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Modulation of G Protein-Coupled Receptors by an Estrogen Receptor that Activates Protein Kinase A

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SUMMARY

17β-Estradiol (E_2) rapidly (<20 min) attenuates the ability of μ-opioids to hyperpolarize guinea pig hypothalamic (β-endorphin) neurons. In the current study, we used intracellular recordings from guinea pig hypothalamic slices to characterize the receptor and intracellular effector system mediating the rapid effects of E_2. E_2 acted stereospecifically with physiologically relevant concentration dependence (EC_{50} = 8 nM) to cause a 4-fold reduction in the potency of a μ-opioid agonist to activate an inwardly rectifying K^+ conductance. Using Schild analysis to estimate the affinity of the μ-opioid receptor for an antagonist (naloxone), we found that estrogen did not compete for the μ-opioid receptor or alter the affinity of the μ receptor. Both the nonsteroidal estrogen diethylstilbestrol and the "pure" antiestrogen ICI 164,384 blocked the actions of E_2, the latter with a subnanomolar affinity. The protein synthesis inhibitor cycloheximide did not block the estrogenic uncoupling of the μ-opioid receptor from its K^+ channel, implying a nongenomic mechanism of action by E_2. The actions of E_2 were mimicked by the protein kinase A (PKA) activators forskolin and cAMP, Sp-isomer triethylammonium salt. Furthermore, the selective PKA antagonists cAMP, Rp-isomer triethylammonium salt and KT5720, which have different chemical structures and modes of action, both blocked the effects of E_2. Thus, estrogen binds to a specific receptor that activates PKA to rapidly uncouple the μ-opioid receptor from its K^+ channel. Because we have previously shown that γ-aminobutyric $acid_B$ receptors are also uncoupled by estrogen, this mechanism of action has the potential to alter synaptic transmission via G protein-coupled receptors throughout the brain.

Classically, the ER is thought to act by increasing transcription at estrogen-response elements (1). However, it has become clear that ER actions are much more complex, involving multiple accessory proteins (2) and complex interactions with other intracellular systems (e.g., protein kinases) (3). Furthermore, there is compelling evidence for the existence of nonclassic steroid receptors, some of which are in the plasma membrane (4–7). Finally, numerous rapid (<30 min), presumably nongenomic effects of E_2 are found in the brain and other tissues (8–11); however, the pharmacology and cellular mechanisms of these effects are often poorly understood. Thus, despite the recent progress toward understanding the complexity of E_2 actions, it remains unclear how these diverse actions work together to regulate cellular physiology.

One well-characterized and vital action of E_2 is regulation of reproduction through negative feedback on the HPG axis. *In vivo* and *in vitro* studies in several species have shown that E_2 rapidly (<30 min) suppresses GnRH/luteinizing hormone release (12–14). Although this estrogenic inhibition is thought to involve β-endorphin neurons that are presynaptic to GnRH cells (15), the cellular mechanism by which β-endorphin neurons mediate the rapid regulation by estrogen of GnRH secretion remains unknown. However, β-endorphin preferentially binds to μ-opioid receptors (16), and the vast majority (>90%) of hypothalamic neurons, including GnRH cells (17), are hyperpolarized by μ-opioid activation of inwardly rectifying K^+ currents (18). Furthermore, a brief (20 min) exposure to E_2 rapidly reduces μ-opioid potency in β-endorphin but not GnRH neurons (17, 18). The EC_{50} value of the μ-opioid agonist DAMGO after the application of E_2 is nearly 4-fold greater than control values with no change in the efficacy. 17α-Estradiol is a biologically inactive isomer of E_2 that is identical to the native steroid except for the configuration of a single hydrogen atom. The inability of this compound to mimic the effects of E_2 helped confirm the specificity of this response. Finally, we have also shown that the actions of E_2 occur at physiologically relevant concentrations.

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ABBREVIATIONS: E_2, 17β-estradiol; DES, diethylstilbestrol; ER, estrogen receptor; BSA-E_2, bovine serum albumin/estrogen; PKA, cAMP-dependent protein kinase; HPG, hypothalamic-pituitary-gonadal axis; GnRH, gonadotropin-releasing hormone; DAMGO, [D-Ala^2-*N*-$MePhe^4$-Gly^5-ol]enkephalin; aCSF, artificial cerebrospinal fluid salt solution; Rp-cAMP, cAMP, Rp-isomer, triethylammonium salt; Sp-cAMP, cAMP, Sp-isomer triethylammonium salt; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; AC, adenylate cyclase; RIA, radioimmunoassay.

In the current study, we characterized the receptor and intracellular effector system mediating the rapid attenuation by E_2 of μ-opioid response. Because PKA activation uncouples purified μ receptors from their G proteins (19), we investigated the possibility that a protein kinase mediates the rapid actions of estrogen. We found that PKA stimulators mimicked the effects of E_2 and that two different PKA antagonists with different chemical structures and mechanisms of action blocked the effects of E_2.

Materials and Methods

Animals. Female guinea pigs (Topeka; 350–600 g) that were born and raised in our colony were maintained on a 14-hr light/10-hr dark lighting schedule (lights on 6:30 a.m. to 8:30 p.m.) and were ovariectomized while under anesthesia with ketamine (33 mg/kg)/xylazine (6 mg/kg) 6–10 days before each experiment. Serum estrogen levels as determined by RIA (steroid RIA core, P30 HD18185) were <12 pg/ml (sensitivity of the RIA was 2.5 pg/ml) at the time of death. Each animal was decapitated between 9:00 and 10:00 a.m.; the brain was removed, the hypothalamus was dissected, and coronal slices of 450-μm thickness were cut with a vibratome (18). A single slice was submerged in an oxygenated (95% O_2/5% CO_2), salt solution (aCSF) at 35 ± 1°; the solution flowed through at a rate of 1.5 ml/min and contained 124 mM NaCl, 5 mM KCl, 1.25 mM NaH_2PO_4, 2 mM $MgSO_4$, 2 mM $CaCl_2$, 26 mM $NaHCO_3$, 10 mM dextrose, and 10 mM HEPES.

