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The Potency of μ-Opioid Hyperpolarization of Hypothalamic Arcuate Neurons Is Rapidly Attenuated by 17β-Estradiol

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The μ-opioid agonist DAMGO (Tyr-D-Ala-Gly-MePhe-Gly-ol) hyperpolarizes the majority of arcuate hypothalamic (ARC) neurons by opening an inwardly rectifying potassium conductance. The EC_{50} for the DAMGO-induced hyperpolarization was 60 ± 3 nM in ARC neurons from ovariectomized guinea pigs. Superfusion of 17β-estradiol (E_2; 100 nM) for 20 min *in vitro* resulted in a significant decrease in DAMGO potency (EC_{50} = 212 ± 16 nM) in 40% of the neurons that were tested. This rapid effect of E_2 on the μ-opioid response was not mimicked by the biologically inactive isomer 17α-estradiol. Multiple concentrations of E_2 were used to generate an E_2 concentration–response curve, with an EC_{50} of 9 nM and a maximal increase in the DAMGO EC_{50} of 411% of controls. The membrane properties and firing rate of E_2-sensitive and E_2-insensitive neurons were not different. Streptavidin-FITC labeling did not reveal any significant morphological differences between the groups, but a higher number of E_2-sensitive cells was found in the lateral ARC and cell-poor zone. Moreover, immunocytochemical staining of the recorded cells revealed that β-endorphin neurons were among those sensitive to E_2. Therefore, E_2 could increase β-endorphin release by decreasing the potency of β-endorphinergic autoinhibition, thus increasing the tonic opioid inhibition of E_2-insensitive cells. Furthermore, the diffuse projections of hypothalamic β-endorphin neurons would allow E_2 to alter processes throughout the brain, as well as having local effects in the hypothalamus.

[Key words: hypothalamus, arcuate nucleus, μ-opioid receptor, K^+ channel, G-protein coupling, immunocytochemistry]

β-Endorphin is an endogenous opioid peptide with widespread action throughout the CNS. Although this transmitter is produced in the nucleus tractus solitarius, nearly all the β-endorphin in the forebrain is found in the projections arising from the arcuate hypothalamus (ARC) (Bloom et al., 1978; Khachaturian et al., 1984; Palkovits et al., 1987; Joseph and Michael, 1988). Acting through G-protein–coupled μ-opioid receptors, the direct effects of this peptide include activation of potassium channels (North and Williams, 1985; North et al., 1987), as well as inhibition of adenylate cyclase (Mulder et al., 1990; Schoffelmeer et al., 1992) and calcium channels (Schroeder et al., 1991). In the ARC, the majority (84%) of neurons are hyperpolarized by μ-opioid receptor activation of an inwardly rectifying potassium conductance (Loose and Kelly, 1989; Kelly et al., 1990; Loose et al., 1991). Among the opioid-responsive ARC cells are β-endorphin neurons, allowing β-endorphin to inhibit its own release (Kelly et al., 1990). Consistent with this, both *in vivo* (Koenig et al., 1986) and *in vitro* (Nikolarakis et al., 1987) experiments have found that activation of μ receptors decreases β-endorphin release from the hypothalamus.

β-Endorphin appears to play a role in many CNS processes, ranging from motivation to control of homeostasis. The rewarding properties are evident by the efficacy of i.c.v. injections of this peptide as a positive reinforcement in conditioned place preference paradigms (Amalric et al., 1987). One of the best-characterized physiological functions of this neuropeptide is the control of the hypothalamic-pituitary-gonadal (HPG) axis. This axis is controlled by a long-loop negative feedback in which gonadally produced estrogen inhibits its own production through a decrease in pituitary gonadotropin release (see Kalra and Kalra, 1983; Ferin et al., 1984). This negative feedback is mediated by increasing the opioidergic tone of the hypothalamus, resulting in a decreased hypothalamic LHRH release, with a subsequent reduction in LH release from the pituitary (Van Vugt et al., 1982; Kalra and Kalra, 1983; Ferin et al., 1984; Millan and Herz, 1985). When estrogen levels are high, injection of the β-endorphin antagonist naloxone increases serum LH levels and can even advance the LH surge in women by an average of 2 d (Rossmanith et al., 1988). In contrast, naloxone has very little effect on LH release when E_2 levels are low (Van Vugt et al., 1983). Ovariectomy in rats results in an increase in basal LH levels that are reduced by injection of estradiol (Legan et al., 1973). This inhibition by estradiol is partially reversed by injection of naloxone (Van Vugt et al., 1982). The mechanism of estrogenic modulation of opioidergic tone does not seem to involve changes in the affinity and/or number of hypothalamic μ receptors (Zhang et al., 1993). Instead, we have previously shown that a 24 hr treatment of ovariectomized guinea pigs with estradiol benzoate *in vivo* leads to a threefold reduction in μ-opioid potency compared with oil-treated controls (Kelly et al., 1992). In the present study, *in vitro* exposure of hypothalamic slices to E_2 was used to characterize further the time course and concentration dependence of this, or a similar, estrogenic effect. The specificity of estrogen's actions, as well as distribution of estrogen-responsive neurons, was also investigated.

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Materials and Methods

Animals. All procedures performed on animals were approved by our institutional animal care committee according to NIH guidelines. Female guinea pigs (Topeka, 350–600 gm), born and raised in our colony, were maintained on a 14/10 hr light/dark lighting schedule (lights on 0630–2030) and were given free access to food and water. Females were ovariectomized under ketamine (33 mg/kg)/xylazine (6 mg/kg) anesthetic 6–10 d before each experiment. Each animal was given a subcutaneous injection of oil (100 μl) 24 hr before it was sacrificed by decapitation to allow comparison with previous experiments. Serum estrogens determined by radioimmunoassay (Steroid RIA Core, P 30 HD18185) were less than 12 pg/ml (sensitivity of the RIA was 2.5 pg/ml) at the time of sacrifice. Each animal was decapitated at 0830–0930; the brain was removed, the hypothalamus was dissected and coronal slices of 450 μm thickness were cut on a vibratome (Loose and Kelly, 1989). A single slice was submerged in an oxygenated (95% O_2, 5% CO_2) salt solution at 35 ± 1°C; the solution flowed through at 1.5 ml/min and contained (in mM) NaCl, 124; KCl, 5; NaH_2PO_4, 2.6; $MgSO_4$, 2; $CaCl_2$, 2; $NaHCO_3$, 26; dextrose, 10; and HEPES, 10.

