding protein designed to mimic the C terminus of the Gα_q subunit were synthesized by PeptidoGenic Research (Livermore, CA). The peptide sequence for Gα_q peptide was Ac–GLNLEKEYNLV–OH, and the peptide sequence for Gqα peptide was CIRCHLQYEL. The α-aminophenoxycetone–N,N,N’,N’-tetra-acetic acid (BAPTA) tetrasodium salt was dissolved in the internal solution at a 10 μM concentration. Aliquots of the stock solutions were stored as appropriate until needed.

Tissue preparation. On the day of experimentation, the animal was decapitated, its brain was removed from the skull, and the hypothalamus was dissected. The resultant hypothalamic block was mounted on a plastic cutting platform that was then secured in a vibratome well filled with ice-cold, oxygenated (95% O2, 5% CO2) artificial CSF (aCSF) (in mM: 124 NaCl, 26 NaHCO3, 10 dextrose, 10 HEPES, 5 KCl, 2.6 NaH2PO4, 2.6 NaHCO3, 11 MgSO4, 1 CaCl2, 124 NaCl, 26 NaHCO3, 10 dextrose, 10 HEPES, 5 KCl, 2.6 NaH2PO4, 2.6 NaHCO3, 11 MgSO4, 1 CaCl2). Four coronal slices (350 μm) through the arcuate were cut. The slices were transferred to a multiwell auxiliary chamber containing oxygenated aCSF and kept there until electrophysiological recording after ~2 hr.

Electrophysiology. Whole-cell patch recordings in voltage clamp were performed as described previously (Wagner et al., 2001). Slices were maintained briefly in a chamber perfused with warmed (35°C), oxygenated aCSF containing the same constituents and respective concentrations, except for CaCl2, which was raised to 2 mM. aCSF and all drug solutions were perfused via a peristaltic pump at a rate of 1.5 ml/min. Drug solutions were prepared in 20 ml syringes by diluting the appropriate stock solution with aCSF, and the flow was controlled via a three-way stopcock.

For whole-cell recordings, electrodes were fabricated from borosilicate glass (1.5 mm outer diameter; World Precision Instruments, Sarasota, FL). Resultant electrodes were then filled with an internal solution containing 0.5% biocytin and consisting of the following (in mM): 128 K+ gluconate, 1 NaCl, 1 MgCl2, 11 EGTA, 10 HEPES, 1.2 ATP, and 0.4 GTP (pH was adjusted to 7.3–7.4 with 1N KOH, 272–315 mM). Voltage pulses were amplified and passed through the electrode using an Axopatch 1D preamplifier (Axon Instruments, Union City, CA). The resultant current deflections were monitored using a digital oscilloscope (Tektronix 2230; Tektronix, Beaverton, OR). After the reduction of the current deflection, negative pressure was applied via a 5 ml syringe connected by polyethylene tubing to the electrode to form a seal (>1 GΩ). After formation of a seal, intracellular access was achieved by suction, followed by perfusion with 1 μM TTX for at least 4–6 min to block spontaneous firing and synaptic potentials before applying the GABA_B receptor agonist baclofen (see Fig. 1). All of the responses to baclofen were measured in voltage clamp as outward currents (Vhold = −60 mV), and only those cells that showed <10% change in access resistance (ac-
cess resistances ranged from 20 to 30 MΩ throughout the recording were included in this study. Membrane currents underwent analog-to-digital conversion via a Digidata 1200 interface coupled to pClamp 7.0 (Axon Instruments). Low-pass filtering of the currents was conducted at a frequency of 2 kHz. The liquid junction potential was −10 mV and was corrected for in subsequent data analysis.