Drugs. All drugs and chemicals were from Sigma Chemical (St. Louis, MO) unless otherwise specified. All drugs were dissolved in aCSF and then superfused over the slice. Drug changes were made using a manual three-way stopcock. Tetrodotoxin (1 μM) was added to all drug solutions before application to ensure a postsynaptic effect. The μ-opioid responses were measured with the selective agonist DAMGO (20 nM–24 μM; Peninsula Laboratories, Belmont, CA; Ref. 16) and antagonized with naloxone (20–320 nM; Ref. 16). E_2, DES, and BSA-E_2 were from Steraloids (Wilton, NH), and ICI 164,384 [*N*-*n*-butyl-11-(3,17β-dihydroxyestra-1,3,5(10)-trien-7α-yl)-*N*-methylundecanamide] was the generous gift of Dr. Alan Wakeling (Zeneca Pharmaceuticals, Cheshire, UK). The E_2 had been recrystallized to ensure purity. E_2, DES, and ICI 164,384 were stored at 4° in a 1 mM 95% ethanol solution and dissolved in aCSF before application. We have shown previously that 17α-estradiol in similar concentrations of ethanol did not alter μ-opioid responses (18). Cycloheximide, forskolin, staurosporine, and KT5720 (Calbiochem, San Diego, CA) were dissolved in ethanol and then diluted in aCSF before application. Rp-cAMP and Sp-cAMP (Calbiochem) were dissolved in deionized H_2O (10 mM) and then diluted in aCSF.

Electrophysiology. Intracellular recordings were made from arcuate neurons using techniques similar to those previously described (18). Microelectrodes were made from borosilicate glass micropipettes (1-mm o.d.; Dagan, Minneapolis, MN) and were filled with a 3% biocytin solution in 1.75 M KCl and 0.025 M Tris, pH 7.4; resistances varied from 100 to 250 MΩ. Intracellular potentials were amplified, and current was passed through the electrode using an Axoclamp 2A (Axon Instruments, Burlingame, CA). Current and voltage traces were recorded on a chart recorder (model 2200; Gould, Cleveland, OH), digitized at 83 Hz, and stored on an IBM PC clone with Axotape software (Axon Instruments). Voltage-current relationships were obtained by applying a series of depolarizing and hyperpolarizing current pulses (1-sec) and measuring the voltage at the end of each step. Voltage-matched voltage-current plots were also done during the drug-induced hyperpolarization to determine the reversal potential of the conductance. Similarly, voltage-current plots were generated before and during the application of all steroids and kinase analogs to ensure that there were no direct effects on membrane conductances.

Pharmacology. Cumulative concentration-response curves were generated by applying increasing concentrations of DAMGO until the drug-induced hyperpolarization reached a new steady level, usually after 6–7 min (e.g., Fig. 1A). The EC_{50} value was calculated using SigmaPlot (Jandel Scientific, Costa Madre, CA) software to determine the best fit to the logistic equation. The K_e value for naloxone was estimated by Schild analysis (20), and the values for control cells and E_2-sensitive cells were compared after the application of 100 nM E_2.

The concentration dependence of estrogen action was assessed by performing DAMGO concentration-response curves after the application of varying concentrations of E_2 (1 nM to 1 μM). Many cells were tested with multiple E_2 concentrations. In another series of experiments, slices were superfused with 1 or 2 nM ICI 164,384 for 15 min before E_2. The response to E_2 was calculated using the following relation: (DAMGO EC_{50} after E_2)/(DAMGO EC_{50} before E_2) × 100%. The data were computer fitted to the logistic equation to generate an E_2 concentration-response curve.

Immunocytochemistry. Hypothalamic slices that had been incubated with aCSF (time in aCSF, 3–6 hr), estrogen (time after E_2, 2–6 hr), or cycloheximide followed by estrogen (time after E_2, 2–6 hr) were prepared for immunocytochemistry as previously described in detail (21). Briefly, after fixation and cryostat sectioning, the sections mounted on slides were incubated with rat ER antibody (H222; a gift from Dr. Geoffrey Greene, University of Chicago School of Medicine) at 1.0 μg/ml for 40 hr at 4°. Next, the slides were washed for 30 min in phosphate buffer, pH 7.4. Then, the sections were incubated with donkey anti-rat IgG conjugated to Cy3 at 1:100 dilution (Jackson ImmunoResearch, West Grove, PA) for 2 hr at room temperature. Finally, the slides were washed in phosphate buffer for a minimum of 2 hr, and the sections were coverslipped with glycine-buffered glycerol, pH 7.4. The ER staining was photographed with Tri-X-Pan film (ASA 400; Eastman Kodak, Rochester, NY) on a Zeiss Axiophot microscope. To determine the number of cells con-