Electrophysiology. Intracellular recordings were made from arcuate neurons using similar techniques to those previously described (Kelly et al., 1990; Loose et al., 1990). Microelectrodes were made from borosilicate glass micropipettes (1.2 mm outer diameter; 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, Foster City, CA). Current and voltage traces were recorded on a chart recorder (Gould 2200, Gould Inc., Glen Burnie, MD), digitized with a Cyberamp 320 (Axon Instruments), 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 (>150 msec) and measuring the voltage at the end of each step. The conductance was calculated from the slope of the current–voltage (I/V) plots in the region between −60 and −80 mV. Voltage-matched I/V plots were also done during the drug-induced hyperpolarization to ascertain the reversal potential of the current and the total conductance change mediating the drug effect. The membrane time constant (τ) was estimated by measuring the time for a voltage deflection (≈10 mV) to reach 63% of its steady-state level on a digital storage scope (Tektronix 2232, Tektronix, Beaverton, OR). Numerical data are expressed as mean ± SEM, except as noted. Comparisons between groups were evaluated using an unpaired Student's t test, and a p value less than 0.05 was considered significant.

Tetrodotoxin (1 μM; Sigma) was added to the solution prior to application of opioids in the majority of cells. A cumulative concentration–response curve was generated to DAMGO (Peninsula Labs, Belmont, CA), an opioid agonist selective for μ receptors (Goldstein and Naidu, 1989). Solutions containing various concentrations of DAMGO were applied until the drug-induced hyperpolarization reached a new steady level, usually after 6–7 min (Fig. 1). The EC_{50} value was calculated using SIGMAPLOT (Jandel Scientific, Corte Madre, CA) software to determine the best fit to the logistic equation

$$V = V_{\text{Max}} \cdot \frac{[\text{DAMGO}]^n}{EC_{50}^n + [\text{DAMGO}]^n},$$

where V is the hyperpolarization seen at each DAMGO concentration and V_{max} is the hyperpolarization seen at the highest DAMGO concentration. EC_{50} is the concentration of DAMGO giving a 50% maximal hyperpolarization, and n is the Hill coefficient. The Hill slope was determined by a linear fit to a Hill plot using at least three points between 20% and 80% maximal response (Taylor and Insel, 1990). After washout of the highest DAMGO concentration, 1–200 nM 17β-estradiol (Steraloids, Wilton, NH) was superfused for 20 min. Both 17α- (Steraloids) and 17β-estradiol were stored at 4°C in a 1 mM 95% ethanol solution. A sample of this stock solution was then dissolved in and applied in the medium (<0.02% ethanol). The 17β-estradiol had been recrystallized to ensure purity. As with DAMGO applications, a current–voltage relationship was determined before and after estrogen application to determine any direct actions of this steroid on conductances. Immediately following E_2 application, a second DAMGO concentration–response relationship was generated. An unpaired Student's t test was used to compute statistical significance between the E_2-sensitive and E_2-insensitive groups, and an ANOVA was used to determine statistical difference between pre-E_2 and post-E_2 within a group. A p value of less than 0.05 was considered significant.

Histology. Following recording, the slices were immersed in 4% paraformaldehyde in 0.03 M Sorensen's phosphate buffer (pH 7.4) for 90–180 min (Ronnekleiv et al., 1990). The slices were then soaked overnight in Sorensen's buffer with 30% sucrose. Sections (16 μm) were cut on a cryostat and mounted on slides coated with poly-L-lysine. Sections were washed with a 0.1 M sodium phosphate buffer (pH 7.4) and then processed with streptavidin-FITC as previously described (Ronnekleiv et al., 1990). After localization of the biocytin-filled neurons, histology data were recorded, which included soma shape, size, distance from the ventral surface and edge of the ventricle, as well as number and morphology of fibers. The slides containing the appropriate sections were then processed with β-endorphin antisera (R13; Weber et al., 1982) at 1:1000 or an affinity-purified tyrosine hydroxylase antiserum (Pel Freeze) at 1:1000 using fluorescence immunohistochemistry (Ronnekleiv et al., 1990). The immunoreactive β-endorphin cells in the slices were most often characterized by clusters of grains surrounding the nucleus in a distinct circular pattern. Cells that appeared to be innervated by β-endorphin–positive fibers had an uneven distribution of finely beaded single fibers over a biocytin-filled cell (Kelly et al., 1990).

Results

One hundred and three cells from the guinea pig arcuate hypothalamus were recorded in current clamp. Figure 1 is an example of a chart record used to generate the DAMGO concentration–response curves for further data analyses. The inset shows the I/V relationship for this cell before and during DAMGO application. As previously reported, the reversal potential of the DAMGO-induced current (−95 mV in this cell) is approximately at E_K. In 40% of the cells thus tested, application of 100 nM estradiol resulted in an approximately fourfold increase in the DAMGO EC_{50} (Fig. 2). The pre-E_2 DAMGO EC_{50} was 60 ± 3 nM (n = 52) with a range of 33–104 nM. The post-E_2 DAMGO EC_{50} values followed a bimodal distribution of E_2 insensitive (62 ± 3, range 35–99 nM, n = 24) and E_2 sensitive (212 ± 16, range 152–394 nM, n = 14), with no overlap between the two groups. The post-E_2 DAMGO EC_{50} values of the E_2-insensitive cells were not different from the pre-E_2 EC_{50} values. The responses to DAMGO in E_2-sensitive and E_2-insensitive neurons are shown in Table 1. The response to DAMGO prior to E_2 was not different between these two groups. The only measured effect of E_2 was increased EC_{50} in E_2-sensitive neurons, with no change in the Hill slope, or the maximal hyperpolarization or conductance change induced by DAMGO. Moreover, there were no differences in the membrane properties or firing rate between E_2-sensitive and E_2-insensitive neurons (Table 2).