Post hoc identification of hypothalamic arcuate neurons. After electrophysiological recording, the slices were fixed with 4% paraformaldehyde in Sorenson’s phosphate buffer, pH 7.4, for 15 min at 37°C. The tissue was then washed four times in 1 vol of Sorenson’s phosphate buffer, pH 7.4, and then cryoprotected with 30% sucrose dissolved in Sorensen’s phosphate buffer, pH 7.4, and coverslips were applied using a glycerolglycine buffer (10 mM Tris, pH 7.5, 10% glycerol, 2 mM DTT, 10% BSA, and 3 nM [2,4,6,7,16,17-3H]estradiol at 4°C). The slices were scanned for the injected neuron with a Nikon (Melville, NY) Eclipse 800 fluorescence microscope. After localization of the injected neuron with buffer, the slices were incubated at 94°C for 2 min, then added to 2.5 ml of scintillant and counted in a liquid scintillation counter. A binding curve was fitted using software package (GraphPad Software, San Diego, CA). The SD was determined to be 0.5 µl of 100 mM DTT, and DEPC-treated water.

In addition, hypothalamic tissue was homogenized, and total RNA was extracted using the RNeasy kit (Qiagen, Valencia, CA) according to the protocol of the manufacturer. The harvested cell solution and 25 ng of hypothalamic total RNA in 1 µl were denatured for 5 min at 65°C and cooled on ice for 5 min, and then single-stranded cDNA was synthesized from cellular RNA by adding 50 U of murine leukemia virus reverse transcriptase (Applied Biosystems, Foster City, CA), 1.5 µl of 10× buffer, 2 mM MgCl₂, 0.2 µl of deoxynucleotide triphosphates (dNTPs), 15 U of RNasin, 10 mM DTT, 100 ng random hexamers, and DEPC-treated water to a final volume of 20 µl. Cells and tissue RNA used as negative controls were processed as described above but without reverse transcriptase. The reaction mixture was incubated for 4°C for 60 min, denatured at 99°C for 5 min, and cooled on ice for 5 min.

PCR was performed using 3 µl of cDNA template from each RT reaction in a 30 µl of PCR reaction volume containing the following: 3 µl of 10× buffer, 2.4 µl of MgCl₂ (2 mM final concentration for TH, POMC, GABAR receptor2 (GABA B R2), PKCβ, adenyl cyclase VII (AC VII), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH)), or 3.6 µl of MgCl₂ (3 mM final concentration for GAD), 0.2 µl of nTTPs, 0.2 µM forward and reverse primers, 2 µl of Taq DNA polymerase (Promega), and 0.22 µg of TaqStart antibody (Clontech, Palo Alto, CA). Taq DNA polymerase and TaqStart antibody were combined and incubated at room temperature for 5 min, and then the remainder of the reaction contents were added to the tube and incubated at 94°C for 2 min. Then, each reaction went through 60 cycles (35 cycles for GAPDH amplification according to the following protocols: 94°C, 45 sec; 55°C, 40°C, 25°C, 15°C), 45 sec; 72°C, 1 min 10 sec; with a final 72°C extension for 5 min. Ten microliters of the PCR products were visualized with ethidium bromide on a 1.5% agarose gel.

All of the primers were synthesized by Invitrogen (Carlsbad, CA) and were as follows: guinea pig GAD65, 207 bp product, forward primer 5'-GGCCTCTGGTGTAGAAATA-3', reverse primer 5'-CAGATACGCGCTGTTGTTT-3'; guinea pig TH, 233 bp product, forward primer 5'-TCTACGTGTTAACGTGTGAC-3', reverse primer 5'-TTGTGTGCTGTTGACAT-3'; guinea pig GABA B R2, 241 bp product, forward primer 5'-GTGCTCGTTGACTGATAGT-3', reverse primer 5'-GGCTCTGGTGATGGAATA-3'; guinea pig POMC (GenBank accession number S78260), 344 bp product, forward primer 5'-CTGCGTTGCTGTCTCCT-3' and reverse primer 5'-AATGGAGATGGACCGGCGTTC-3'; guinea pig GAPDH, 212 bp product (GenBank accession number CP151527), forward primer 5'-CATCCACTGTTGCGTCCCAAG-3' and reverse primer 5'-GTTCCCTGTTGGTACCCAAG-3'; human protein kinase Cβ, 254 bp product (GenBank accession number S78260), 344 bp product, forward primer 5'-TTGAGGTGTTACCATCAG-3' and reverse primer 5'-TGACGGCCACAGACACT-3'.

