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Estradiol-17β and μ-Opioid Peptides Rapidly Hyperpolarize GnRH Neurons: A Cellular Mechanism of Negative Feedback?

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ABSTRACT Control of the HPG axis involves a rapid (30 min) inhibition of LH (GnRH) release by E_2. The time course of this effect is faster than expected for a purely transcriptional mechanism of E_2 action. To elucidate the mechanism of E_2 action, intracellular recordings in TTX were performed in guinea pig hypothalamic GnRH neurons. These neurons were directly hyperpolarized by both the μ-opioid agonist, DAMGO (Tyr-D-Ala-Gly-MePhe-Gly-ol, 9 mV) and the $GABA_B$ agonist, baclofen (18 mV) by opening K^+ channels. Schild analysis with naloxone (K_e=2.4 nM) confirmed that μ-opioid receptors mediated the effect of DAMGO. E_2 also directly hyperpolarized GnRH neurons by opening K^+ channels. Coupled with previous work showing a rapid effect of E_2 to alter μ-opioid potency (1), a model is presented in which E_2 rapidly inhibits GnRH neurons through parallel, possibly synergistic pathways.

INTRODUCTION

Control of the female reproductive cycle involves complex interactions among the gonads, pituitary and hypothalamus. The final effector of hypothalamic control of the pituitary/gonadal (HPG) axis is GnRH. Regulation of this peptide is poorly understood but appears to involve both positive and negative feedback control by ovarian 17β-estradiol (E_2). The classical mechanism of E_2 action is the alteration of gene transcription; a process that affects cellular physiology over a period of hours to days (2). However, studies in a number of ovariectomized animal models have shown that E_2 injections can quickly reduce serum LH levels (<30 min) (3,4) and portal blood GnRH (5). The rapidity of this estrogenic effect has been difficult to reconcile with a genomic mode of E_2 action. Moreover, GnRH neurons do not contain the classical E_2 receptor (6), thereby suggesting that GnRH release is controlled by E_2-sensitive neurons that are presynaptic to GnRH neurons (see (7)). During the menstrual cycle, negative feedback control of LH secretion by E_2 appears to involve the hypothalamic opioid systems (8). Morphine attenuates the LH surge and prevents ovulation in the rat (9,10). Conversely, the opioid antagonist, naloxone potentiates the duration and magnitude of the LH surge (9,10), increases GnRH levels in portal blood in monkeys (11) and advances ovulation in women (12,13). Although direct synaptic contacts between β-endorphin and GnRH neurons have been found in monkeys (14) and rats (15), a direct action of opioids (opiates) on GnRH neurons has not been demonstrated. Moreover, the opioid receptor subtype or effector system to which it is coupled is not known.

To elucidate the mechanism of estrogenic negative feedback, we made intracellular electrophysiological recordings from the hypothalamus of ovariectomized guinea pigs. Our laboratory has recently described a rapid *in vitro* effect of E_2 (20 min) which alters the pharmacodynamics of μ-opioids in β-endorphin neurons (1). Since β-endorphin binds to the μ-opioid receptor with high affinity (16), we hypothesized that the μ receptor is the postsynaptic receptor responsible for opioid actions. In the present studies we evaluated the response of GnRH neurons to μ-opioid agonists and the modulation of this response by E_2.

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METHODS

Animals. All procedures performed on animals were approved by our Animal Care and Use Committee following NIH guidelines. Female guinea pigs (Topeka; 350-600 g) were ovariectomized 6-10 days prior to sacrifice. E_2 concentrations at the time of sacrifice were less than 12 pg/ml as determined by RIA (sensitivity of the RIA was 2.5 pg/ml). Coronal hypothalamic slices (450 μm thick) were cut on a vibratome and a single slice was submerged in an artificial CSF (aCSF) (1).

Electrophysiology. Intracellular recordings from arcuate neurons were performed with biocytin-filled (3%) electrodes using techniques similar to those described previously (17). Voltage-current relationships were obtained by applying a series of current pulses (1 s) and measuring the voltage at the end of each step. The conductance was calculated from

