Estrogenic activation of cAMP-dependent protein kinase rapidly uncouples G-protein coupled receptors in the hypothalamus

by

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INTRODUCTION

Review of the female reproductive axis

Control of the reproductive cycle involves a complex interaction between neurotransmitters in the CNS and hormones from the pituitary and gonads. A key mediator in the function of this hypothalamic-pituitary-gonadal (HPG) axis is gonadotropin-releasing hormone (GnRH). This decapeptide is produced in cells ranging from the preoptic region to the mediobasal hypothalamus (MBH) (Silverman, 1976; Silverman et al. 1982). However, in primates and guinea pigs, the cells that are critical for reproduction are found in the arcuate nucleus within the MBH (Plant et al. 1978). Ablation of this region abolishes the reproductive cycle (Plant et al. 1978), but pulsatile gonadotropin release is maintained following surgical deafferentation of the MBH from higher centers (Krey et al. 1975). GnRH cells send projections down the median eminence, where they release GnRH in a regular, pulsatile fashion (Carmel et al. 1976). Acting through stimulation of phospholipase C (Janovick, Conn, 1994), this peptide stimulates gonadotrophs in the anterior pituitary to release luteinizing hormone (LH) and follicle stimulating hormone (FSH) (Horn et al. 1991). These two proteins then act on the gonads to regulate the reproductive cycle (see (Major, Speroff, 1982)) (Figure 0.1). Without pulsatile GnRH stimulation, the pituitary does not release gonadotropins (Belchetz et al. 1978; Loh et al. 1976). Although there are fewer than 2000-4000 of these broadly distributed GnRH neurons in the primate hypothalamus (Silverman et al. 1982), they are tightly regulated and show a remarkable ability to synchronize their firing (Marshall et al. 1991). Furthermore, the frequency of GnRH stimulation strongly regulates the pituitary responses to GnRH. In monkeys, the midcycle

pulsatile release of GnRH is every hour. If the frequency is increased, the pituitary response is lost (Belchetz et al. 1978). In fact, constant administration of a GnRH analog (leuprolide) is used clinically to suppress the HPG axis in steroid hormone-sensitive cancers (Karten, Rivier, 1986; Plosker, Brogden, 1994). Conversely, when the frequency of GnRH pulses are decreased, the amplitude of LH and FSH pulses get larger (Loh et al. 1976). However, because serum LH and FSH are metabolized at different rates (half lives of 60 and 180 minutes, respectively), the net effect is to decrease LH levels and increase FSH levels (Loh et al. 1976). In contrast to the desensitizing actions of GnRH, hourly pulses of this peptide are able to sensitize pituitary gonadotrophs to release more gonadotropins after each pulse in a process known as "self-priming" (Terasawa et al. 1980). In the ovariectomized state, the release of LH after each GnRH pulse is highly reproducible. In guinea pigs treated 12-48 hours previously with estradiol benzoate (EB), the first response to exogenous GnRH is approximately one-half the size of the response seen in ovariectomized animals. However, subsequent hourly GnRH pulses result in consecutively increasing LH release, up to levels that are four times greater than those seen in EB-untreated animals (Terasawa et al. 1980). Although the timing of these synchronized GnRH pulses is critical, the underlying cellular mechanism of this pulsatility remains unknown. Many studies investigating the regulation of GnRH release have used the technique of push-pull perfusion to measure peptide release into the portal blood (Terasawa et al. 1988). This technique involves stereotaxically placing a canula where the neuroregulatory neurons terminate in the median eminence (ME) extracellular space below arcuate hypothalamus. One pump pushes perfusion media through the canula into the ME. Samples of the extracellular fluid are collected with a second pump

that pulls the media back out. These experiments have provided evidence that the "pattern generator" of GnRH release involves transynaptic control of GnRH activity, possibly by norepinephrine (NE) fibers from the A1 group (Terasawa et al. 1988) and local neuropeptide Y (NPY) neurons (Terasawa, 1994). Both neurotransmitters stimulate GnRH release and endogenous GnRH release is inhibited by either anti-NPY antibodies (Woller et al. 1992) or the α-adrenergic antagonists prazocin (Terasawa et al. 1988) or phenoxybenzamine (Knobil, 1974). Furthermore, the same experiments (Terasawa et al. 1988, Terasawa, 1994) showed that every GnRH pulse is preceded 5-10 minutes by NE and NPY pulses. There is also evidence that synchronized pulsatile activity may be intrinsic to GnRH cells. These neurons make multiple cell-cell contacts with each other (Leranth et al. 1985; Thind, Goldsmith, 1988). Indeed, immortalized GnRH-producing GT1 cells produce pulsatile GnRH release in the absence of any exogenous signal (Martínez de la Escalera et al. 1992; Stojilkovic et al. 1994). Thus, there appears to be both intrinsic and extrinsic control of this important synchronous activity.

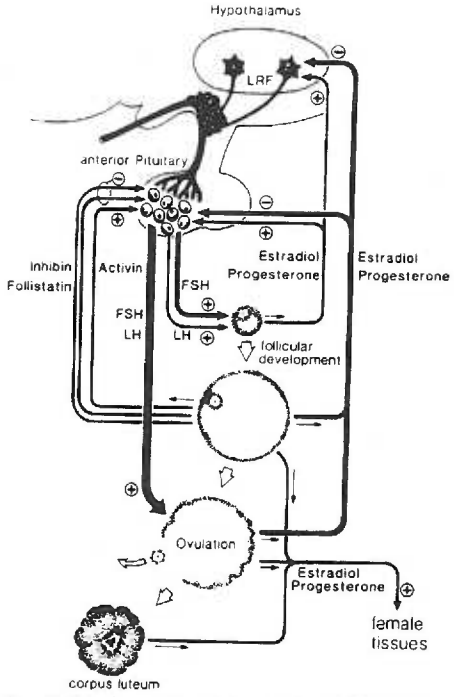


Figure 12–18. A diagrammatic representation of the hypothalamic-pituitary-ovarian axis. LRF, luteinizing-releasing factor. (From SY Ying, Inhibins, activins, and follistatins: gonadal proteins modulating the secretion of follicle-stimulating hormone, Endocr Rev 9, 267–293, 1988, © by The Endocrine Society.)