Statistical analyses. Comparisons between groups were performed using a one-way ANOVA (with post hoc (Newman–Keuls) paired analysis). Differences were considered statistically significant if the probability of error was <.05.

Results

E₂ and SERMs rapidly attenuate the GABA B response in hypothalamic dopamine and POMC neurons

Whole-cell recordings were made in arcuate neurons (n = 195) from ovariecтомized female guinea pigs (Fig. 1). A subgroup of these neurons (n = 55) was identified using dual-labeling immunocytochemistry (Fig. 2). This revealed that 41% of the cells were TH positive (i.e., dopamine neurons) and 39% were β-endorphin positive (i.e., POMC neurons). Moreover, on the basis of dual-immunocytochemical staining and in situ hybridization for GAD 65, a subgroup of arcuate dopamine neurons coexpressed GABA (O. K. Ronnekleiv, unpublished findings), which was
stantiated by the scRT-PCR data (see below). For the electrophysiology analysis, only cells with gigaohm or better seals were included in this study. The mean resting membrane potential was $-54.3 \pm 0.4$ mV at a 0 pA holding current, and the mean input resistance was $1.9 \pm 0.3$ GΩ. Moreover, 50% of A12 dopamine neurons exhibited a T-type Ca$^{2+}$ current and a hyperpolarization-activated cation current ($I_h$), as we described previously (Loose et al., 1990). Seventy-one percent of the POMC neurons exhibited $I_h$ and a transient outward K$^+$ current ($I_o$), as we described previously (Kelly et al., 1990). Therefore, the passive membrane properties measured with whole-cell patch recording are similar to what we described using single-electrode voltage-clamp recordings (Kelly et al., 1990; Loose et al., 1990).

We used the whole-cell recording method to measure the rapid effects of E$_2$ on the activation of the GIRK conductance by the GABAB$_R$ receptor agonist baclofen. We showed previously that E$_2$ rapidly attenuates both $\mu$-opioid and GABAB$_R$ receptor-mediated responses in hypothalamic arcuate neurons (Lagrange et al., 1994, 1996, 1997). Therefore, for measuring E$_2$ modulation of the GABAB$_R$ response, we used an EC$_{50}$ concentration (5 μM) of baclofen and the protocol depicted in Figure 1. A robust outward current was measured in response to baclofen that subsided after washout (Fig. 3A, H). The application of baclofen 20 min later elicited the same robust response, suggesting that desensitization and rundown were not occurring in response to successive applications of 5 μM baclofen. However, if E$_2$ (100 nM) was applied during the interim period (i.e., after the washout of the first application of baclofen), there was a significant ($p < 0.005$) decrease of 41% in the response to a second application of baclofen (Fig. 3B, H). Current–voltage relationships generated before and during the application of 100 nM E$_2$ showed that this steroid did not change the reversal potential for the baclofen-mediated response: control $E_{rev_{baclofen}} = -88.8 \pm 3.6$ mV, $n = 13$; versus after E$_2$, $E_{rev_{baclofen}} = -85.4 \pm 3.9$ mV, $n = 12$ (Fig. 1C, D). The effects of E$_2$ were stereospecific such that the biologically inactive stereoisomer 17α-estradiol (100 nM) had no effect on the baclofen response (Fig. 3C, H). Furthermore, the effects of E$_2$ were blocked by the anti-estrogen ICI 182,780 when coperfused with E$_2$ (Fig. 3H). Treatment with ICI 182,780 alone had no effect on the baclofen response (data not shown).