Estrogen's actions are concentration dependent and stereoisomer specific

To investigate the specificity of this estrogenic effect, 12 cells were tested with the biologically inactive isomer 17α-E_2; 100 nM 17α-E_2 was unable to mimic the effects of the 17β isomer in 12 cells tested ($p < 0.05$), including three cells that were subsequently shown to have a higher DAMGO EC_{50} following perfusion of 100 nM or 200 nM 17β-E_2. Furthermore, the estrogen concentration-dependence was explored using similar protocols with concentrations of 17β-E_2 ranging from 1 to 200 nM. Following superfusion of 1, 10, 100, or 200 nM E_2 the potency of DAMGO was decreased with EC_{50} values of (in nM) 96 ± 14 (n = 5), 158 ± 20 (n = 5), 212 ± 16 (n = 14), and 226 ± 53 (n = 3), respectively. The response to 200 nM E_2 was not significantly different from 100 nM. From this data a concentration–response curve to estrogen was generated. As shown in

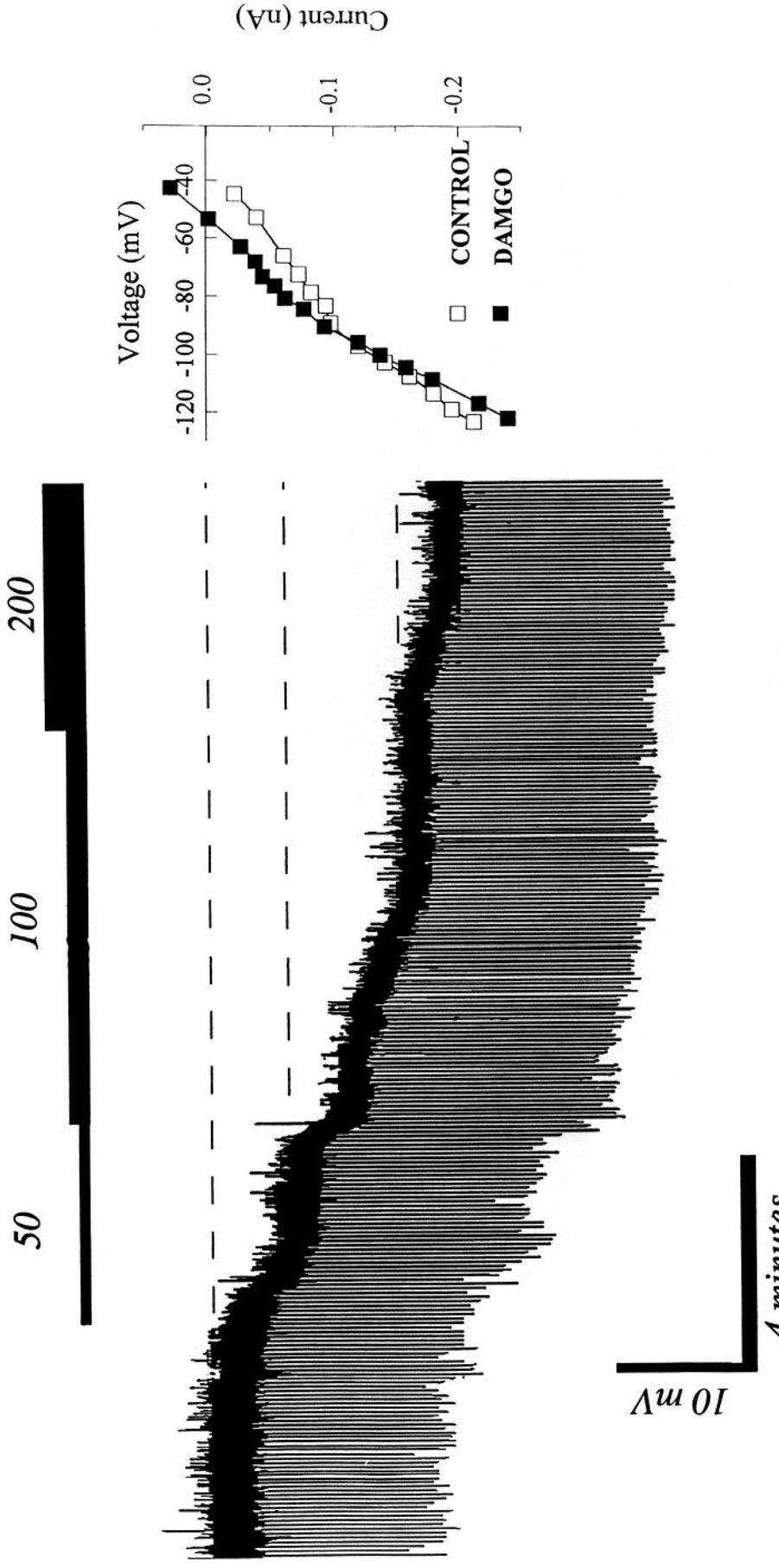

Figure 1. An example of hyperpolarization in response to increasing concentrations of DAMGO in an arcuate neuron. This cell was hyperpolarized by 5, 14, 17 mV by 50, 100, 200 nM DAMGO, respectively. There was no further response to 300 nM DAMGO. Fitting this data to the logistic equation resulted in an EC_{50} of 64 nM. *Inset,* During 300 nM DAMGO, a voltage-matched *I/V* (+40 pA) was performed and compared to an *I/V* performed prior to DAMGO. The effect of DAMGO reverses at approximately −95 mV, with a conductance increase of 0.56 nS measured in the range of −60 to −80 mV. This is an example of an E_2-insensitive neuron tested following 1 nM E_2. A second DAMGO concentration–response curve following 200 nM E_2 gave similar results. The resting membrane potential was −51 mV, and returned to baseline following washout of DAMGO (not shown).