FIGURE 0.1

The LH and FSH released from the pituitary following GnRH stimulation acts at the ovaries to regulate growth of the follicles and production of hormones (see (Major, Speroff, 1982)). The gonadal steroid hormones, particularly estrogens, are responsible for many of the extragonadal aspects of reproduction (Knobil, Neill, 1988). These include cervical and uterine changes necessary for successful fertilization and implantation, breast development and even regulation of reproductive behaviors via actions in the CNS (Etgen et al. 1992). Perhaps one of the most important actions of estrogen is at the hypothalamus and pituitary

to regulate the release of GnRH and LH, respectively (Figure 0.2). In contrast to the circhoral regulation of the central GnRH neurons, the timing of the monthly menstrual cycle is determined by gonadally-produced hormones, especially estrogen and progesterone (Major, Speroff, 1982). The peptides inhibin, activin and follistatin are also crucial for normal follicular development and act to alter FSH release in a steroid-dependent fashion (see (Woodruff, Mather, 1995) for review). The first half of the menstrual cycle (follicular phase) is concerned primarily with the development of an ovarian follicle. During this phase, there are regular small pulses (about every 90-120 minutes in primates) of GnRH and LH/FSH (Major, Speroff, 1982) and serum estrogen levels are low (< 0.1 nM) (Croix, Franchimont, 1975). Estrogen secretion by the follicular granulosa cells rises quickly midway through the cycle, resulting in a massive release of LH, which causes the extrusion of the ovum from the follicle and ovary, allowing that egg to be fertilized (Weick et al. 1973). Following the LH surge, the remaining follicular cells are converted to luteal cells which secrete estrogen and progesterone. These steroid hormones convert the reproductive system to a state that is conducive to implantation (Major, Speroff, 1982) and act to inhibit LH and FSH release, thus preventing other follicles from developing/ovulating (Knobil, 1974).

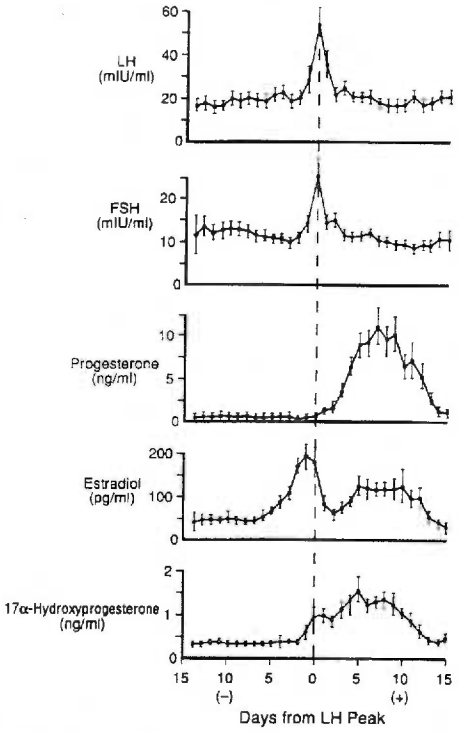


FIG. 10. Mean values of LH, FSH, progesterone, estradiol, and 17α-hydroxyprogesterone in daily serum samples of nine women during ovulatory menstrual cycles. Data from various cycles are combined, using the midcycle peak of LH as the reference point (day 0). *Vertical bars* indicate standard error of mean. Increased production of 17α-hydroxyprogesterone may simply reflect increased progesterone formation; no physiological role has been established for 17α-hydroxyprogesterone. (Data from ref. 5, with permission.)

Thorneycroft IH, Mishell DR, Stone SC Nakamura RM Am J Obstet Gynecol 111:947 (1971)

Figure 0.2

Estrogenic control of reproduction is multifaceted

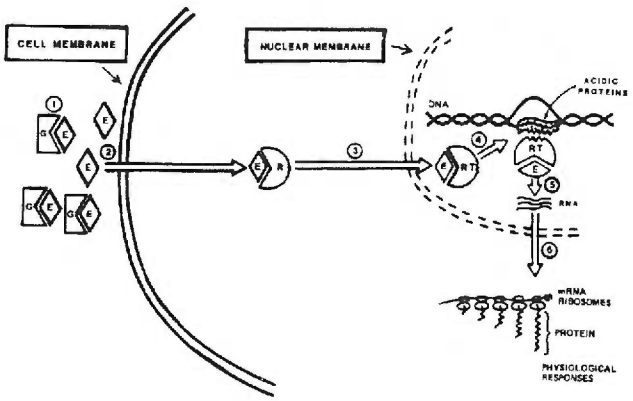
The stimuli for these cyclic changes in LH secretion are unknown, however estrogen plays a critical role. When estrogen production is interrupted by either ovariectomy or

menopause, gonadotropin levels quickly rise (Atkinson et al. 1970). Administration of exogenous E_2 to ovariectomized individuals has complex effects, with actions that are both time- and concentration-dependent. There is a very rapid (30 min) inhibition of LH levels, followed a day later by an LH surge (Terasawa et al. 1979; Yamaji et al. 1972; Levine et al. 1985). Although estrogen can only cause an LH surge when serum E_2 levels reach about 0.5 - 1 nM for at least 24 hours (Karsch et al. 1973), inhibition of GnRH/LH happens within a few minutes and requires ten-fold lower E_2 concentrations (Yamaji et al. 1972). The mechanism(s) by which estrogen exerts these qualitatively different actions on the reproductive axis remains unknown.

Biochemical basis for the multifaceted nature of estrogen action?

Classically, steroid hormones are thought to have a single mechanism of action, namely regulation of transcription (O'Malley, Tsai, 1992). Following diffusion across the cell membrane, steroids bind intracellular receptors. These complexes then stabilize transcription complexes at specific segments of DNA (e.g., estrogen-response elements, EREs) thereby activating transcription of specific genes (Figure 0.3). This view of steroid hormone action persisted for decades while work in other fields began to reveal the diverse action of other intercellular signals (e.g., neurotransmitters). Although the effects of individual steroid hormones have been thought to be mediated by a single receptor type, peptide and amine neurotransmitters are well-known to activate multiple, structurally-unrelated receptors. For example, γ-amino butyric acid (GABA) can directly activate a membrane delimited chloride channel or stimulate the G-protein coupled $GABA_B$ receptor to alter intracellular cascades