We next investigated the cellular localization of this estrogen receptor using the membrane-impermeable estrogen conjugate E$_2$–BSA. Interestingly, E$_2$–BSA (100 nM) was fully efficacious in inhibiting the baclofen response, indicating that this estrogen receptor-mediated response is initiated at the plasma membrane (Fig. 3D, H). We checked the integrity of the E$_2$–BSA preparation by performing an E$_2$ radioimmunoassay of the slice perfusate. We
Estrogen and SERMs rapidly attenuate the GABA_B response. A–F, Representative traces of the GABA_B responses before and after steroid treatment. Experiments were conducted as described in Figure 1. G, The chemical structures of E_2 and the SERMs. H, Bar graphs summarizing the effects of E_2 and SERMs on the baclofen response. E_2 (100 nM) attenuated the GABA_B receptor-mediated outward current by 41%. The inhibitory effects of E_2 on the baclofen response were blocked by the estrogen receptor antagonist ICI 182,780 (1 μM). E_2–BSA (100 nM), 4-OH tamoxifen (1 μM), raloxifene (1 μM), GW-5638 (1 μM), and STX (10 nM) also inhibited the baclofen response; however, 17α-E_2 (1 μM) had no effect. Error bars represent the mean ± SEM of 4–11 cells tested per group. *p < 0.05, **p < 0.01, and ***p < 0.005, versus vehicle control group.
Table 1. Relative binding affinities of ligands to full-length ERα or ERβ

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<tr>
<th>Ligand</th>
<th>RBA ERα</th>
<th>ERβ</th>
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<tr>
<td>17β-Estradiol</td>
<td>100</td>
<td>100</td>
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<tr>
<td>4-Hydroxytamoxifen</td>
<td>36</td>
<td>43</td>
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<tr>
<td>Raloxifene</td>
<td>34</td>
<td>76</td>
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<tr>
<td>GW-5638</td>
<td>5</td>
<td>8</td>
</tr>
<tr>
<td>STX</td>
<td>4.3E-6</td>
<td>9.0E-6</td>
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Relative binding affinities (RBA) are expressed as a percentage of the potency of 17β-estradiol. Under the experimental conditions described in Materials and Methods, 17β-estradiol was found to have an RBA of 5 nM for ERα and 3 nM for ERβ.

found no unbound E2 in the media (data not shown), indicating that E2–BSA conjugate did not contain contamination-free E2.

We also characterized the estrogen receptor by using several SERMs. Tamoxifen (1 μM) was inactive (p > 0.05 vs control) and did not attenuate the effects of E2 on the baclofen activation of GIRK (R2/R1 for E2, 58.6 ± 3.4%, n = 10; vs tamoxifen plus E2, 60.4 ± 6.6%, n = 5). However, 4-OH tamoxifen (1 μM) did partially mimic the actions of E2 by blocking the baclofen response by 25% (Fig. 3E, H). Because 4-OH tamoxifen always exists as an E/Z mixture of olefin isomers (Katzenellenbogen et al., 1985), we suspect that only one of the isomers is active at mediating this novel estrogen response. Raloxifene (1 μM), another SERM with a hydroxylated aromatic ring, mimicked completely the actions of E2 in terms of efficacy in the suppression of the baclofen response (Fig. 3H). In contrast, the nonhydroxylated SERM GW-5638, which structurally resembles the triphenylethylene core of tamoxifen, was found to be significantly more efficacious than E2 at inhibiting GIRK channel activation by baclofen (Fig. 3H).

**STX is a SERM devoid of nuclear ER activity that selectively attenuates rapid responses**

All of the above-mentioned compounds are high-affinity ligands for the nuclear estrogen receptors (Table 1), which complicates the interpretation of the observed pharmacology and makes it difficult to exclude unequivocally a role for nuclear ERs. Therefore, we next tested a novel diphenylacrylamide compound (STX) (Fig. 3G), which is a close structural analog of 4-OH tamoxifen. However, unlike 4-OH tamoxifen, STX is geometrically stable and does not exist as a mixture of E/Z olefin isomers. An additional distinction between STX and other SERMs is that STX has an ~1 million-fold reduced binding affinity for the nuclear ERα or ERβ compared with that of E2 (Table 1). In addition, this compound has no uterotrophic actions, even at five times the dose of E2 (Fig. 4), confirming in vivo that STX has no 4-OH tamoxifen-like estrogenic activity mediated by the nuclear ERs. However, in the whole-cell electrophysiological assay, 10 nM STX was as efficacious as 100 nM E2 in attenuating the GABA B response (Fig. 3F, H).