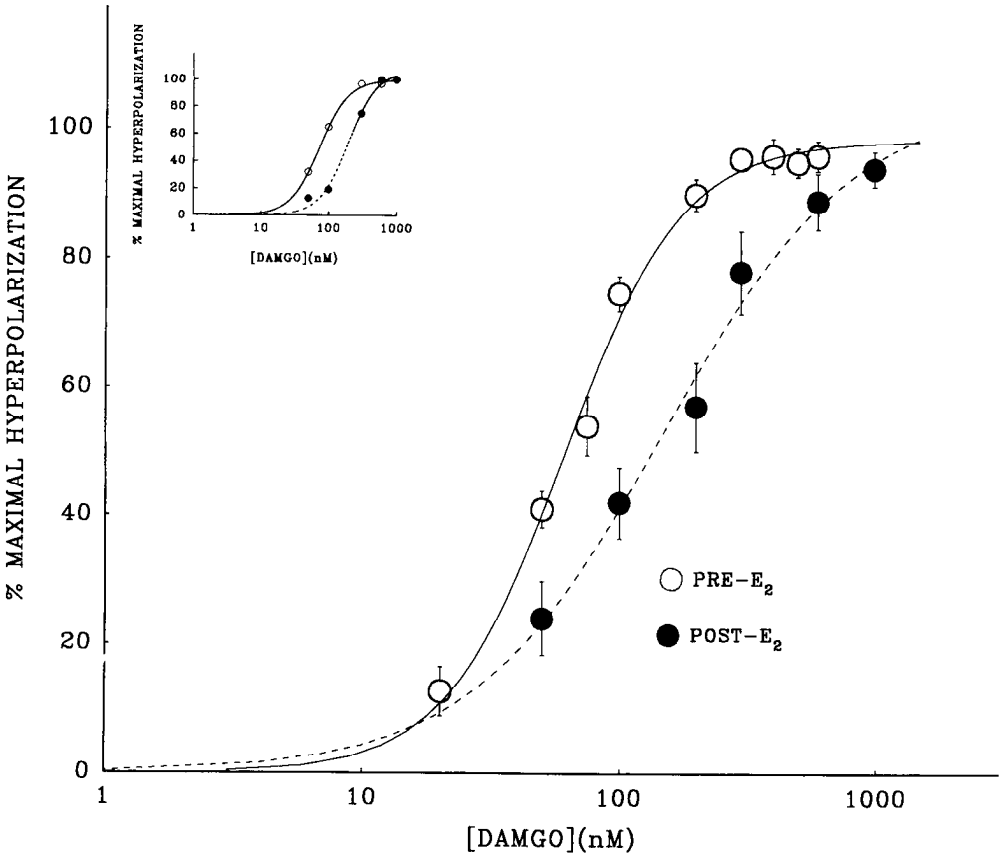

Figure 2. A composite of the concentration–response curves before E_2 application in all cells tested and after E_2 in E_2-sensitive cells. The mean DAMGO EC_{50} was 60 ± 3 nM ($n = 52$) before E_2, and 212 ± 16 nM ($n = 14$, $p < 0.001$) after E_2, with no overlap in the pre- and post-E_2 EC_{50} values. *Inset,* One of the β-endorphin cells in which the DAMGO EC_{50} was determined both before and after E_2. Prior to E_2 the DAMGO EC_{50} was 73 nM, with a maximal hyperpolarization of 9 mV, and following E_2 the DAMGO EC_{50} was 230 nM, with an 8 mV maximal hyperpolarization.

Figure 3, 17β-E_2 decreased μ-opioid potency over fourfold, with an EC_{50} of 9 nM. Multiple concentrations of E_2 were applied to two cells. The DAMGO EC_{50} was 73 and 136 nM following 1 nM E_2 and 188 and 228 nM, following 100 nM E_2, respectively.

Estrogen's effects are not homologous desensitization

Many ARC cells ($n = 12$) were found to have an increased DAMGO EC_{50} following superfusion of 100 or 200 nM E_2 without prior exposure to DAMGO, thus excluding homologous desensitization as a mechanism for this E_2 effect. As with all E_2-sensitive neurons, the post-E_2 DAMGO EC_{50} values did not overlap with the DAMGO EC_{50} values of either the pre-E_2 controls or the post-E_2 E_2-insensitive cells. The decreased DAMGO potency was maintained for as long as we were able to record from the cells (up to 4.5 hr following cessation of E_2 application). The duration of this effect is likely to be intrinsic to the mechanism of E_2 action rather than a lack of E_2 washout, as similar electrophysiologic studies on LHRH neurons have shown a rapid washout of E_2 effects (Kelly et al., 1984). Moreover, the actions of E_2 were long lasting even when superfused with low (1 nM) concentrations of E_2 ($n = 5$; e.g., one cell had a DAMGO EC_{50} of 93 nM 4.5 hr following 1 nM E_2).

Since estrogen is usually thought to work through a genomic mechanism, it is conceivable the E_2-insensitive cells did not have enough time to manifest the effects of estradiol. Although E_2-responsive cells had a decreased DAMGO potency immediately following E_2 superfusion, those cells not responding to E_2 retained a low DAMGO EC_{50} for up to 6.5 hr following E_2 superfusion. Figure 4 is an example of such a nonresponsive cell. This cell was used to generate one pre- and three post-E_2 concentration–response curves, all of which were similar to each other.