(Bowery et al. 1981; Alger, Nicoll, 1982). Furthermore, any given G-protein coupled receptor (e.g., μ-opioid receptors) may alter multiple intracellular effector systems (e.g., adenylate cyclase, potassium channels, calcium channels) (see North, 1993 for review). However, the possibility of other mechanisms of steroid action has been implied by many physiological studies in which steroids have qualitatively different actions depending on the duration and concentration of steroid exposure, as is seen in estrogenic regulation of reproduction (Yamaji et al. 1972; Karsch et al. 1973). Recent biochemical and molecular biological experiments have revealed that steroid actions are much more complex and diverse than originally suspected. In addition to binding transcription factors, activity of steroid receptors requires interactions with several other proteins. The unliganded estrogen receptor (ER) appears to be associated with multiple heat shock proteins, which dissociate upon receptor activation but are critical for normal receptor function (Church et al. 1994; Kimura et al. 1995). Furthermore, the ER itself has multiple actions including regulation of genes that are not under control of EREs. Under certain conditions E_2 can alter mRNA synthesis at cAMP-responsive elements (CREs) or at AP-1 sites (Umayahara et al. 1994; Aronica et al. 1994). Both effects are rapid and not blocked by protein synthesis inhibitors and therefore are thought to reflect direct action at these enhancers, rather than an ERE-mediated increase in transcription factors (Webb et al. 1995; Hagiwara et al. 1993). Thus, it is becoming clear that a single mechanism of action may be insufficient to explain the multitudinous effects of steroid hormones.



From Hsueh, A.J.W. in Endocrinology (3rd ed) ed byL.J. DeGroot Published by W.B. Saunders 1995

Molecular mechanism of estrogen action in target tissues. Nuclear membrane is depicted as dashed lines to denote the uncertain cellular localization of the unbound "free" receptors. E, estrogen; G, serum steroid-binding globulin; R, receptor; RT, transformed receptor; SRE, steroid-responsive element.

FIGURE 0.3

There are multiple types of steroid receptors

In addition to the myriad of actions of classical steroid receptors, there is compelling evidence for the existence of multiple classes of steroid receptors. For example, steroid binding sites have been found in the plasma membrane of cells (Towle, Sze, 1983). Progesterone derivatives bind and modulate $GABA_A$ receptor function in a manner that is

similar to barbiturates (Mellon, 1994). There are also specific extracellular steroid receptors, such as the G-protein coupled corticosterone receptor found in newt brains (Orchinik et al. 1992). Progesterone stimulates the acrosome reaction in sperm and is critical for fertilization. Not only do sperm not have the intracellular machinery for genomic steroid actions, an extracellular progesterone receptor has been found in the plasma membrane of these cells (Meizel, Turner, 1991). Electrophysiological measurements in the hippocampus have discovered a pertussis toxin-sensitive inhibition of calcium channels by derivatives of pregnenolone and cortisol (Ffrench-Mullen et al. 1994). There is even evidence from *in situ* hybridization studies for the existence of isoforms of the classical estrogen receptor in the brain and pituitary (Skipper et al. 1993; Friend et al. 1995), although the function of these transcripts remains unknown. Therefore, besides multiple actions of the classical estrogen receptor, the existence of novel steroid receptors serves to expand the realm of estrogen action.

Cellular mechanism for estrogen's regulation of reproduction

Control of the reproductive axis provides a physiological milieu within to study the various actions of estrogen. Although E_2 is known to be a critical regulator of the HPG axis, the mechanisms of estrogenic control remain unclear. It has long been debated whether estrogen E_2 works at the level of the hypothalamus, pituitary, or both (Negro-Vilar et al. 1973; Chappel et al. 1981; Condon et al. 1988). The sensitivity of the pituitary to GnRH changes over the reproductive cycle, being highest at the time of the LH surge and remaining somewhat elevated during the luteal phase (Wang et al. 1976). These changes appear to be

estrogen-dependent (Lasley et al. 1975) and may involve, among other things, alterations in the ability of GnRH to self-prime at the pituitary (Terasawa et al. 1980). Because of the proximity of the portal blood system to the brain, *in vivo* measurements of GnRH release have been wrought with technical difficulties. One commonly used method to study neural control of the HPG axis is extracellular recordings of the hypothalamic "pulse generator" (Kaufman et al. 1985). Electrodes positioned within the hypothalamus can measure a "volley" of multiunit electrical activity preceding every LH pulse. This electrical activity is thought to arise from some hypothalamic cell group that is important in regulating LH release from the pituitary, possibly GnRH neurons. In this model, E_2 injection into ovariectomized monkeys decreases both LH pulses and the electrical activity of the "pulse generator" within two hours, implying a CNS-mediated effect (Kesner et al. 1987). However, experiments directly measuring GnRH release with push-pull perfusion in ovariectomized monkeys following E_2 have yielded inconsistent results (Levine et al. 1985; Pau et al. 1990). In fact, when GnRH release was abolished by MBH ablation, LH and FSH fall to undetectable levels. However, ovulatory cycles were reinitiated when these same animals were given regular hourly pulses of exogenous GnRH (Knobil et al. 1980). These results were interpreted as being conclusive evidence that GnRH release plays a merely permissive role in the regulation of the HPG axis. GnRH was thought to be secreted in an invariably regular fashion and estrogenic control of reproduction was asserted through actions at the pituitary to alter the sensitivity to GnRH (Knobil et al. 1980). However, push-pull perfusion experiments have since shown that the hypothalamic release of GnRH does indeed change over the cycle, in a manner that parallels LH release (Pau et al. 1993).

Furthermore, although the pituitary responsiveness to GnRH is elevated at the LH surge, it remains high into the early luteal phase, after the surge is over (Wang et al. 1976). Similarly, although injection of EB with progesterone in women during the follicular phase results in the expected increase in pituitary sensitivity to GnRH (Lasley et al. 1975), these steroids are unable to mimic the midcycle surge of LH (Taylor et al. 1995). Indeed, closer examination of the data from the MBH-lesioned monkey experiments reveals that the LH surges artificially induced by regular GnRH pulses are one-half to one-third the normal size and are not very reproducible from cycle to cycle (Knobil et al. 1980). Furthermore, speculation has arisen that the hypothalamic-pituitary separation in these experiments was not truly complete. When the same experiments are done with a Teflon barrier inserted between the cut ends of the stalk, regular pulses of GnRH are not able to stimulate ovulation (Norman et al. 1982). However, if the GnRH is given constantly for 24 hours once the serum estrogens reached 0.5 nM, a robust LH surge is initiated (Norman et al. 1982). Therefore, modulation of hypothalamic input to the pituitary is crucial for normal reproduction, although the subtleties of these interactions remain unclear.