**The rapid effect of E2 on the GABA B response involves protein kinase A**

We next examined the involvement of specific signaling proteins in the E2–mediated modulation of GABA B. If activation of the PKA pathway is involved, then the effect of E2 on GABA B responses should be inhibited by blocking PKA and mimicked by stimulating PKA. To test this, we applied selective PKA activators and inhibitors. As shown in Figure 5, A and E, forskolin (10 μM) could mimic the actions of E2 to attenuate the GABA B response. However, the specific PKA inhibitor H-89 (10 μM) blocked the E2-induced suppression of the GABA B response (Fig. 5B, E). To confirm the involvement of PKA in E2 modulation of GABA B responses further, we dialedyzed neurons with the specific PKA-inhibitory peptide PKI (PKA inhibitor 6–22 amide, 20 μM) or the nonhydrolyzable cAMP analog Rp-cAMPS (200 μM) that blocks PKA activation. After ~15 min of dialysis with PKI or Rp-cAMPS, the E2-induced reduction of the GABA B response was abolished (Fig. 5C, E). CTX, which is a bacterial exotoxin secreted by vibrio cholerae, elevates intracellular cAMP levels in a variety of tissues by ADP ribosylating the G-protein Gs, thereby stimulating adenyl cyclase activity in an apparently irreversible manner. Intracellular dialysis with the active unit of CTX into individual cells occluded the rapid inhibition of GABA B response by estrogen (Fig. 5D, E). These results indicate that the suppression of the GABA B response by E2 requires the activation of PKA.

**Attenuation of the GABA B response involves protein kinase Cδ**

We next examined whether activation of PKC is also critical for E2 modulation of GABA B. As shown in Figure 5, A and E, forskolin (10 μM) could mimic the actions of E2 to attenuate the GABA B response. However, the specific PKA inhibitor H-89 (10 μM) blocked the E2-induced suppression of the GABA B response (Fig. 5B, E). To confirm the involvement of PKC in E2 modulation of GABA B responses further, we dialedyzed neurons with the specific PKC-inhibitory peptide PKI (PKC inhibitor 6–22 amide, 20 μM) or the nonhydrolyzable cAMP analog Rp-cAMPS (200 μM) that blocks PKA activation. After ~15 min of dialysis with PKI or Rp-cAMPS, the E2-induced reduction of the GABA B response was abolished (Fig. 5C, E). CTX, which is a bacterial exotoxin secreted by vibrio cholerae, elevates intracellular cAMP levels in a variety of tissues by ADP ribosylating the G-protein Gs, thereby stimulating adenyl cyclase activity in an apparently irreversible manner. Intracellular dialysis with the active unit of CTX into individual cells occluded the rapid inhibition of GABA B response by estrogen (Fig. 5D, E). These results indicate that the suppression of the GABA B response by E2 requires the activation of PKA.

**Figure 4.** Uteri are enlarged after estradiol treatment but not after STX or oil vehicle treatment. After a 48 hr treatment period, the uteri of wild-type C57BL/6 mice were collected and examined. A, In E2-treated mice, there was a noticeable increase in uterine size after estradiol benzoate (EB) compared with oil vehicle or STX treatment. B, Bar graph shows the uterine weights. **p < 0.01 versus oil-treated females (n = 3–5 mice per group).
Inhibition of the GABA<sub>B</sub> response by E<sub>2</sub> involves G<sub>q</sub>. Although the specific PKC inhibitor BIS blocked the E<sub>2</sub> effect, forskolin (10 μM) was found to mimic the effects of estrogen in the presence of BIS blockade (Fig. 6 A–D). This indicated that the action of PKC is upstream of the activation of PKA. Therefore, we focused on pathways upstream of PKC to elucidate further the E<sub>2</sub>-mediated signaling pathway.