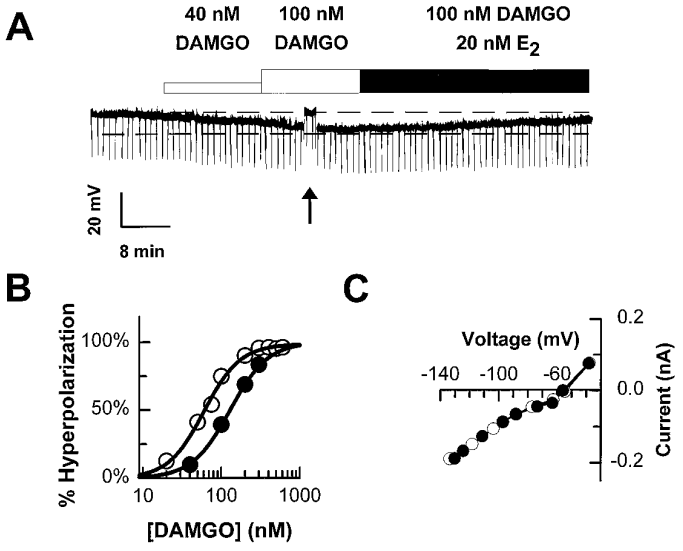

Fig. 1. E_2 rapidly alters the response of hypothalamic neurons to the μ-opioid agonist DAMGO. A, Concentration-dependent hyperpolarization to 40 nM and 100 nM DAMGO in this cell. The response to 100 nM DAMGO was reduced to 50% by the addition of 20 nM E_2. *Upper dashed line,* predrug membrane potential (−51 mV). *Lower dashed line,* maximal DAMGO-induced hyperpolarization (−62 mV). Transient downward deflections represent the response to current pulses (100 msec, 50 pA) used to monitor input resistance. *Arrow,* membrane potential was current-clamped to the predrug resting potentials to verify that DAMGO caused a decreased input resistance. B, After washout of the drugs, a cumulative concentration-response curve was performed (●) and compared with pre-E_2 control responses to DAMGO (○). *Line through data*, computer-generated fit to the logistic equation. C, Current-voltage plot performed by applying current steps (1 sec, 0.2 Hz) before (○) and during (●) E_2 showed that this steroid did not alter resting conductances.

taining immunoreactive ERs, the cells within 250 μm^2 on each section were counted under fluorescent illumination using an eyepiece square grid reticle on a Leitz Laborlux microscope. Two or three sections from the arcuate area of each slice were counted without knowledge of the treatment groups. The total number of cells that were counted from the different sections were averaged, and the mean number of cells was used for further analysis.

Statistical analysis. Numerical data are expressed as mean ± standard error. Electrophysiological data were compared using an unpaired two-tailed Welch *t* test, except as noted. A value of $p < 0.05$ was considered significant. The mean DAMGO EC_{50} value (114 ± 9 nM, 65 cells) after E_2 (including both E_2-sensitive and -insensitive cells) was significantly different from controls ($p < 0.0001$) and was compared with the DAMGO EC_{50} values after E_2 plus kinase/estrogen antagonists with the use of a Mann-Whitney test to evaluate those agents. In the immunocytochemical studies, statistical differences among these groups were determined using an analysis of variance with a Tukey-Kramer *post hoc* test.

Results

Estrogen rapidly attenuates the μ-opioid response in hypothalamic neurons. E_2 rapidly (20 min) reduces the potency of the μ-opioid agonist DAMGO, causing a nearly 4-fold, parallel shift in the DAMGO dose-response curve in approximately one third of hypothalamic cells (53 cells) (18). To further characterize this time course, we applied submaximal concentrations of DAMGO and E_2 simultaneously (Fig. 1A). Although E_2 does not reduce the maximal response to DAMGO (18), it reduced the response to a submaximal μ-opioid concentration as the cell re-equilibrated to the lower potency state. The response to 100 nM DAMGO before E_2 was −9 mV hyperpolarization (82% maximum). However, when 20 nM E_2 was added, the DAMGO response was diminished within 7 min, and after ~12 min, the DAMGO response equilibrated to −4 mV (36% maximum response) below the resting membrane potential. There was no desensitization when this same cell was subsequently tested with higher concentrations of DAMGO (up to 300 nM for 18 min).[1] In our preparation, the response to even higher DAMGO concentrations (1 μM) caused no obvious desensitization in hypothalamic cells (mean change = 0.12 ± 0.08 mV; 13 cells), and E_2 did not increase the desensitization to 1 μM DAMGO in E_2-sensitive cells (mean change = 0.15 ± 0.04 mV; 17 cells). Furthermore, we have shown previously that E_2 alters DAMGO potency without prior exposure to μ-opioids (18). Thus, the attenuated DAMGO response after E_2 does not appear to be due to homologous desensitization of the μ-opioid receptor.

After the washout of DAMGO and E_2, a complete DAMGO concentration-response curve showed that the DAMGO EC_{50} value (177 nM) was shifted from pre-estrogen controls (EC_{50} = 59 ± 3 nM; 43 cells) (Fig. 1B). Furthermore, current/voltage relationships generated before and during the application of 20 nM E_2 alone showed that this steroid did not directly alter ion channels (Fig. 1C). Therefore, estrogen rapidly attenuates the DAMGO response by altering the potency at the μ-opioid receptor.

Estrogen acts via a specific receptor. The parallel, rightward shift in the DAMGO concentration-response curve induced by E_2 is consistent with a competitive block of the μ-opioid receptor, similar to what has been seen with pharmacological concentrations of E_2 (≈200 μM; Ref. 22). This possibility was investigated using Schild analysis (20) to determine the affinity of the receptor for the opioid antagonist naloxone (16) before and after estrogen. As seen in Fig. 2, the K_e value for naloxone in cells treated with 100 nM E_2 was not different from that of control cells. Furthermore, both Schild plots have a slope of −1.0, which is consistent with competitive blockade of the μ-opioid receptor with naloxone. This would not be true if E_2 were competing with both naloxone and DAMGO for the μ-opioid binding site (23). Thus, E_2 neither alters the affinity of the μ-opioid receptor for antagonist nor competitively blocks it.