Estrogenic inhibition of GnRH release involves endogenous opioid peptides

The current studies sought to elucidate the mechanism of estrogen action on hypothalamic neurons. My research focused on estrogenic inhibition of the HPG axis because LH levels are rapidly (< 30 min) suppressed following E_2 (Chappel et al. 1981, Condon et al. 1988) and may therefore imply a novel mechanism of estrogen action. GnRH neurons do not concentrate E_2 (Shivers et al. 1983), and so estrogenic regulation of these

cells is thought to be mediated by presynaptic neurons. One of the most likely candidates is the hypothalamic β-endorphin neurons (Ferin et al. 1984). This peptide activates μ-opioid receptors, with multiple inhibitory actions including inhibition of adenylate cyclase and calcium channels, as well as opening potassium channels (Di Chiara, North, 1992). β-endorphin neurons make direct synaptic contacts onto GnRH cells (Thind, Goldsmith, 1988), and although morphine does not alter the pituitary responsiveness to GnRH (Ferin et al. 1982), administration of morphine or other μ-opioid agonists mimic the effects of E_2 to inhibit LH (Leadem, Kalra, 1985). Estrogen-sensitive release of endogenous opioids is implied by the fact that the μ-opioid antagonist naloxone increases basal LH levels when E_2 levels are high (e.g., luteal phase), with almost no effect during the early to mid-follicular phase (Millan, Herz, 1985). Furthermore, direct measurements of hypothalamic release of β-endorphin have shown that release is high when estrogenic negative feedback on the HPG axis predominates (Wehrenberg et al. 1982; Ortega et al. 1993; Frautschy, Sarkar, 1995). Finally, *in vivo* electrophysiological recordings combined with simultaneous measurements of serum LH levels have shown that E_2 can rapidly inhibit firing of the hypothalamic "pulse generator" neurons and LH secretion, and these actions are reversible with naloxone (Grosser et al. 1993). In addition to the slower, luteal phase estrogenic inhibition, β-endorphin also appears to be involved in the more acute inhibition immediately before the LH surge (Kato et al. 1994). In fact, administration of naloxone can advance the LH surge by several days in women (Rossmanith et al. 1988). This acute opioidergic inhibition may serve to restrain the LH surge until the GnRH neurons are synchronized and the nonneural components (e.g., pituitary, ovary, etc.) are ready to respond. Alternatively, this brief inhibitory phase may be

an integral part of the synaptic changes necessary to stimulate the LH surge. The importance of opioidergic modulation of the HPG axis is illustrated by the disorder, hypothalamic amenorrhea, most commonly seen after starvation, stress or even strenuous exercise (Marshall et al. 1991). In this condition the sensitivity of the pituitary is normal, but there is a disruption of the hypothalamic release of GnRH. Although the gonadotropin and steroid levels are low, LH pulse frequency resembles that of the luteal phase rather than the follicular phase or ovariectomized state (Marshall et al. 1991). Administration of the opioid antagonist naloxone results in a prompt restoration of gonadotropin secretion and ovulatory cycles in 70% of the women treated (Wildt et al. 1993).

Basis for estrogenic regulation of hypothalamic opioids

It remains to be determined exactly how E_2 serves to increase the "opioidergic tone" of the hypothalamus. Since modulation of β-endorphin physiology appears to play a critical role in estrogen's regulation of reproduction, it is important to elucidate the cellular basis for these actions. Furthermore, because these estrogen actions include an unusually rapid component (Chappel et al. 1981), they may represent a novel, nongenomic action for this steroid. We chose the guinea pig for our animal model because, among nonprimates, its reproductive cycle most closely resembles the human cycle (Knobil, Neill, 1988). These animals have a 15-16 day cycle, with an estrogen-dependent surge, followed by a luteal phase in which the HPG axis is suppressed by estrogen and progesterone (Croix, Franchimont, 1975). Rabbits ovulate upon sensory stimulation, ungulates (like sheep) have an annual pattern (Knobil, Neill, 1988). Much work has been done on the reproduction of

the rat because these animals have a very regular and short four- to five-day cycle (Knobil, Neill, 1988). However, this brief cycle does not have a true luteal phase in which steroid production by the corpus luteum serves to regulate the timing of the next menstrual cycle. Instead, there is a strong circadian component, presumably mediated by the CNS (Knobil, Neill, 1988). For example, there is a critical window in which the LH surge may occur in rats (Everett, Sawyer, 1950), and when ovariectomized rats are exposed to constant, elevated E_2, they exhibit daily afternoon LH surges (Fink, 1979). In contrast, the timing of reproductive cycle in guinea pigs and primates depends primarily on ovarian factors, such as E_2 (Terasawa et al. 1979; Karsch et al. 1973). The differences between rats and guinea pigs are further highlighted by neuroanatomical studies showing that the reproductively important GnRH neurons in rats are located in the preoptic hypothalamus (Dyer et al. 1978), while these cells are found in the arcuate hypothalamus in guinea pigs and primates (Krey et al. 1975; Krey, Silverman, 1978).

Within the guinea pig hypothalamus, the vast majority (>90%) of neurons, including β-endorphin cells, are hyperpolarized by μ-opioid activation of inwardly-rectifying potassium channels ($I_{K(ir)}$) (Kelly et al. 1990). A brief (20 min) exposure to E_2 acts to rapidly reduce μ-opioid potency in a subpopulation of hypothalamic neurons (Lagrange et al. 1994). The EC_{50} of the μ-opioid agonist [D-Ala^2-N-Me-Phe^4-Gly^5-ol]-enkephalin (DAMGO) following E_2 is nearly four-fold greater than control values, with no change in the efficacy. E_2 actions are stereospecific and occur at physiologically relevant-concentrations. As expected for a heterogenous region like the hypothalamus, this response is only seen in approximately one-third of the hypothalamic cells, with a preponderance of cells in the

caudal ventrolateral mediobasal hypothalamus. The present studies provide a description of this phenomena and a characterization of the receptor and signal transduction mechanism(s) mediating the rapid effects of E_2. Furthermore, immunocytochemical identification of cell phenotypes was used to elucidate the physiological significance of these actions.