Inhibition of the GABA<sub>B</sub> response by E<sub>2</sub> involves G<sub>q</sub>. Although the specific PKC inhibitor BIS blocked the E<sub>2</sub> effect, forskolin (10 μM) was found to mimic the effects of estrogen in the presence of BIS blockade (Fig. 6 A–D). This indicated that the action of PKC is upstream of the activation of PKA. Therefore, we focused on pathways upstream of PKC to elucidate further the E<sub>2</sub>-mediated signaling pathway. To examine whether the estrogen receptor-mediated inhibition of the GABA<sub>B</sub> response depended on the activation of G<sub>q</sub>, arcuate neurons were dialyzed with a peptide (11 amino acids) that mimics the C-terminal binding site of G<sub>q</sub> (Akhter et al., 1998). This peptide blocks the
interaction between G-protein-coupled receptors and Gα proteins. In cells dialyzed with this peptide (200 μM), the E2-mediated reduction of the GABA<sub>B</sub> response was blocked significantly (Fig. 7A,E) compared with cells dialyzed with a control peptide (11 amino acids) that mimics the C-terminal domain of Gα<sub>q</sub> (Fig. 7B,E).

In light of these results for a primary role for Gα<sub>q</sub> in E2-mediated rapid inhibition, we tested whether the activation of PLC, a well known Gα<sub>q</sub> effector, might also play a role. To determine whether the activation of PLCβ is required for the estrogen-induced inhibition of GABA<sub>B</sub> response, neurons were treated with the broad-spectrum PLC inhibitor U73122 (10 μM). U73122 (10 μM) was perfused in the extracellular bathing media. Under these conditions, the estrogen-mediated reduction of GABA<sub>B</sub> response was blocked (Fig. 7C,E), whereas the less active PLC inhibitor U73343 at the same concentration had no effect (Fig. 7D,E).

The attenuation of the GABA<sub>B</sub> response does not involve MAP kinase

Recent studies have shown that 17β-E<sub>2</sub> rapidly activates the MAP kinase pathway in primary neuronal cortical cultures and in organotypic cerebrocortical explant cultures (Watters et al., 1997; Singh et al., 1999, 2000). We therefore tested whether inhibition of MAP kinase activity could prevent estrogen modulation of the baclofen response. Treatment with MAP kinase inhibitors PD98059 (10 μM, in the pipette) or U0126 (5 μM) did not affect E2 inhibition of baclofen responses (R2/R1 for E<sub>2</sub>, 58.6 ± 3.4%, n = 10; vs PD98059 plus E<sub>2</sub>, 66.1 ± 11.8%, n = 5).

Expression of GABA<sub>B</sub> receptor PKCδ and adenylate cyclase VII transcripts in arcuate (GABA, dopamine, and POMC) neurons

Using single-cell RT-PCR from 75 acutely dispersed arcuate neurons, we found that 90% of the neurons expressed GAD<sub>65</sub> transcripts, including TH-expressing and POMC-expressing neurons (data not shown). Most importantly, 92% of the neurons expressed GABA<sub>B</sub>-R2 transcripts, which correlates with the 90% response rate to baclofen. Furthermore, we also determined that dopamine and POMC neurons express PKCδ and adenylyl cyclase VII transcripts using single-cell RT-PCR. In one group of cells (n = 22), we found that PKCδ and adenylyl cyclase VII transcripts are expressed in the majority (70%) of TH neurons (Fig. 8A), including those that coexpress GAD<sub>65</sub>. TH and GAD were colocalized in 60% of this population of neurons because of a limited amount of cDNA from individual neurons, POMC expression was determined in another group of cells (n = 29), and we found that PKCδ and adenylyl cyclase VII transcripts were expressed in the majority (75%) of POMC neurons, including those that coexpress GAD<sub>65</sub> (Fig. 8B). POMC and GAD were colocalized in 28% of this population of neurons. Therefore, the single-cell RT-PCR data support the electrophysiological findings that dopamine and POMC neurons express the critical transcripts for rapid estrogen signaling.

Discussion

A unique membrane estrogen receptor mediates the rapid effects of E2

Estrogen suppresses the action of the GABA<sub>B</sub> receptor agonist baclofen to activate GIRK channels in GABA, POMC, and dopamine neurons. This E2 effect is rapid, with measurable suppression occurring within minutes after addition of E2. The kinetics of this response support the notion that a membrane E2 receptor is mediating the response and argue against the involvement of the classical nuclear estrogen receptors operating by transcription regulation.