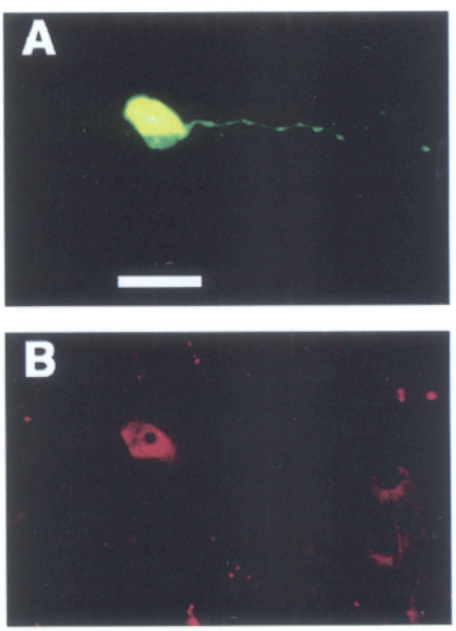

Figure 1: **Double-labelling of GnRH neurons** A. Streptavidin-FITC staining of a biocytin-filled cell following electrophysiological recording. B. Immunocytochemical identification of this cell using an anti-GnRH primary antibody (EL-14) and a Texas Red labelled secondary antibody. Bar = 20 μm.

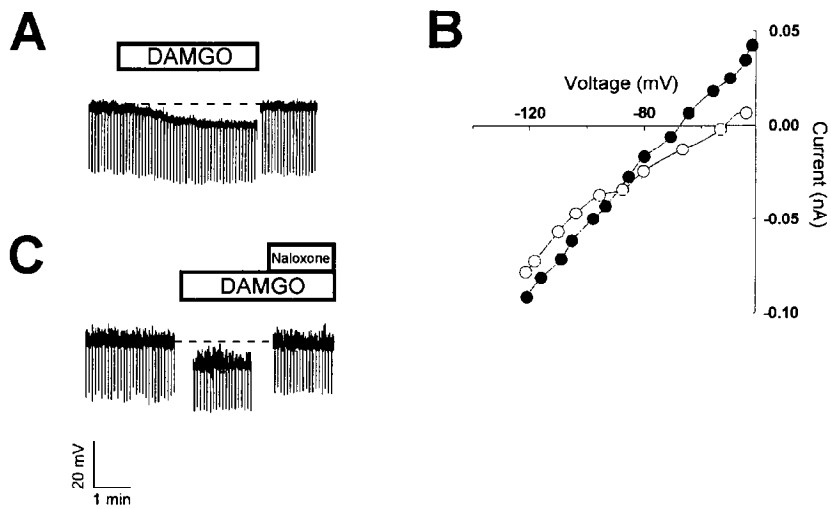

Figure 2: **DAMGO hyperpolarizes GnRH neurons**. A. 200 nM DAMGO hyperpolarized this cell by 7 mV. The RMP = -55 mV (dotted line) Break in recording represents the generation of I/V plots followed by washout. B. Current-voltage relationship (I/V) for the cell in A during DAMGO (●) crosses the control I/V (○) at -90 mV. The Δg_{DAMGO} was 0.5 nS. C. In another cell, application of 300 nM DAMGO resulted in a 12 mV hyperpolarization that was reversed by application of 20 nM naloxone. RMP = -60 mV (dotted line). Breaks in recordings represent the generation of I/V plots followed by drug equilibration.

the slope of the current-voltage (I/V) plots between -60 and -80 mV. Voltage-matched I/V plots were obtained during the drug-induced hyperpolarization. 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. Numerical data are expressed as mean ± SEM, except as noted. Groups were compared using an unpaired, two-tailed Student's t-test, and a $p < 0.05$ was considered significant.

Tetrodotoxin (TTX, 1 μM, Sigma, St. Louis) was added to the solution prior to application of drugs in order to block synaptic input. A cumulative dose-response curve was generated to DAMGO (Peninsula Labs, Belmont, CA), an opioid agonist selective for μ-receptors (19) and baclofen (Sigma), a $GABA_B$-selective agonist. Increasing concentrations of DAMGO were applied until the drug-induced hyperpolarization equilibrated (≈ 6-7 min). The EC_{50} value was calculated using Sigmaplot (Jandel Scientific, Corte Madre, CA) software to determine the best fit to the logistic equation. 17β-estradiol (Sigma) was stored at 4° in a 1 mM 95% ethanol solution and then dissolved in aCSF to make a 100 nM solution. The tissue was superfused with this solution for 20 minutes followed by another DAMGO concentration-response determination. Schild analysis was performed with the opioid antagonist, naloxone (Sigma) (20).

Histology. Following recording, the slices were processed for immunocytochemistry as previously described (21). Briefly, slices were fixed in 4% paraformaldehyde, sectioned on a cryostat and processed with streptavidin-FITC. After localization of the biocytin-filled neurons, histological data was collected and the appropriate slides were then processed with EL-14 GnRH antisera (22) at 1:2,500 using fluorescence immunohistochemistry.