Specific Aims:

I. To study the type of receptor mediating the rapid estrogenic modulation of μ-opioid potency:

1. To ensure the specificity of this response using the biologically inactive isomer, 17∝-E_2.
2. To determine the concentration-dependence of estrogen's rapid actions.
3. To determine if the membrane-impermeant 17β-E_2 -albumin conjugate mimics free E_2.
4. To ascertain whether the antiestrogen ICI 164,384 blocks the rapid effects of E_2, and to use Schild analysis to estimate the affinity of the estrogen receptor for that antagonist.
5. To determine whether the nonsteroidal estrogenic ligand diethylstilbestrol mimics E_2.

II. To investigate the mechanisms by which estrogen modulates opioidergic potency:

1. To determine whether the nonselective kinase inhibitor staurosporine blocks estrogenic action.
2. To ascertain if stimulation of adenylate cyclase with forskolin mimics the actions of E_2.
3. To measure whether KT5720, a selective PKA antagonist, blocks the effects of E_2.
4. To test the ability of cAMP derivatives to mimic (Sp-cAMP) and block (Rp-cAMP) E_2 actions.

5. To measure the stimulation of PKA-induced ^{32}P-incorporation by E_2 in a biochemical assay.

III. To elucidate the site of estrogen's modulation of μ-opioids:

1. To determine whether estrogen alters the interaction of the μ-opioid receptor with DAMGO.
2. To determine if estrogen uncouples the $GABA_B$ response in the estrogen responsive cells.
3. To ascertain whether E_2 directly affects ion conductances or the reversal potential of the μ-opioid response

IV. To elucidate the physiological significance of estrogen's rapid actions in the hypothalamus by immunocytochemically identifying the cell type of recorded neurons.

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 g), born and raised in our colony, were maintained on a 14 hr light/10 hr 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 days 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 (Kelly et al. 1992). 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, Kelly, 1989). A single slice was submerged in an oxygenated (95% O_2, 5% CO_2), artificial cerebrospinal fluid (aCSF) 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; HEPES, 10.

Drugs. All drugs and chemicals were from Sigma (St. Louis, MO) unless otherwise specified. Tetrodotoxin (1 μM, TTX) was added to all drug solutions prior to application to ensure a postsynaptic effect. $GABA_A$ postsynaptic potentials were blocked with bicuculline

(5 - 10 μM). $GABA_B$ and μ-opioid responses were investigated with the selective agonist baclofen (0.3 - 40 μM) (Bowery et al. 1981) and [D-Ala^2, N-Me-Phe^4, Gly-ol^5]-enkephalin (DAMGO, 50 nM - 1 μM, Peninsula Labs) (Goldstein, Naidu, 1989), respectively. DAMGO responses were antagonized with naloxone (20 - 320 nM, (Goldstein, Naidu, 1989)). 17β-estradiol, DES and BSA-E_2 were from Steraloids (Wilton, NH) and ICI 164,384 was the generous gift from Dr. 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° C in a 1 mM 95% ethanol solution. E_2 solutions (1 nM - 1 μM) were made by diluting this stock solution into aCSF. Aliquots of BSA-E_2 were dissolved directly into aCSF. The estimates of total E_2 concentrations (100 nM to 4 μM) were based on the fact that there are 35 E_2 molecules per BSA molecule. A sample of this stock solution was then dissolved in the media to make a 100 nM solution (0.01% ethanol). Forskolin (13 mM) was also kept in a 95% ethanol solution at 4° C and dissolved in aCSF prior to application. Staurosporine and KT5720 (Calbiochem, San Diego, CA) were dissolved in 95% EtOH (1 mM) and then diluted in aCSF 10-15 min prior to application. Rp-cAMP and Sp-cAMP (Calbiochem) were dissolved in dH_2O (10 mM) and then diluted in aCSF. Tetrodotoxin (1 μM, TTX) was added to the solutions prior to agonist and E_2 applications to ensure postsynaptic effects.

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 mm outer diameter, Dagan, Minneapolis) 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 amplified with a Cyberamp 320 (Axon Instruments) and recorded on a chart recorder (Gould 2200, Cleveland, OH). This data was also digitized at 83 Hz and stored on an IBM compatible computer with Axotape software (Axon Instruments). Voltage-current relationships were obtained by applying a series of depolarizing and hyperpolarizing current pulses (1 s) and measuring the voltage at the end of each step. The conductance (g) and apparent input resistance of the cell (R_{in}) were 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 ($E_{Baclofen}$) of the conductance and the total conductance change (Δg) mediating the drug effect. The membrane time constant was estimated by measuring the time for a voltage deflection ($\approx$ 10 mV) to reach 63% of its steady-state level on a digital storage scope (Tektronix 2232, Beaverton, OR). Action potential data was acquired at 10 kHz on Axotape and used to determine firing rate, amplitude, and duration at one-third height.

Pharmacological Techniques. Cumulative concentration response curves were generated to baclofen and DAMGO by applying increasing drug concentrations until the drug-induced hyperpolarization reached a new steady level, usually after 5-6 minutes. The EC_{50} value was calculated using Sigmaplot (Jandel Scientific, Corte Madre, CA) software to determine the best fit to the logistic equation :

$$V = V_{Max} * \frac{[agonist]^n}{EC_{50}^n + [agonist]^n}$$

V is the hyperpolarization seen at each agonist concentration and V_{max} is the hyperpolarization seen at the highest agonist concentration. EC_{50} is the concentration of agonist 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 20-80% maximal response (Taylor, Insel, 1990). After washout of the highest DAMGO or baclofen concentration, 17β-estradiol was superfused for 20 minutes. A current-voltage relationship was generated before and during estrogen application to detect any direct actions of this steroid on conductances. Immediately following E_2 application, a second baclofen concentration-response curve was generated.

Schild analysis (Schild, 1947) was performed by first generating a concentration-response to DAMGO. With the highest concentration of DAMGO still present, the opioid antagonist, naloxone (20-320 nM) was superfused until the membrane potential re-equilibrated (usually 15-20 min). Then another DAMGO concentration-response profile was generated in the presence of naloxone. Because competitive antagonists do not activate intracellular effectors, their ability to antagonize receptor activation can be used to estimate

the affinity of the receptor for the antagonist. If an antagonist is applied at its K_d , mass action suggests that twice as much agonist would be required to get the same effect. Schild analysis involves performing DAMGO concentration-response curves in the presence of multiple concentrations of naloxone and interpolating the concentration of antagonist required to double the agonist EC_{50} (K_e for the antagonist). The K_e for naloxone was estimated by Schild analysis and was compared between control cells and E_2-sensitive cells following E_2.