The receptor mediating the rapid effects of E_2 was further characterized using the antiestrogen ICI 164,384 and the nonsteroidal estrogen DES. Although DES binds to the classical ER and is an agonist for the genomic effects of E_2, this compound did not alter the response to μ-opioids (100 nM DES, DAMGO EC_{50} = 69 ± 5 nM; seven cells). However, DES (100 nM) blocked the actions of 20 nM E_2 when these two compounds were superfused together (DAMGO EC_{50} = 53 ± 4 nM; nine cells; $p < 0.0001$) (Fig. 3). Thus, DES acts as an estrogenic agent, although it is an antagonist rather than an agonist in this system. To our knowledge, this is the first described system in which DES is an estrogen antagonist. However, a similar mixed agonist/antagonist action at classic ERs has been described for other nonsteroidal estrogens, such as tamoxifen (24). ICI 164,384 is a well-characterized, competitive estrogen antagonist (25). As seen in Fig. 4A, ICI 164,384 (100 nM) blocks E_2 (20 nM) action (DAMGO EC_{50} = 61 ± 4 nM; seven cells; $p < 0.0001$). Further studies generated E_2 concentration-response curves by superfusing E_2 (1 nM to 1 μM; 53 cells) followed by a complete concentration-response profile to DAMGO. In several cases, multiple con-

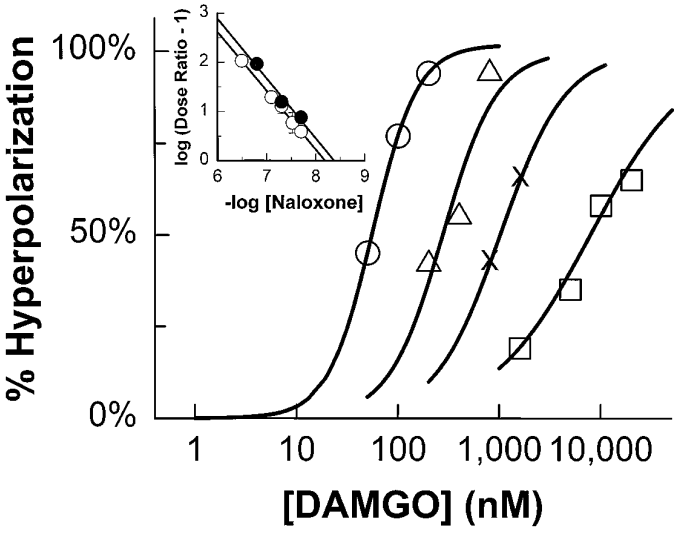

Fig. 2. Estrogen does not compete for the μ-opioid receptor. A representative response in a cell before E_2 (○) in which the DAMGO EC_{50} was shifted from 56 nM to 275, 1047, and 9126 nM by (△) 20, (×) 80, and (□) 320 nM naloxone, respectively. *Inset,* Schild analysis was used to estimate the K_e value for naloxone. *Lines*, linear regression fit of the data. The slopes of both lines are −1.0, which satisfies the criterion for Schild analysis with a single competitive antagonist (20). The K_e value for naloxone before E_2 (○, 4.0 nM; four cells) was not different from that for E_2-sensitive cells after E_2 (●, 3.2 nM; six cells) despite a 3-fold shift of the DAMGO EC_{50} value (from 57 ± 2 to 171 ± 12 nM, respectively).

[1] A. H. Lagrange, O. K. Rønnekleiv, and M. J. Kelly, unpublished observations.

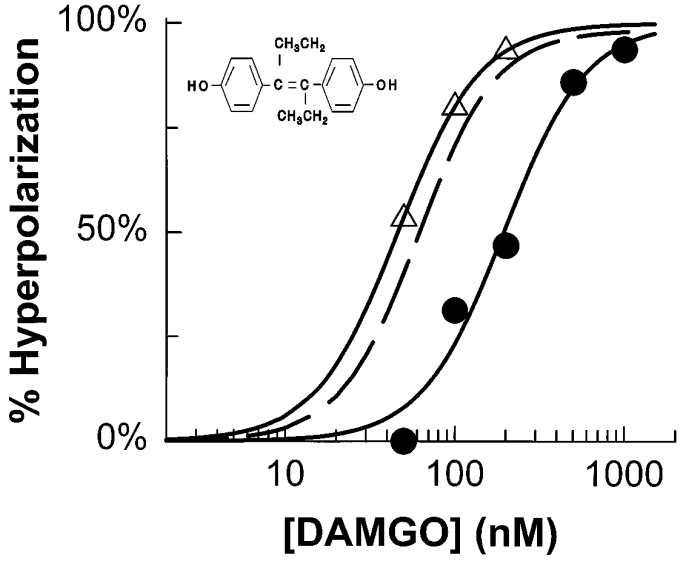

Fig. 3. The nonsteroidal estrogen DES antagonized the effects of E_2. When DES (100 nM; nine cells) was superfused for 10 min before and during E_2 (20 nM, 20 min), the effects of E_2 were blocked. The DAMGO EC_{50} value in this representative cell was 46 nM after the application of DES plus E_2 (△). However, when E_2 alone was applied to the same cell, the DAMGO EC_{50} value was increased to 194 nM (●). *Dashed line*, summary of pre-E_2 DAMGO concentration-response curves (EC_{50} = 59 ± 3 nM). *Inset,* Molecular structure of DES.