RESULTS

Electrophysiological recordings were obtained from eight immunocytochemically identified GnRH neurons (Fig. 1). The morphology of the GnRH neurons was similar to previously published descriptions (23a). The cells were small (11 ± 0.6 by 18 ± 1.2 μm diameter) oval to fusiform in shape, with 2-3 varicose fibers per cell. One cell was pyramidal and smaller than the other seven (10x12 μm). The passive membrane properties of GnRH neurons which did not differ significantly from other arcuate neuronal types (24,25), were the following (n=8):Resting Membrane Potential (RMP)=-55 ± 3 mV; τ = 23 ± 5 ms; R_{in} =483 ± 65 MΩ. In addition, GnRH neurons expressed several conductances that are pertinent to bursting in parvocellular neurosecretory neurons (17), including a pacemaker current (I_h) and a transient outward K^+ current.

To elucidate the site of opioidergic control of GnRH neurons, 7 of these cells were tested with the μ-opioid agonist, DAMGO in the presence of 1μM TTX. In all cells tested, DAMGO caused an increased conductance (Δg_{DAMGO} = 0.30 ± .04 nS) that resulted in a mean hyperpolarization of 9 ± 3 mV. As previously reported for other hypothalamic cells (18) the reversal potential of the DAMGO-induced conductance (E_{DAMGO}) in GnRH cells was close to E_K (-95 ± 4 mV, n=5). Figure 2 shows the DAMGO-induced hyperpolarization and its reversal by naloxone. Subsequent Schild analysis revealed the K_e for naloxone to be 2.4 ± 1.2 nM (n=2), confirming a μ-opioid receptor mediated effect of DAMGO. GnRH neurons also are contacted by GABAergic terminals (26), and perfusion with the $GABA_A$ antagonist, bicuculline (10 μM), significantly reduced the postsynaptic potentials in five of six cells tested (data not shown). Furthermore, μ-opioid and $GABA_B$ receptors are coupled to the same K^+ channels in the hypothalamus (28). Like DAMGO, the $GABA_B$ agonist, baclofen, hyperpolarized GnRH neurons (n=5) with a V_{max} of 18 ± 3 mV, an $E_{Baclofen}$ of -93 mV ± 8 mV, and a $\Delta g_{Baclofen}$ of 0.7 ± 0.2 nS. The EC_{50} for baclofen was 4.2± 1.3 μM.

To characterize the role of E_2 in regulating GnRH

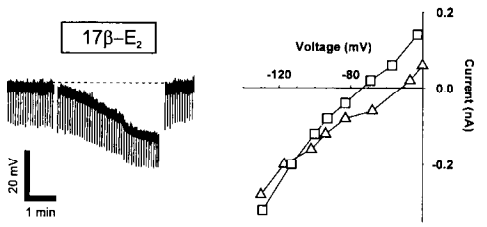

Figure 3: **E_2 can hyperpolarize GnRH neurons.** A. 100 nM E_2 caused an 18 mV hyperpolarization that washed out within 10 minutes. I/V plots were done during the two breaks in the trace with a third I/V done after E_2 washout. RMP = -53 mV (dotted line). B. Summary of the I/V plots done with (□) and without (▲) E_2. E_2 caused a 2.0 nS conductance increase with a reversal potential near E_K (-100 mV).

function, cells were perfused with E_2 (100 nM, 20 min) *in vitro*. As reported previously (23a), E_2 hyperpolarized GnRH neurons by opening K^+ channels (Fig. 3). The E_2-induced hyperpolarization was a direct effect on GnRH cells, as these experiments were performed in the presence of 1 μM TTX. Another rapid effect of E_2 is to cause a fourfold decrease in the potency of μ-opioids in a subset of hypothalamic neurons (1), but this effect was not seen in GnRH neurons (Fig 4). Although both the hyperpolarization and decreased opioid potency can be seen with 1 nM E_2 (1, 23a,b), 100 nM was chosen to elicit the maximal effect.

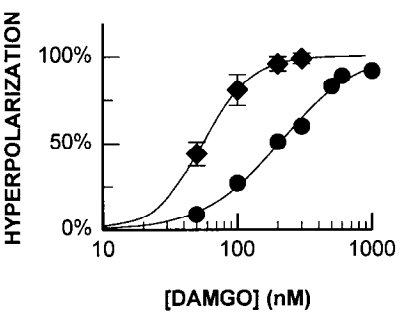

Figure 4: **Potency of DAMGO is not altered by E_2** Summary of the dose response curves generated from the DAMGO-induced hyperpolarization after 100 nM E_2 in GnRH neurons (EC_{50} = 64 nM ± 8, n=6, ◆) and other hypothalamic neurons including β-endorphin neurons (205 ± 12 nM, n=24, ●). The potency of DAMGO in GnRH neurons following E_2 was not different from pre-E_2 controls (60 ± 3 nM, n=49, not shown).