Mechanism of estrogen's actions

Estrogen appears to be working through a postsynaptic mechanism, as 88% of cells were recorded in the presence of 1 μM TTX. Direct effects of E_2 to alter cell conductances are unlikely, as E_2 did not induce a change in the resting membrane potential

Table 1. The pharmacodynamics of DAMGO in E_2-insensitive and E_2-sensitive neurons before and after 100 nM E_2

		Post-E_2	
	Pre-E_2[a]	E_2 sensitive	E_2 insensitive
EC_{50} (nM)	60 ± 3 ($n = 52$)	212 ± 16** ($n = 14$)	60 ± 3 ($n = 22$)
ΔV_{max} (mV)	11.9 ± 0.6 ($n = 74$)	12.1 ± 1.1 ($n = 14$)	13.4 ± 0.9 ($n = 23$)
Δg_{max} (nS)	1.0 ± 0.2 ($n = 20$)	1.0 ± 0.4 ($n = 8$)	0.8 ± 0.2 ($n = 12$)
Hill slope	1.6 ± 0.4 ($n = 17$)	1.4 ± 0.2 ($n = 8$)	1.3 ± 0.2 ($n = 7$)

[a] The pre-E_2 responses to DAMGO were not different between these two groups and were pooled for the table only. This includes data from cells that were not subsequently tested after E_2. For the ANOVA analysis the pre-E_2 values were analyzed separately for each group.

** $F_{1,70} = 195$; $p < 0.001$ vs pre-E_2.

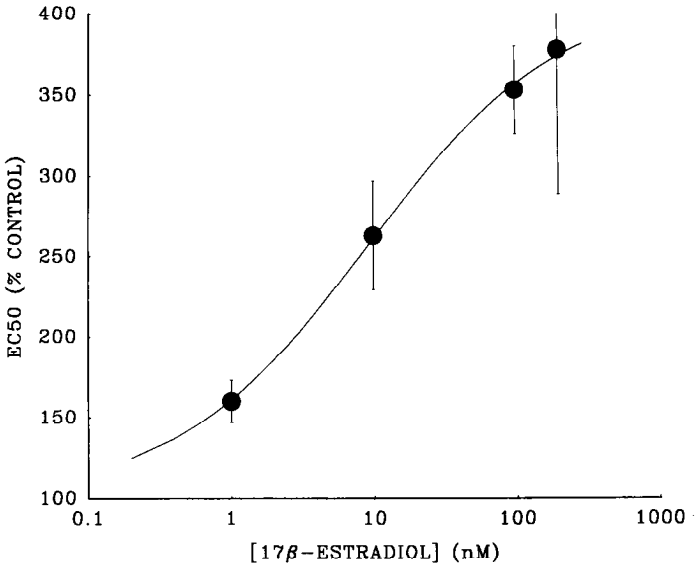

Figure 3. An estrogen concentration–response curve. The DAMGO EC_{50} data are expressed as the average percentage of control levels (60 ± 3 nM, $n = 52$) as a function of E_2 concentration. The DAMGO EC_{50} following 1, 10, 100 and 200 nM E_2 were (in nM) 96 ± 14 ($n = 4$), 158 ± 20 ($n = 5$), 212 ± 16 ($n = 14$), and 226 ± 37 ($n = 4$), respectively. The EC_{50} following 200 nM was not different from 100 nM. The calculated EC_{50} was 9 nM E_2 with a maximum increase in DAMGO EC_{50} of 411% of pre-E_2 controls.

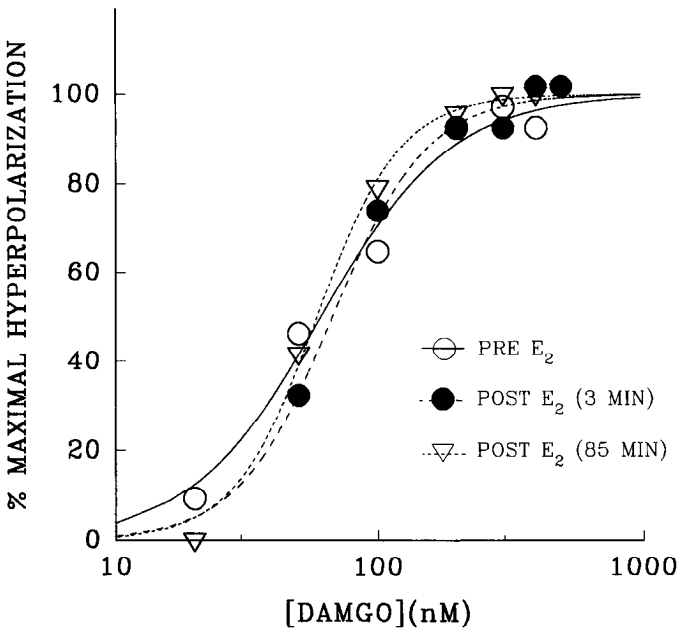

Figure 4. A concentration–response curve in a neuron that did not show a response to E_2 up to 3 hr following 100 nM E_2. Prior to E_2, DAMGO had an EC_{50} of 61 nM, with a V_{max} of 11 mV. Following E_2, two successive concentration–response curves to DAMGO are shown. The DAMGO EC_{50} was 67 and 59 nM, and V_{max} was 11 and 12 mV for the first and second post-E_2 curves, respectively. A third post-E_2 concentration–response curve that was done 3 hr after E_2 application gave similar results (not shown).

or R_{in} in any cells with a high post-E_2 DAMGO EC_{50}. Furthermore, the maximal hyperpolarization and conductance change are the same both before and after E_2 in individual neurons, as well as between the pre- and post-E_2 groups. The reversal potential for the DAMGO-induced hyperpolarization was not different between E_2-sensitive and E_2-insensitive neurons (−94 ± 4 mV, $n = 8$, vs −97 ± 1 mV, $n = 23$, respectively). The reversal potential was also not different from cells tested prior to E_2 exposure (−94 ± 2 mV, $n = 29$). In three cells from each post-E_2 group, DAMGO application resulted in a parallel shift in the *I/V* curves; this is assumed to be the result of poor space clamp in these cells.