The pharmacology we observed for this rapid estrogen re-
functions as an agonist of the nuclear ERs, albeit with slightly negative control (Allerand et al., 2002). However, we did not see any effects of activating the MAP kinase pathway (Wade et al., 2001; Toran-Delgado et al., 1999, 2000; Fitzpatrick et al., 2002). Interestingly, ERX also has a distinct pharmacology in that 17α-estradiol was much less efficacious than E2 in reducing L-type calcium currents in neostriatal neurons. Therefore, it appears that the membrane estrogen receptor that modulates channel activity in neurons via the PKC–PKA pathway is pharmacologically distinct from the receptor that is coupled to activation of ERK1 and ERK2 that promotes growth and survival.

Although the GABA_B ionotropic receptor has been identified as a target for 5α-reduced progesterone metabolites in CNS neurons (Harrison et al., 1987; Lambert et al., 1995; Rupprecht and Holsboer, 1999), the nature of the transmembrane receptor for estrogen that uncouples GABA_B (μ-opioid) receptors from GIRQ is not known. In sea trout oocytes, prostaglandins inhibit adenylyl cyclase via a pertussis toxin-sensitive mechanism to initiate oocyte maturation, and recently, a high-affinity progestin, GPCR, has been cloned from spotted seatrout oocytes (Zhu et al., 2003). However, whether there is an estrogen GPCR with similar homology needs to be determined.

E2 activates PKCδ and PKA to alter the coupling of GPCRs to K+ channels in hypothalamic neurons

A number of studies have shown that protein kinase pathways affect GABA_B receptor-mediated signaling in CNS neurons. Activation of protein kinase C suppresses the GABA_B receptor activation of GIRQ channels in the hippocampal CA1 pyramidal neurons (Dutar and Nicoll, 1988) and attenuates the GABA_B receptor-mediated inhibition of norepinephrine release from cerebellar slices (Taniyama et al., 1992).

Currently, there are 12 known members of the PKC family (Way et al., 2000). The family is divided into three groups on the basis of sequence homology and biochemical regulation. Class A, or conventional PKCs (PKCα, β, and γ) are the well known, Ca2+-dependent PKCs. Class B, or novel PKCs (PKCδ, ε, η, and θ), are Ca2+-independent. Finally, class C PKCs, or atypical PKCs (PKCζ and τ), are the most divergent class. Atypical PKCs are also Ca2+ independent and do not require diacylglycerol for activation (Way et al., 2000). We found that the rapid GABA_B₉-suppressing effects of estrogen in hypothalamic neurons were sensitive to the broad-spectrum PKC inhibitor BIS but not to Gö6976, suggesting the involvement of a PKC not belonging to the conventional PKC class. In addition, the inhibition of estrogen of the GABA_B response was not altered by inclusion of 10 μM BAPTA in the intracellular recording patch pipette, providing additional evidence that the Ca2+-dependent conventional PKCs are not involved. However, the selective PKCδ inhibitor rottlerin blocked the actions of E2, suggesting that this novel-class PKC is a mediator of the rapid E₂ response. Moreover, our scRT-PCR data on the expression of PKCδ transcripts in arcuate neurons support the involvement of PKCδ in the E₂-mediated inhibition of the GABA_B response. Likewise, PKCδ is involved in the estrogen-
mediated inhibition of K⁺ channels and fluid retention in female distal colonic epithelial cells, although the upstream signaling pathway is not known (Doolan et al., 2000).

**PKC activation is in the upstream of PKA activation**

In our study, internal perfusion of BIS could completely block the inhibition of the baclofen response by E₂ but could not attenuate the inhibition of the baclofen response by forskolin applied via bath perfusion. PKC is known to activate adenylyl cyclases (Jacobowitz et al., 1993; Yoshimura and Cooper, 1993; Lin and Chen, 1998); moreover, when AC is activated by PKC instead of by Go₉, or forskolin, it is resistant to inhibition by Go₉ (Pieroni et al., 1993). To date, nine AC isozymes have been cloned (AC types I–IX). Notably, AC VII has a potential binding site for PKCδ that is not present in the sequences of the other adenylyl cyclases, which would allow PKCδ to directly phosphorylate AC VII (Nelson et al., 2003). Interestingly, GABA neurons in the cortex, hippocampus, striatum, and cerebellum are immunoreactive for AC VII (Mons et al., 1998), and, in the present study, we show that hypothalamic GABA, TH, and POMC neurons express AC VII transcripts.