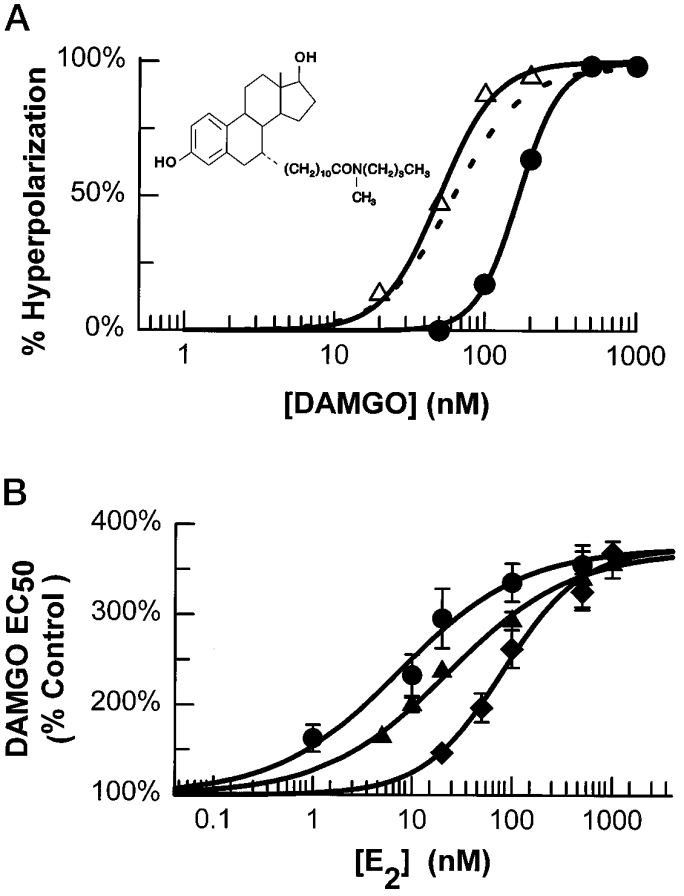

Fig. 4. The antiestrogen ICI 164,384 blocked the rapid effects of E_2. A, ICI 164,384 (100 nM; seven cells) was applied for 10–15 min before E_2 and then cosuperfused with E_2 (20 nM, 20 min). As represented by this cell, the resulting DAMGO EC_{50} value was not different from that of controls. When E_2 was superfused alone, the DAMGO EC_{50} value shifted from 50 nM (ICI 164,384 + E_2, △) to 166 nM (E_2 alone, ●). *Dashed line*, summary of pre-E_2 DAMGO concentration-response curves (EC_{50} = 59 ± 3 nM). *Inset,* Molecular structure of ICI 164,384. B, DAMGO concentration-response curves were performed before and after various concentrations of E_2 (1 nM–1 μM; 53 cells). The data were used to generate an E_2 concentration-response curve (●, EC_{50} = 7.5 nM). The addition of 1 nM ICI 164,384 (▲, EC_{50} = 23 nM; six cells) and 2 nM ICI 164,384 (♦, EC_{50} = 81 nM; eight cells), shifted E_2 concentration-response curve to the right.

centrations of E_2 were applied to the same cell, each followed by a DAMGO concentration-response curve. As seen in Fig. 4B, E_2 had a maximal effect of increasing the DAMGO EC_{50} value (V_{max}) by 376%. The EC_{50} value for the actions of estrogen was 7.5 nM, and the Hill slope was 0.7. A second estrogen concentration-response curve was then generated in which 1 nM ICI 164,384 was superfused before and during E_2 (six cells). The resulting concentration-response curve had an EC_{50} value of 23 nM, a Hill slope of 0.6, and a V_{max} of 372%. A third E_2 concentration-response curve was generated in the presence of 2 nM ICI 164,384, resulting in an EC_{50} value of 81 nM, a Hill slope of 1.1, and a V_{max} of 375%. A modified, single-point Schild analysis (23) estimated the K_e value of ICI 164,384 to be 0.48 and 0.20 nM after treatment with 1 and 2 nM ICI 164,384, respectively. This is very similar to what has been reported for the classic ER (K_d = 0.7 nM; Ref. 25).

Inhibition of protein synthesis does not block the effects of E_2. The question arises of what is the biochemical mechanism of the rapid effects of E_2. A genomic mechanism seems unlikely because E_2 requires ≥30–60 min to alter protein synthesis (26) and probably a longer time to affect cellular physiology. Moreover, cycloheximide did not block the rapid effects of E_2. Slices were superfused with 200 μM cycloheximide for 30 min before, during, and 30 min after E_2 (100 nM, 20 min). This treatment has been shown to block >90% of protein synthesis in brain slices (27) but was unable to block the effects of E_2 (DAMGO EC_{50} = 115 ± 28 nM; eight cells with three cells having a DAMGO EC_{50} value of >160 nM) (Fig. 5). To help confirm that this cycloheximide treatment did indeed block protein synthesis, we made use of the fact that E_2 causes a protein synthesis-dependent down-regulation of the ER (28). Consistent with these previous findings, we found that E_2 caused a robust decrease in the number of cells stained with an anti-ER antibody (77 ± 3 cells/250-μm^2 field in control slices versus 12 ± 4 cells in E_2-treated slices; $p < 0.001$) and that this effect was blocked with prior cycloheximide treatment (78 ± 7 cells; $p < 0.001$ versus controls; Fig. 6).

PKA stimulators mimic the effects of E_2. Research in other systems has shown that protein kinases can uncouple opioid receptors from their effector systems (19), and the rapid effects of E_2 have been shown to be mediated by increases in intracellular cAMP levels in neural (29) and non-neural (9) tissues. We tested the hypothesis that the rapid effects of estrogen are mediated by nongenomic stimulation of PKA. Stimulation of AC with forskolin (1–25 μM) decreased DAMGO potency (DAMGO EC_{50} = 105–221 nM; six cells). Furthermore, direct PKA activation by superfusion of the nonhydrolyzable cAMP analog Sp-cAMP (Fig. 7A) mimicked E_2 action in a concentration-dependent manner. A concentration-response curve for Sp-cAMP (similar to the E_2 concentration-response curve shown in Fig. 4B) estimated the EC_{50} value for Sp-cAMP to be 84 μM, with a maximal

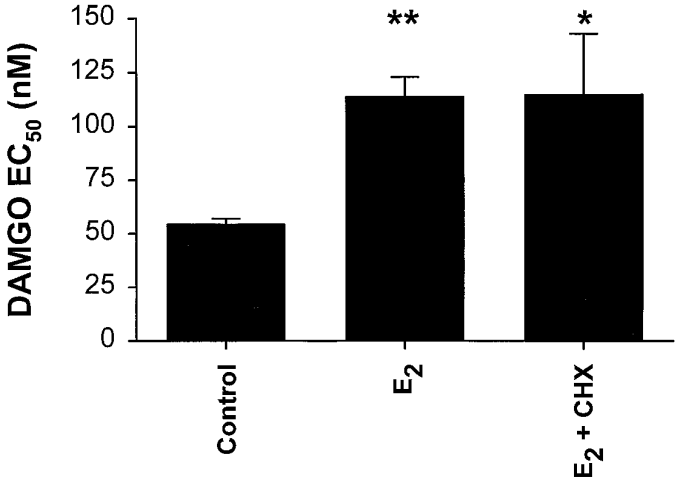

Fig. 5. Pretreatment of the slice with cycloheximide (*CHX*; 200 μM) had no effect on the ability of estrogen to attenuate μ-opioid potency. *, $p < 0.03$ versus controls; **, $p < 0.0001$ versus controls.