Distribution and type of estrogen-responsive cells

There was no morphological difference between E_2-sensitive and E_2-insensitive cells as revealed by biocytin-streptavidin-FITC labeling of the cells. However, the E_2-sensitive neurons had a distribution that was different from E_2-insensitive cells. As shown in Figure 5, the E_2-responsive neurons were clustered more laterally ($p < 0.01$) than the E_2-insensitive cells. The mean distance from the edge of the ventricle was 394 ± 29 μm ($n = 22$) for E_2-sensitive neurons, but only 306 ± 23 μm ($n = 36$) for E_2-insensitive neurons. Also, a subpopulation of the E_2-sensitive neurons were identified as β-endorphin containing. Figure 6 is photomicrograph of one of the five β-endorphin–positive neurons that responded to E_2. None of the E_2-sensitive neurons stained positively for TH ($n = 5$). Not all β-endorphin neurons were E_2 sensitive. Among those cells that did not respond to E_2, five were TH-containing and seven were β-endorphin–containing neurons.

Discussion

The present study provides the first example of a rapid effect of steroids to alter the pharmacodynamics of a ligand-gated G-protein–coupled conductance. With a brief (20 min) exposure, 17β-E_2 (100 nM) decreased the potency of the μ-opioid ligand DAMGO approximately fourfold in about one-third of guinea pig hypothalamic neurons tested. The biologically inactive stereo-

Table 2. Membrane properties of E_2-insensitive and E_2-sensitive neurons

	RMP (mV)	R_{in}[a] (MΩ)	τ (msec)	AP duration[b] (msec)	Resting firing rate (Hz)
E_2 sensitive	−50 ± 2 ($n = 27$)	487 ± 87 ($n = 26$)	17.8 ± 2.0 ($n = 25$)	1.7 ± 0.1 ($n = 16$)	6.7 ± 1.1 ($n = 23$)
E_2 insensitive	−52 ± 1 ($n = 42$)	540 ± 45 ($n = 42$)	21.3 ± 1.3 ($n = 43$)	1.5 ± 0.1 ($n = 29$)	8.0 ± 1.4 ($n = 41$)

[a] Measured as the voltage deflection (≈10 mV) seen with a hyperpolarizing current step (>150 msec).

[b] Measured at one-third height.

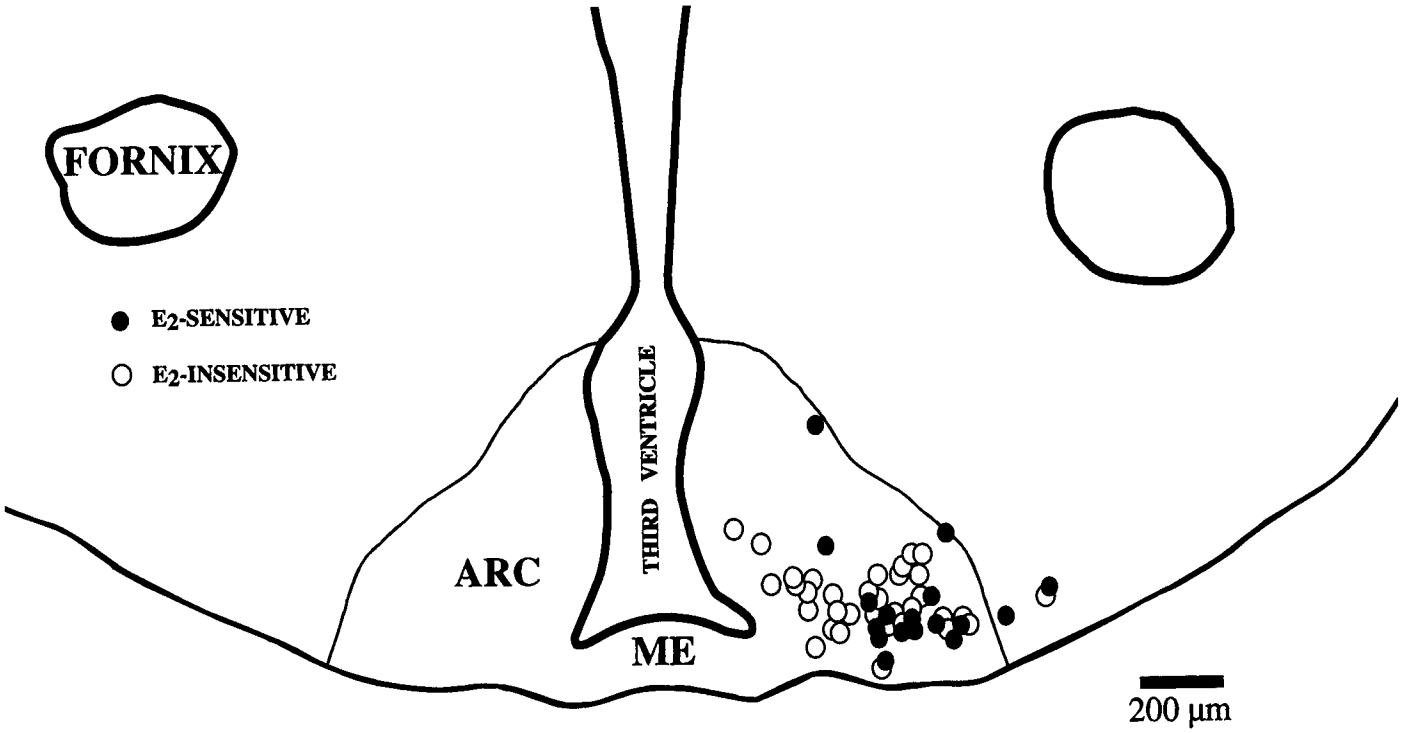

Figure 5. Histological map of the distribution of E_2-sensitive and E_2-insensitive neurons superimposed upon a camera lucida drawing of a representative ARC slice. *Open circles* represent E_2-insensitive neurons, while E_2-sensitive cells are represented by *solid circles*. Coordinates were determined by measuring the distance of the biocytin-streptavidin-FITC–labeled neuron from the edge of the third ventricle and ventral surface of the hypothalamus. The approximate locations of the arcuate nucleus (*ARC*) and the median eminence (*ME*) are shown.

isomer 17α-estradiol was unable to mimic the effect of 17β-E_2, supporting the idea that this modulation is not an artifact of either the steroid or the vehicle used to dissolve it. The reduction in DAMGO potency following estrogen was seen without previous exposure to DAMGO, thus excluding homologous desensitization as a mechanism for this steroidal effect. The ability of E_2 to reduce the tonic opioidergic inhibition of a subset of ARC neurons could produce diverse changes in the physiology of individual neurons as well as the hypothalamus as a whole.