The concentration dependence of estrogen action was assessed by performing DAMGO concentration-response curves following various concentrations of E_2 (1 nM to 1 μM). The response to E_2 was measured as the ((DAMGO EC_{50} after E_2)/(DAMGO EC_{50} before E_2)-1)) × 100%. The data were computer fitted to the logistic equation to generate an E_2 concentration-response curve.

A modified Schild analysis (Tallarida et al. 1979) was used to estimate the K_e of the estrogen receptor for ICI 164,384. These experiments were performed by superfusing 1 nM ICI 164,384 for 10-15 min, followed by various concentrations of E_2 with 1 nM ICI 164,384. The E_2 concentration-response curve in the presence of 1 nM ICI 164,384 was then compared to the previously generated E_2 concentration-response curve, followed by Schild analysis.

Biochemical determination of protein kinase activity. Hypothalamic slices were divided into two symmetrical pieces by a midline cut through the third ventricle. One half of the slice was bathed in control aCSF, the other in aCSF containing 100 nM E_2 for 10-15 minutes.

Following homogenization in 0.25 ml of 10 mM KH_2PO_4 (pH = 6.8), 10 mM EDTA, 500 μM IBMX and 150 mM NaCl, these brain slices were assayed for ^{32}P incorporation into an exogenous substrate (Kemptide) to determine protein kinase activity. This assay was done as previously described (Tan et al. 1994) (2 ng protein/tube), except that 1 μM PKC inhibitory peptide was included in the assay mixture. Basal activation of protein kinase was 34 ± 2% of maximum.

Histology. Following recording, the slices were immersed in 4% paraformaldehyde in 0.03 M Sorensen's phosphate buffer (pH 7.4) for 90-120 minutes (Rønnekleiv et al. 1990). The slices were then soaked overnight in this phosphate 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 (Rønnekleiv et al. 1990). After localization of the biocytin-filled neurons, histology data was recorded. This included soma shape, size, distance from the ventral surface and edge of the third ventricle, as well as number and morphology of fibers (e.g., spines, varicosities, etc.). The slides containing the appropriate sections were then processed with β-endorphin antisera (R13; (Weber et al. 1982)) at 1:1,000, EL-14 GnRH antisera (Ellinwood et al. 1985) at 1:2,500 or an affinity-purified tyrosine hydroxylase antiserum (Pel Freeze) at 1:1,000 using fluorescence immunohistochemistry (Rønnekleiv et al. 1990).

Statistics. Statistical analysis was done with InStat computer software (GraphPad Software,

Inc, San Diego) and numerical data are expressed as mean ± SEM. Comparisons between groups were evaluated using an unpaired two-tailed t-test, a Welch's approximate t-test, or a Mann-Whitney U test, as noted. A paired t-test was used to compare pre- and post-E_2 values taken from the same cell. A Fisher's Exact Probability test was used to compare E_2-modulation of baclofen and DAMGO responses. The mean DAMGO EC_{50} (115 ± 10 nM, n = 65) following E_2 (including both E_2 sensitive and insensitive cells) was significantly different from controls ($p < 0.0001$) and was compared to the DAMGO EC_{50}'s after E_2 + kinase/estrogen antagonists to evaluate those agents. The biochemical data were analyzed by comparing the activation ratio of cAMP-dependent protein kinase (PKA) (Corbin, 1983) between control and E_2-treated half slices with a one-tailed, paired Student's t-test. A p-value ≤ 0.05 was considered significant.

MANUSCRIPT #1

The potency of μ-opioid hyperpolarization of hypothalamic arcuate neurons is rapidly attenuated by 17β-estradiol

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ABSTRACT

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 minutes *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 labelling did not reveal any significant morphological differences between the groups, but a higher number of E_2-sensitive cells were 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.

INTRODUCTION

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, Michael, 1988). Acting through G-protein coupled μ-opioid receptors, the direct effects of this peptide include activation of potassium channels (North, 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, Kelly, 1989; Kelly et al. 1990; Loose et al. 1991). Among the opioid-responsive ARC cells are ß-endorphin neurons, allowing ß-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. injection of this peptide as a positive reinforcement in conditioned place preference paradigms (Mucha, Herz, 1985). One of the most well-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,

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, Kalra, 1983; Ferin et al. 1984; Millan, 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 two days (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 (Kelly et al. 1994). Instead, we have previously shown that a 24 hour treatment of ovariectomized guinea pigs with estradiol benzoate *in vivo* leads to a three-fold 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 further characterize 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, were also investigated.

RESULTS

Estrogen rapidly decreases the potency of μ-opioids

One hundred and three cells from the guinea pig arcuate hypothalamus were recorded in current clamp. Figure 1.1 is an example of a chart record used to generate the DAMGO concentration-response curves for further data analyses. 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 4-fold increase in the DAMGO EC_{50} (Figure 1.2). 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}'s 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}'s 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 1.1. 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 1.2).

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), 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 1.3, 17β-E_2 decreased μ-opioid potency over 4 fold, 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}'s did not overlap with the DAMGO EC_{50}'s 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 hours 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).

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 hours following E_2 superfusion. Figure 1.4 is an example of such a nonresponsive cell. This cell was used to generate 1 pre- and 3 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 eighty-eight percent of cells were recorded in the presence of 1μM 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 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's; 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 labelling of the cells. However, the E_2-sensitive neurons have a distribution that was different from E_2-insensitive cells. As shown in Figure 1.5, 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, 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 1.6 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 minute) exposure, 17β-E_2 (100 nM) decreased the potency of the μ-opioid ligand, DAMGO, approximately four fold in about one-third of guinea pig hypothalamic neurons tested. The biologically inactive stereoisomer, 17α-estradiol, was unable to mimic the effect of 17β-E_2, supporting the idea that this modulation is not an artefact 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. Non-homogenous 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 - 1 nM (Croix, Franchimont, 1975). Considering the capacity of the hypothalamus to concentrate E_2 over plasma levels (Eisenfeld, 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 which is physiologically relevant, 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 imply 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 minutes. 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^{++} from intracellular stores within seconds of E_2 exposure, perhaps via a "non-classical" receptor (Morley et al. 1992). In another population of guinea pig hypothalamic neurons, E_2 directly alters 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 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, Katzenellenbogen, 1991). Estradiol-induced release of Ca^{++} 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 receptors (Towle, 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, getting 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 hour exposure to estradiol results in a reduced DAMGO potency in all of the ARC neurons that were tested (Kelly et al. 1992). So 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 hours (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 et al has 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, 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, 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, non-genomic mechanism of action for this steroid hormone.