393% increase in the DAMGO EC_{50} value. Thus, activation of PKA either directly (Sp-cAMP) or via increasing intracellular cAMP levels (forskolin) mimicked the actions of E_2.

PKA inhibitors block the effects of E_2. To further assess the involvement of protein kinases in modulating μ-opioid responses, the nonselective protein kinase inhibitor staurosporine (100 nM) was superfused before (10 min) and during (20 min) E_2 (100 nM) (Fig. 7B). Staurosporine blocked the effects of E_2, with a mean DAMGO EC_{50} value of 45 ± 6 nM (11 cells) that was significantly lower than in cells treated with E_2 alone ($p < 0.0001$). Similarly, in an E_2-sensitive cell (post-E_2 DAMGO EC_{50} = 143 nM), application of staurosporine (10 nM) after E_2 reduced the DAMGO potency (DAMGO EC_{50} = 46 nM). Thus, staurosporine both blocked the induction and reversed a previously established estrogenic modulation of μ-opioid potency. To confirm that PKA is the protein kinase mediating E_2 action, we used chemically dissimilar compounds that selectively inhibit PKA through different mechanisms. Rp-cAMP is a nonhydrolyzable cAMP analog that blocks PKA activation by binding the regulatory subunit (30). In contrast, KT5720 is an analog of staurosporine that selectively inhibits PKA at its catalytic site (31). Prior application of either agent blocked E_2 action. The DAMGO EC_{50} value (47 ± 7 nM; seven cells) in cells treated with KT5720 (60 nM) plus E_2 (100 nM) was not different from that of controls but was significantly less than that of E_2-treated cells ($p < 0.0005$). Similar effects were seen when Rp-cAMP (100 μM) was used instead of KT5720 (DAMGO EC_{50} = 57 ± 7 nM; eight cells; $p < 0.0001$). After these experiments, the same cells were superfused with E_2 alone, which reduced the DAMGO potency, confirming the E_2 sensitivity of these cells. Finally, the actions of E_2 were reversed by Rp-cAMP and mimicked by Sp-cAMP in the same cell (Fig. 8). In a different cell, KT5720 also reversed the actions of E_2. Thus, PKA inhibitors block the induction of the rapid actions of estrogen and reverse a previously established effect.

Discussion

The current results describe the receptor and intracellular effector system that mediates a novel action of E_2 to rapidly alter synaptic transmission. E_2 seems to act via a specific receptor because the actions of E_2 are saturable, with a physiologically relevant concentration dependence and are not mimicked by the biologically inactive isomer 17α-estra-