Concentration and time dependence of E_2 effect

In studying E_2 concentration–response relationships, the present paradigm has advantages over *in vivo* administration of E_2. Nonhomogeneous tissue distribution, binding to serum globulins, and metabolism of steroid hormones render dose–response curves to exogenous estrogen questionable when considering the concentration–response relationship of endogenous steroid. The rapid effects of E_2 in an *in vitro* slice preparation allowed the generation of a 17β-E_2 concentration–response curve to help ensure the physiological relevance of this estrogenic effect. The peak plasma levels of E_2 in the guinea pig are about 0.5 nM (A. H. Lagrange, O. K. Ronnekleiv, and M. J. Kelly, unpublished observation). Considering the capacity of the hypothalamus to concentrate E_2 over plasma levels (Eisenfeld and Axelrod, 1965), the potency of this E_2 effect (EC_{50} = 9 nM) would appear to be within the physiological range. Furthermore, the prevailing model of a genomic mechanism for E_2 action has been difficult to reconcile with *in vivo* experiments in which exogenous E_2 causes a rapid decrease in plasma LH levels in several species (Negro-Vilar et al., 1973; Ferin et al., 1984; Condon et al., 1988). Not only does the estrogenic modulation of μ-opioid potency have a concentration dependence that is physiologically relevant, but the time course of E_2 action is similar to that seen *in vivo*.

Potential mechanisms of E_2 action

The rapidity of E_2's actions observed in the present study implies the possibility of a nongenomic mode of action. Although increased transcription may be seen within minutes after exposure to estrogen (Maurer, 1982), it seems unlikely that a protein could be transcribed, spliced, translated, modified, and trafficked within 20 min. There is precedence for rapid, nongenomic estrogenic effects in a variety of tissues. In the uterus, E_2 increases adenylate cyclase activity with a time course and concentration dependence similar to that seen in our system (Bergamini et al., 1985). The ovary in several species releases Ca^{2+} from intracellular stores within seconds of E_2 exposure, perhaps via a "nonclassical" receptor (Morley et al., 1992). In another population of guinea pig hypothalamic neurons, E_2 directly modulates a potassium conductance, possibly via modulation of intracellular nucleotides (Kelly et al., 1984; Nabekura et al., 1986; Minami et al., 1990). A similar effect was seen in some neurons in the present study, but there was no decrease in DAMGO potency in any of these neurons following E_2 application (n = 8). Nonetheless, a similar intracellular pathway such as activation of cyclic nucleotide–dependent kinases may be involved.

Although the mechanism of E_2 action remains to be elucidated, the electrophysiological data suggest that this is a postsynaptic effect working at the level of receptors/G-proteins, rather than a direct alteration of the μ-opioid–gated channel, or other conductances. Perhaps E_2 is altering the affinity of μ receptors or the number of spare receptors. It is also possible that E_2 interacts directly with the G-proteins mediating the μ-opioid