TABLE 1.1: The pharmacodynamics of DAMGO in E_2-insensitive and E_2-sensitive neurons before and after 100 nM E_2.

	pre-E_2[†]	E_2-sensitive post-E_2	E_2-insensitive post-E_2
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)

[†]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

TABLE 1.2: Membrane properties of E_2-insensitive and E_2-sensitive neurons.

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

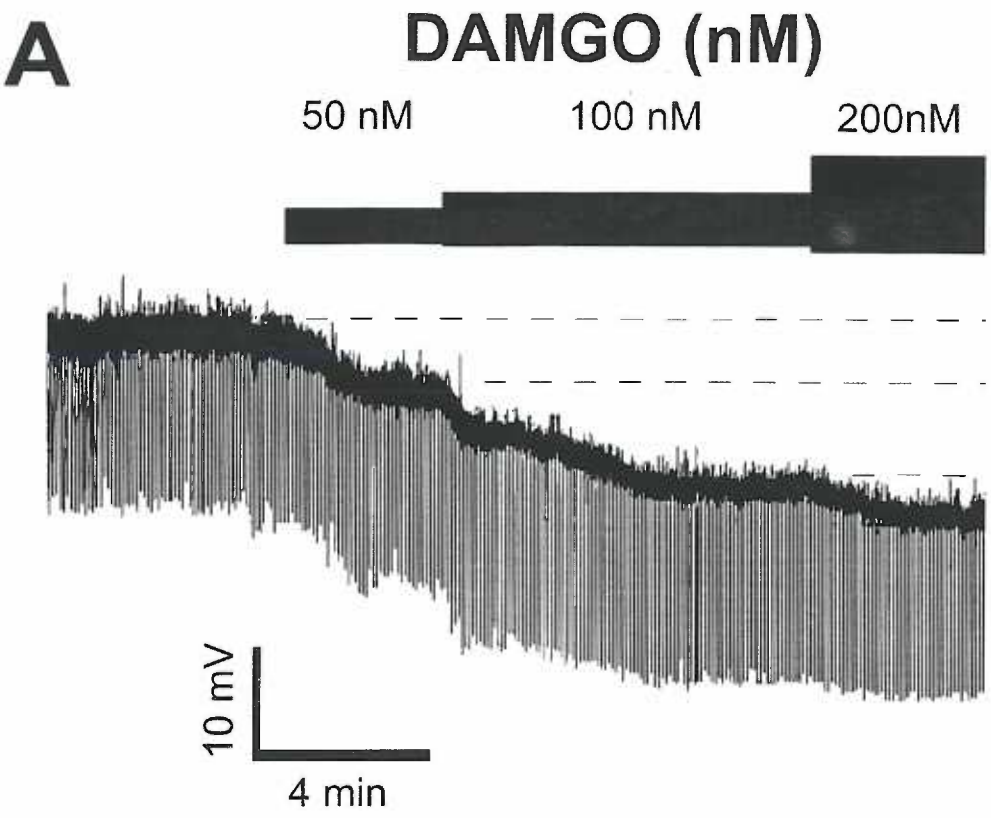
* Measured as the voltage deflection (≈10 mV) seen with a hyperpolarizing current step (>150 ms)

‡ Measured at $^1/_3$ height

FIGURE 1.1: Concentration-response of the μ-opioid agonist DAMGO

A, 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.

B, 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


A
DAMGO (nM)
50 nM
100 nM
200nM
10 mV
4 min

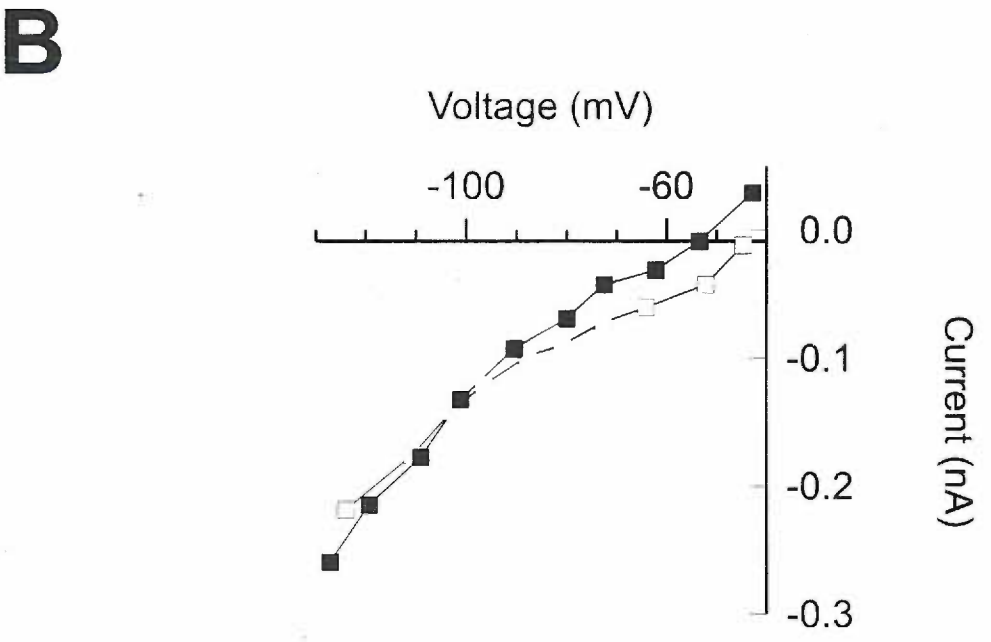

B
Voltage (mV)
-100
-60
0.0
-0.1
-0.2
-0.3
Current (nA)

FIGURE 1.2: 17β-estradiol rapidly attenuates DAMGO potency

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, $p<.001$) after E_2 with no overlap in the pre- and post-E_2 EC_{50}'s.

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.