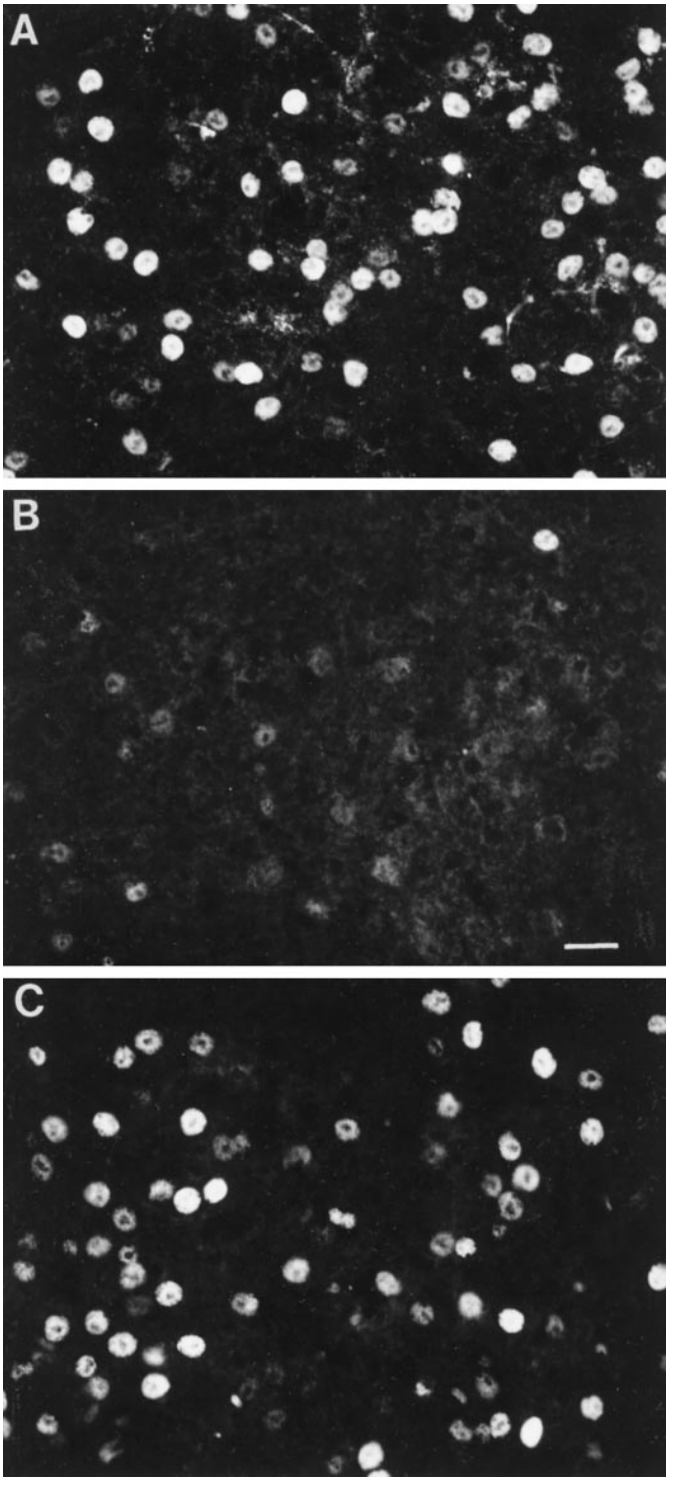

Fig. 6. Cycloheximide treatment blocked the down-regulation of ERs measured immunocytochemically. Coronal sections through the ventral basal hypothalamus illustrate immunoreactive ERs in the arcuate nucleus of slices that were fixed (A) after 4 hr 40 min in aCSF, (B) at 4 hr 30 min after 17β-estradiol (100 nM), or (C) at 4 hr after cycloheximide (200 μM) followed by 17β-estradiol (100 nM). The number of immunoreactive ER cells were significantly reduced after estrogen treatment compared with aCSF-treated or cycloheximide/estrogen-treated slices. *Bar*, 25 μm.

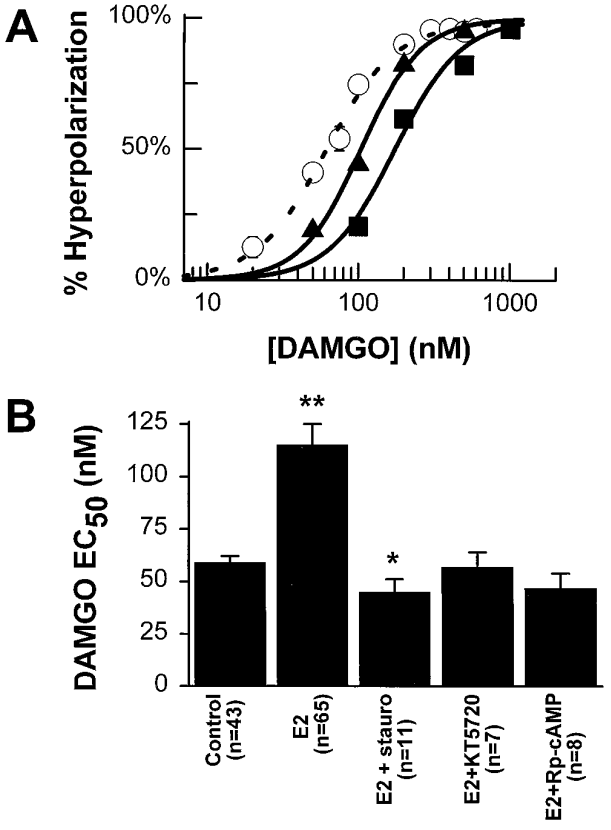

Fig. 7. PKA is necessary and sufficient to alter μ-opioid potency. A, The effects of E_2 were mimicked by the PKA activator Sp-cAMP. DAMGO concentration-response curves were generated in this cell after superfusion of the slice with 50 μM Sp-cAMP (▲, DAMGO EC_{50} = 104 nM) and then subsequently with 150 μM Sp-cAMP (■, DAMGO EC_{50} = 175 nM). *Dashed line with open circles*, summary of pre-E_2 DAMGO concentration-response curves. A similar effect was seen in 3 of 10 cells. B, The rapid attenuation by estrogen of DAMGO potency is blocked by PKA inhibitors. When E_2-sensitive and -insensitive cells were combined, the DAMGO EC_{50} value (114 ± 9 nM; 65 cells) was significantly higher than that of controls. Staurosporine (100 nM) and the more selective PKA inhibitors KT5720 (60 nM; seven cells) and Rp-cAMP (100 μM; eight cells) blocked the effects of E_2 (100 nM, 20 min) when these agents were superfused for 10 min before and during E_2. After the generation of these DAMGO concentration-response curves, E_2 was subsequently applied alone and shown to reduce DAMGO potency (data not shown), thus confirming that each of these kinase inhibitors blocked the actions of estrogen in E_2-sensitive neurons. Staurosporine caused a small but significant reduction in the DAMGO EC_{50} value compared with that for controls. However, none of these agents had any other effect on either the passive or DAMGO-induced properties of these cells. **, $p < 0.0001$; *, $p < 0.01$ compared with controls.

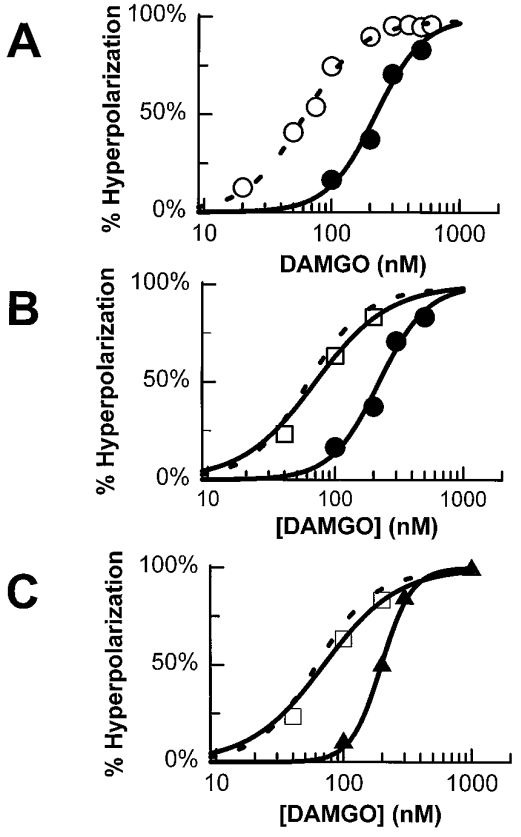

Fig. 8. cAMP analogs modulate E_2 action. A, E_2 shifted the DAMGO potency (●, EC_{50} = 221 nM) from control levels (○, *dashed line*). B, Subsequent superfusion of this cell with Rp-cAMP (□, 50 μM, 15 min) reversed the effect of E_2 (DAMGO EC_{50} = 70 nM). *Dashed line*, controls. C, The inhibition of PKA by Rp-cAMP was overcome by Sp-cAMP (▲, 200 μM, 15 min; DAMGO EC_{50} = 256 nM). *Dashed line*, controls. All of these studies were done within the same cell over a 5-hr period, during which there was <10% rundown of the DAMGO response.