**Go₉ mediates the inhibition of the GABAB response by E₂ through PLC**

Most PKCs are activated by diacylglycerol, and some require the presence of Ca²⁺. Thus, PKCs are downstream of the PLC–inositol triphosphate–diacylglycerol signaling cascade. Because different forms of PLC can be activated by various messengers, including Go₉, Gβγ (PLCβ), and tyrosine kinases (PLCγ), the PKC family is involved in a diverse array of signaling cascades (Tanaka and Nishizuka, 1994; Battaini, 2001). Our results show that a membrane ER is specifically coupled to Go₉ protein. This conclusion is based on experiments in which intracellular dialysis with a peptide fragment of Go₉ blocked the receptor interaction with G-protein. This Go₉ peptide has been used to block Go₉ signaling pathways in cortical pyramidal neurons (Carr et al., 2002). In addition, the estrogen-mediated reduction of the GABAB response was significantly reduced by the phospholipase C inhibitor U73122 compared with cells perfused with the less active inhibitor U73343.

Therefore, from the collective results of this study, we formulate the signal transduction pathway for the rapid response to estrogen in hypothalamic neurons depicted in Figure 9. The sequence of events in this model are as follows: (1) E₂ binds to a novel transmembrane estrogen receptor; (2) ligand binding activates Go₉; (3) activated Go₉ in turn activates PLC; (4) activated PLC liberates DAG; (5) free DAG stimulates PKCδ; (6) PKCδ activates adenyl cyclase (VII); (7) cAMP levels are elevated; (8) cAMP stimulates PKA; and (9) PKA phosphorylates membrane targets critical for K⁺ channel function.

**Functional significance of rapid membrane effects of E₂ in CNS neurons**

It was discovered previously that E₂ could rapidly modulate synaptic efficacy via activation of PKA (Gu and Moss, 1996, 1998; Lagrange et al., 1997; Kelly et al., 1999). Presently, we delineate the upstream components of this signaling pathway that includes G₉, phospholipase C, and PKCδ activation (Fig. 9). This is a novel signaling pathway for E₂ to rapidly modulate hypothalamic neuronal excitability, and there is most likely a similar E₂ signaling pathway in hippocampal CA1 neurons (Gu and Moss, 1996, 1998). Therefore, we believe that this pathway is important for increasing synaptic efficacy not only in hypothalamic neurons but also in other neurons in the CNS. In addition, we identified a specific ligand (STX) that is selective for activating this pathway. The consequences of STX effects in hypothalamic neurons are evident in that these neurons are involved in controlling the oscillatory cycle, lactation, stress responses, temperature, and energy balance, all of which require rapid feedback regulation by estrogen. Furthermore, having a selective E₂ agonist for rapid signaling is critical because SERMs such as tamoxifen and raloxifene increase the incidence of hot flashes in women, suggesting that they act as E₂ antagonists in the hypothalamus (Stearns et al., 2002; Sherwin, 2003). In addition, raloxifene treatment is no better than placebo treatment in maintaining cognitive performance of postmenopausal women (Sherwin, 2003), which suggests that
raloxifene is not an $E_2$ agonist in hippocampus. Most importantly, raloxifene and tamoxifen bind to ERα and ERβ with high affinity (Barkhem et al., 1998). In contrast, the STX ($E_2$) receptor is similarly coupled as the serotonin 5HT2A,C receptor (Carr et al., 2002) in CNS neurons, which may explain the ability of serotonin uptake inhibitors (SSRIs) to prevent hot flashes in postmenopausal women (Stearns et al., 2002). Hence, we would predict that STX would prevent hot flashes, maintain sleep cycles, elevate mood, etc. Therefore, this rapid PLC–PKC–PKA signaling of $E_2$ may synergize with CNS transmitter systems to enhance synaptic efficacy in brain circuits that are critical for maintaining homeostatic functions.

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