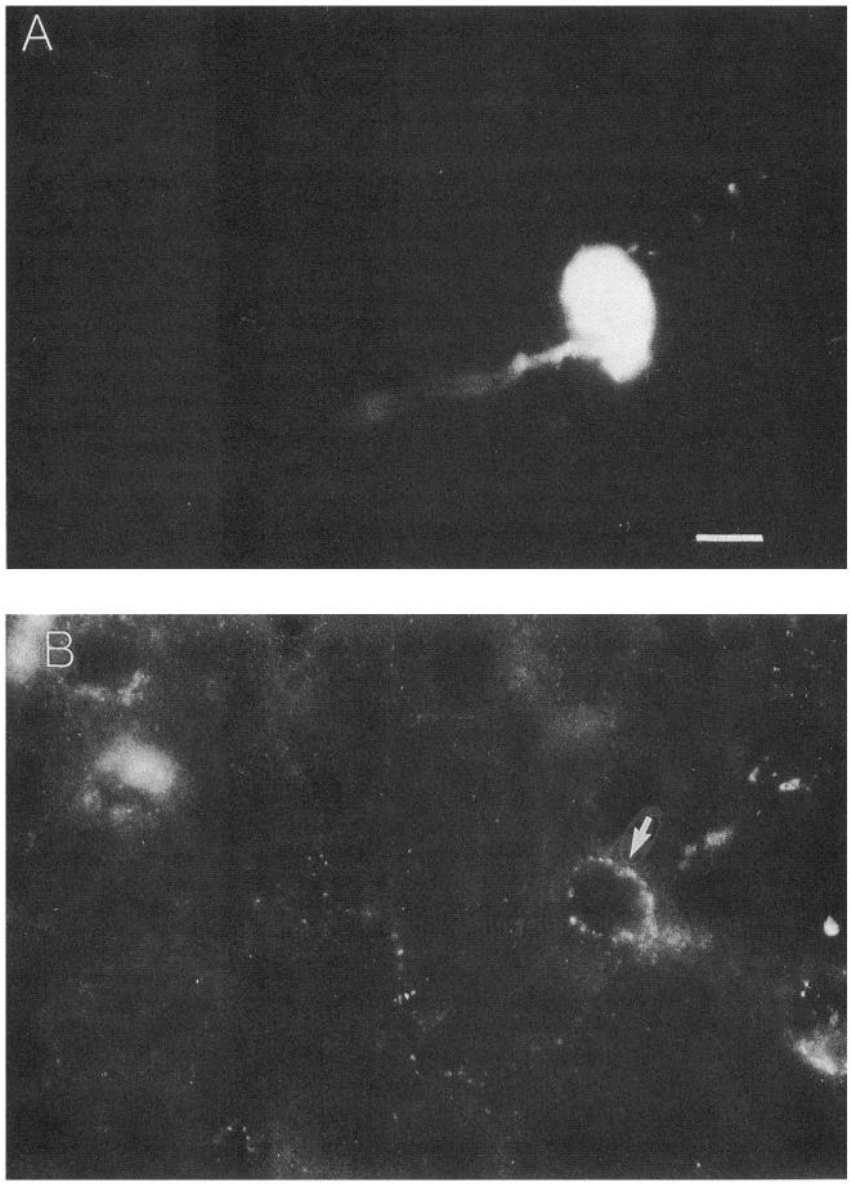

Figure 6. A photomicrograph of a double-labeled β-endorphin neuron that was sensitive to E_2 (see Fig. 2 inset). *A*, Photomicrograph of the biocytin-filled cell labeled with streptavidin-FITC. *B*, Photomicrograph of the same cell immunocytochemically stained for β-endorphin (*arrow*). Four other E_2-sensitive cells stained positively for β-endorphin. Scale bar, 10 μm.

transduction pathway. Equally plausible would be a role of E_2 in modulating receptor/G-protein coupling. Both protein kinase A (PKA) and protein kinase C (PKC) have been shown to be involved in uncoupling opioid receptors from their G-proteins (Harada et al., 1990; Louie et al., 1990). As previously mentioned, adenylate cyclase activity is rapidly stimulated by E_2, and the ability of E_2 to regulate progesterone receptor levels is blocked by inhibitors of PKA (Aronica and Katzenellenbogen, 1991). Estradiol-induced release of Ca^{2+} from intracellular stores could serve to activate PKC. Furthermore, E_2 can induce an isoform of phospholipase C that would serve to increase the activation of this kinase (Mobbs et al., 1991).

There are several possible mechanisms whereby E_2 might alter intracellular messenger pathways. The binding of E_2 to the "classical" estrogen receptor has been shown to stimulate a tyrosine kinase (Auricchio et al., 1987), possibly altering the activity of other kinase systems. Furthermore, the purified E_2 receptor has also been shown to be associated with a serine kinase (Baldi et al., 1986). Alternatively, perhaps E_2 is working through a membrane-associated receptor, such as a synaptosomal steroid receptor (Towle and Sze, 1983) or a membrane-associated G-protein–coupled steroid receptor (Orchinik et al., 1991).

Potential physiological significance of opioidergic modulation

Since a subset of the E_2-responsive neurons were identified as β-endorphin containing, E_2 could serve to enhance opioidergic tone in the hypothalamus and other brain areas by diminishing autoinhibition of β-endorphin release. Not all β-endorphin neurons are E_2 sensitive, and it is reasonable to hypothesize other neuronal types may exhibit this estrogenic effect. Thus, the complexity of the hypothalamus allows the actions of E_2 to have manifold potential consequences. A nonresponsive cell receiving input from an E_2-responsive β-endorphin neuron would be subjected to higher concentrations of this peptide, and thus be more inhibited. An E_2-responsive neuron with input from a nonresponsive β-endorphin neuron would be released from some

of its tonic inhibition and thus would be more excitable. Finally, an E_2-responsive neuron subjected to increased extracellular β-endorphin might not have much change in excitability with E_2 treatment. In this way, E_2 could have widely different actions, depending on cell type and innervation. Furthermore, our previous work has shown that a 24 hr exposure to estradiol results in a reduced DAMGO potency in all of the ARC neurons that were tested (Kelly et al., 1992). Thus, there may also be a time dependence to this regulation of endogenous opioids. We have previously shown that the μ-opioid and $GABA_B$ receptors are G-protein coupled to the same potassium conductance in ARC neurons (Loose et al., 1991). As with DAMGO, the potency of the $GABA_B$ agonist baclofen is decreased following exposure to E_2 for 24 hr (Kelly et al., 1992). Therefore, E_2's ability to alter the potency and/or efficacy of neurotransmitters is not limited to the μ-opioid system. Studies are currently underway to determine if E_2 rapidly modulates the $GABA_B$ system.

Although the connection between estrogenic action and control of the HPG axis is the most obvious, a role for E_2 in regulating the opioidergic tone in other regions of the brain may also be important. Morrell and co-workers have shown that a population of estrogen-concentrating neurons in the ventrolateral arcuate send long projections throughout the midbrain (Morrell et al., 1992) and that β-endorphin neurons of this region concentrate E_2 (Morrell et al., 1985). Therefore, E_2's actions in the hypothalamus could result in altered β-endorphin release throughout the brain. For example, the aromatization of testosterone in the male (Roselli and Resko, 1987) would allow anabolic steroids to have an estrogenic effect on opioidergic tone. This may explain some of the morphine-like aspects of anabolic steroid abuse, including drug craving and a withdrawal syndrome similar to that seen in morphine-deprived addicts (Kashkin and Kleber, 1989). Interestingly, in one case report, the opiate antagonist naloxone given to an anabolic steroid abuser precipitated a morphine-like withdrawal, including autonomic, cardiovascular, and psychological symptoms, with drug craving (Tennant et al., 1988). The estrogenic modulation of opioidergic tone may provide a mechanism for the addictive properties of anabolic steroids, as well as some of the other side effects of their abuse.

In summary, this is the first demonstration of a rapid steroidal modulation of the pharmacodynamics of a G-protein–coupled neurotransmitter system. Besides providing a possible mechanism for the estrogenic feedback on the HPG axis, the rapid effects of E_2 to modulate opioidergic tone may be involved in a variety of physiological and pathophysiological processes in the CNS. Furthermore, the unusual rapidity with which E_2 is able to effect these changes implies the possibility of a novel, nongenomic mechanism of action for this steroid hormone.

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