diol (18). This steroid did not compete for μ-opioid receptors. Furthermore, the lipophilic estradiol diffuses freely across cell membranes, but by covalently linking E_2 at its C6 position to BSA-E_2, the steroid is rendered cell-impermeant. Although this conjugate binds to extracellular E_2 receptors and is biologically active in other cell types (7), BSA-E_2 did not alter the response of hypothalamic neurons (eight cells) to μ-opioids.[2] In addition, pharmacological characterization revealed that the antiestrogen ICI 164,384 blocked the effects of E_2 with a subnanomolar affinity (25). Furthermore, although the molecular structures of E_2 and DES are quite different, DES serves as an estrogenic agent at both the classic ER and the currently described ER. However, DES mimics the genomic effects of E_2, but this compound was an antagonist in our system. Although the currently described receptor is similar to the classic receptor, the pharmacodynamics of E_2 and the antagonism by DES imply that a different ER is mediating this effect. Perhaps these E_2 effects are mediated by one of the isoforms of the classic ER that have been found in the brain (4). Therefore, the currently described phenomenon may be mediated by a novel ER or a novel action of the classic ER.

PKA mediates the rapid modulation by estrogen of μ-opioid potency. PKA did not play a simply permissive role in E_2 action because PKA activators altered μ-opioid response in the absence of added steroid. Conversely, inhibition of PKA by two chemically and mechanistically different compounds confirmed that PKA is mediating (rather than merely mimicking) E_2 action. Finally, preliminary data indicate that treatment of hypothalamic slices with E_2, but not with 17α-estradiol, for 10 min stimulates ^{32}P-incorporation into a PKA substrate peptide.[3] Although the estrogenic activation of PKA is clear, the mechanism of this stimulation remains to

[2] A. H. Lagrange, O. K. Rønnekleiv, and M. J. Kelly, unpublished observations.

[3] A. H. Lagrange and H. Enslen, unpublished observations.

be determined. However, the ability of Rp-cAMP to reverse E_2 action suggests that estrogenic activation of PKA involves increases in cAMP levels rather than direct stimulation of the kinase (10). Perhaps E_2 stimulates the activity of AC or inhibits a phosphodiesterase. Finally, it remains to be determined whether other intracellular effectors (e.g., protein kinase C) are also involved in transducing the rapid effects of estrogen. Nevertheless, along with the well-described genomic and plasma membrane-delimited actions of E_2, the present intracellular messenger broadens our understanding of how E_2 regulates cellular physiology.

In addition to heterologous control by E_2, PKA may be involved in homologous regulation of μ-opioid receptors. Chronic exposure to morphine causes a similar uncoupling of μ-opioid receptors from their potassium channels (32). Furthermore, μ-opioids inhibit AC, and chronic inhibition by morphine results in a compensatory up-regulation of AC and PKA (33). Changes in PKA have been correlated with the development of morphine tolerance and dependence (33). However, previous studies have been unable to show a PKA-induced reduction in the maximal μ-opioid response (34, 35). To our knowledge, the current study is the first report that PKA decreases μ-opioid potency in neurons. Perhaps the up-regulation of PKA seen with chronic morphine causes an uncoupling of μ receptors from their effector systems, similar to what has been shown for β-adrenergic receptors (36). Because β-endorphin neurons develop tolerance to chronic morphine (37) and are sensitive to rapid E_2 effects (18), it may be that acute E_2 and chronic morphine share some of the same mechanisms (i.e., increased PKA activity). Studies are under way to examine the effects of PKA modulators in morphine-tolerant animals.

In addition, the currently described phenomenon provides a cellular substrate for the rapid inhibition by estrogen of the HPG axis. We have found that both β-endorphin and GnRH neurons are hyperpolarized by μ-opioids (17, 18) and have proposed a model for negative feedback of estrogen on GnRH release. Because the μ receptor is an autoreceptor on β-endorphin neurons, a given β-endorphin cell would be hyperpolarized by its own neurotransmitter. Therefore, the rapid attenuation by E_2 of μ-opioid potency in β-endorphin neurons (18) would uncouple β-endorphin autoinhibition. This would cause increased opioid peptide release with subsequent inhibition of GnRH neuronal activity (17). Furthermore, modulation of μ-opioid potency occurs within a few minutes and requires nanomolar E_2 concentrations, whereas genomic actions of E_2 require hours to days to alter cellular physiology and act with subnanomolar potency (26, 38). Thus, E_2 may have different actions depending on the time and concentration of E_2, as has been predicted by research in animal models (39). Finally, because we have recently shown that E_2 can rapidly alter the potency at the γ-aminobutyric $acid_B$ receptor (40), estrogen may modulate a variety of G protein-coupled receptors that participate in regulation of the HPG axis.

The genomic effects of E_2 have often been assumed to be the sole pathway for steroid actions. The recent discovery of membrane-delimited estrogen actions has added to the complexity of E_2 physiology, resulting in a dichotomy between extremely rapid membrane effects and slow nuclear actions. Estrogenic activation of PKA is a mechanism for rapid alteration of synaptic transmission that may both complement and complete the other modes of E_2 action. These findings extend the range of E_2 actions from months to minutes and from the nucleus to the extracellular membrane. Although we must further characterize the pharmacology and physiology of these various actions and the interactions among them, we are beginning to develop a more comprehensive picture of how E_2 actually works.

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