DISCUSSION

The mechanism of opioidergic control of GnRH neurons has been a matter of ongoing debate. It has not been clear whether opioids directly inhibit GnRH neurons, or act presynaptically through some other neurotransmitter system (see (7,8)). Previous studies have used i.v. or i.c.v. injections of pharmacological doses of opioid agonists and antagonists, thereby excluding assessment of receptor subtype and cell type involved. For the first time, we have been able to show a direct action of opioids, like β-endorphin, on electrically isolated (in TTX) GnRH neurons. Moreover, we have determined that these opioids bind to μ-opioid receptors and hyperpolarize GnRH neurons by opening inwardly-rectifying K^+ channels. Activation of these K^+ channels increases the cell's conductance two fold, which not only hyperpolarizes the cell but also shunts any other synaptic input (18). These findings differ from reports using immortalized GnRH cells in which μ-opioid receptors have not been identified (27). Furthermore, we have previously shown that the μ-opioid receptor and $GABA_B$ receptor are coupled to the same K^+ channel (28). Our present findings demonstrate that GnRH neurons, like other parvocellular neurosecretory neurons, also express functional $GABA_B$ receptors.

Estrogen has the potential to exert both presynaptic and postsynaptic effects that ultimately hyperpolarize GnRH neurons by opening K^+ channels. Consistent with our previous findings, E_2 directly inhibited these cells by opening K^+ channels. Furthermore, we have previously shown that E_2 rapidly decreases the potency of μ-opioids to hyperpolarize β-endorphin neurons. The μ-receptor functions as an inhibitory autoreceptor on β-endorphin neurons. By decreasing autoinhibition, E_2 disinhibits β-endorphin neurons, allowing greater release of this inhibitory peptide. However, GnRH neurons did not show this change in μ-opioid potency. By maintaining their sensitivity to μ-opioids (even after E_2), GnRH neurons are inhibited by the increased opioidergic tone, in addition to being directly hyperpolarized by E_2 (Fig. 5). The direct, independent inhibitory actions of E_2 and opioids on GnRH neurons explains why naloxone cannot completely block the estrogenic inhibition of the "GnRH pulse generator" in monkeys (29), and why naloxone stimulation of GnRH release is not attenuated following ovariectomy in rats (30).

Several interesting questions remain that warrant further investigation. Firstly, by what mechanism does E_2 exert its rapid effects? Does it work through a membrane (32,33) or an intracellular receptor? Perhaps E_2 is exerting a nongenomic action of the classical estrogen receptor (34). It also remains to be seen how these inhibitory estrogenic actions give way to a positive feedback on GnRH neurons. A prominent effect of long term (24 hr) exposure to E_2 is an increase in excitatory (α_1) noradrenergic drive to GnRH neurons (35) that may be mediated by an increase in α_1-noradrenergic receptors (36). From the present studies we now know that there are at least three qualitatively different modes of estrogenic action, each with its own time course and cell-specificity: 1) a direct hyperpolarization of GnRH neurons occurring within seconds, 2) modulation of opioidergic tone on β-endorphin cells seen within minutes, and 3) numerous genomic effects on several cell types requiring hours to days.

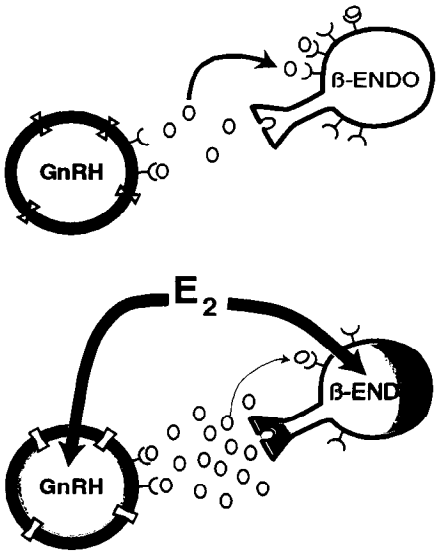

Figure 5: **Model of E_2 's dual inhibitory actions on GnRH neurons.** By decreasing autoinhibition of β-endorphin neurons, E_2 increases the opioidergic tone of the hypothalamus. Furthermore, E_2 can directly inhibit GnRH neurons by opening a K^+ channel, thus hyperpolarizing the cell.

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