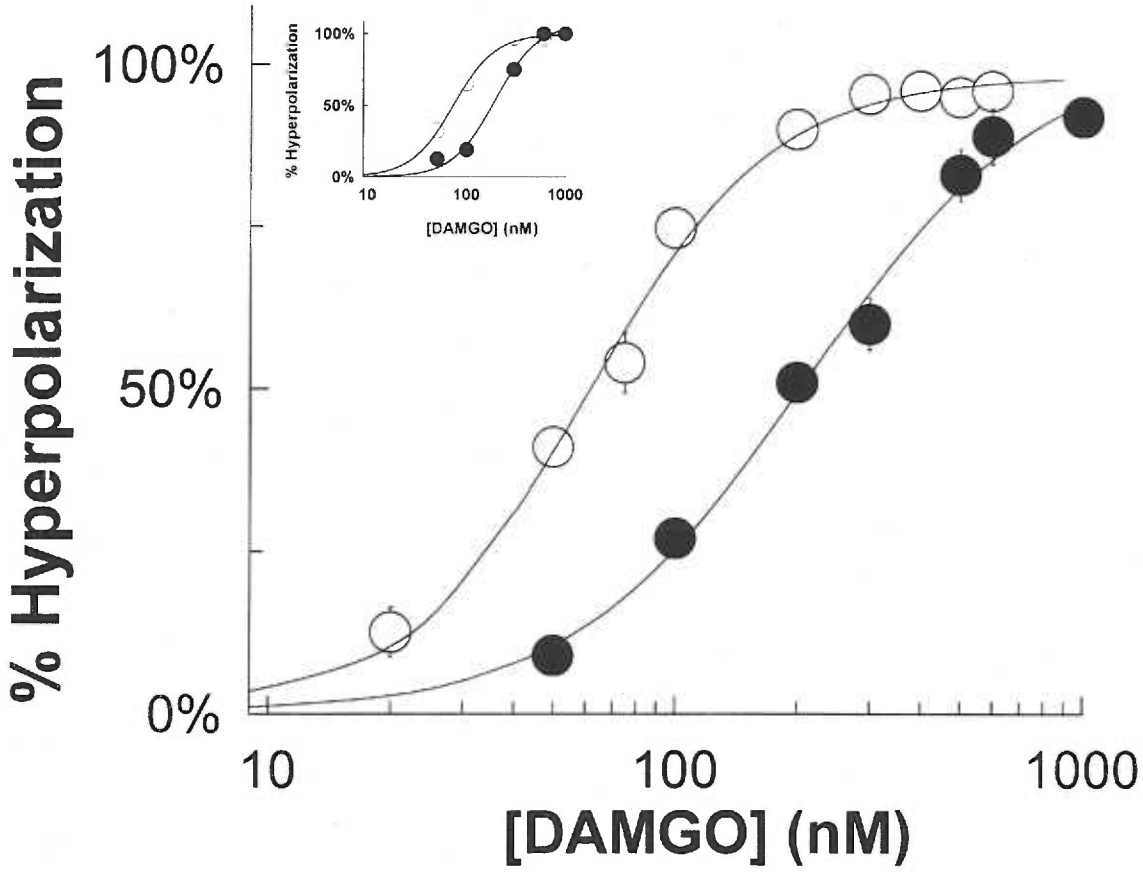

% Hyperpolarization
100%
50%
0%
10
100
1000
[DAMGO] (nM)
% Hyperpolarization
100%
50%
0%
10
100
1000
[DAMGO] (nM)

FIGURE 1.3: The rapid actions of E_2 have a physiologically-relevant concentration dependence.

An estrogen concentration-response curve. The data are expressed as the average per cent DAMGO EC_{50} 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.

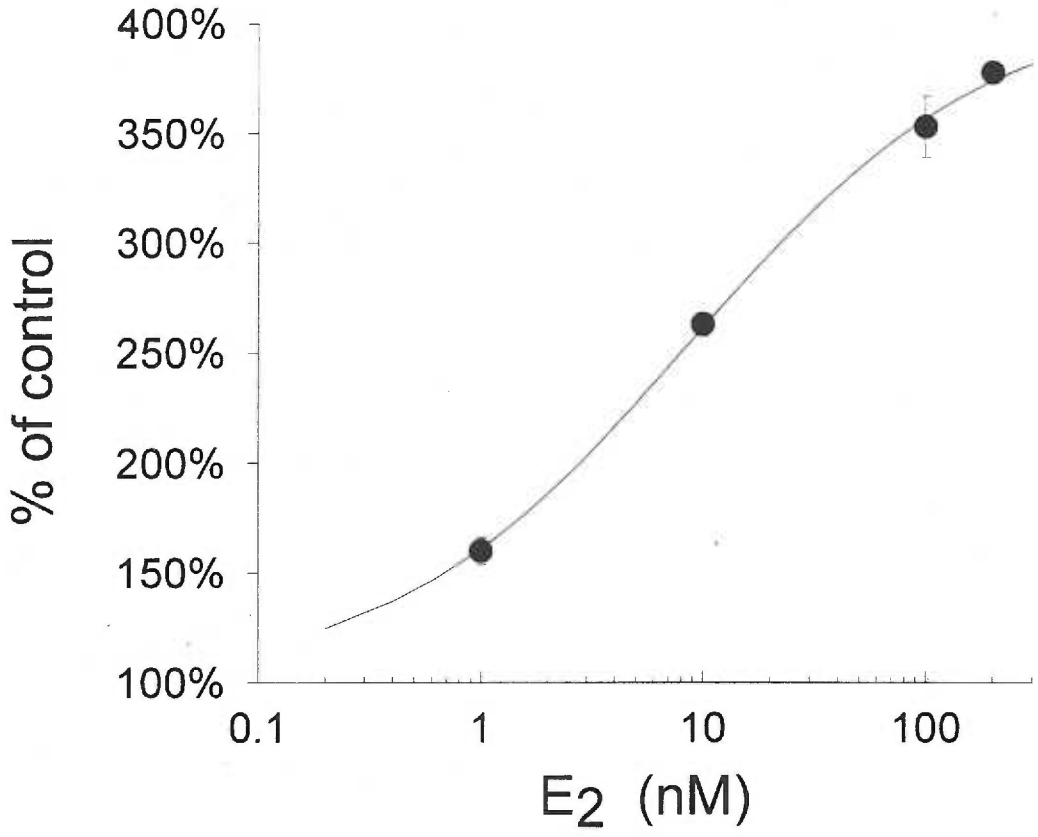

400%
350%
300%
250%
200%
150%
100%
% of control
0.1
1
10
100
E2 (nM)

FIGURE 1.4:

A DAMGO concentration-response curve in a neuron that did not show a response to E_2 up to 3 hours 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 three hours after E_2 application gave similar results (not shown).

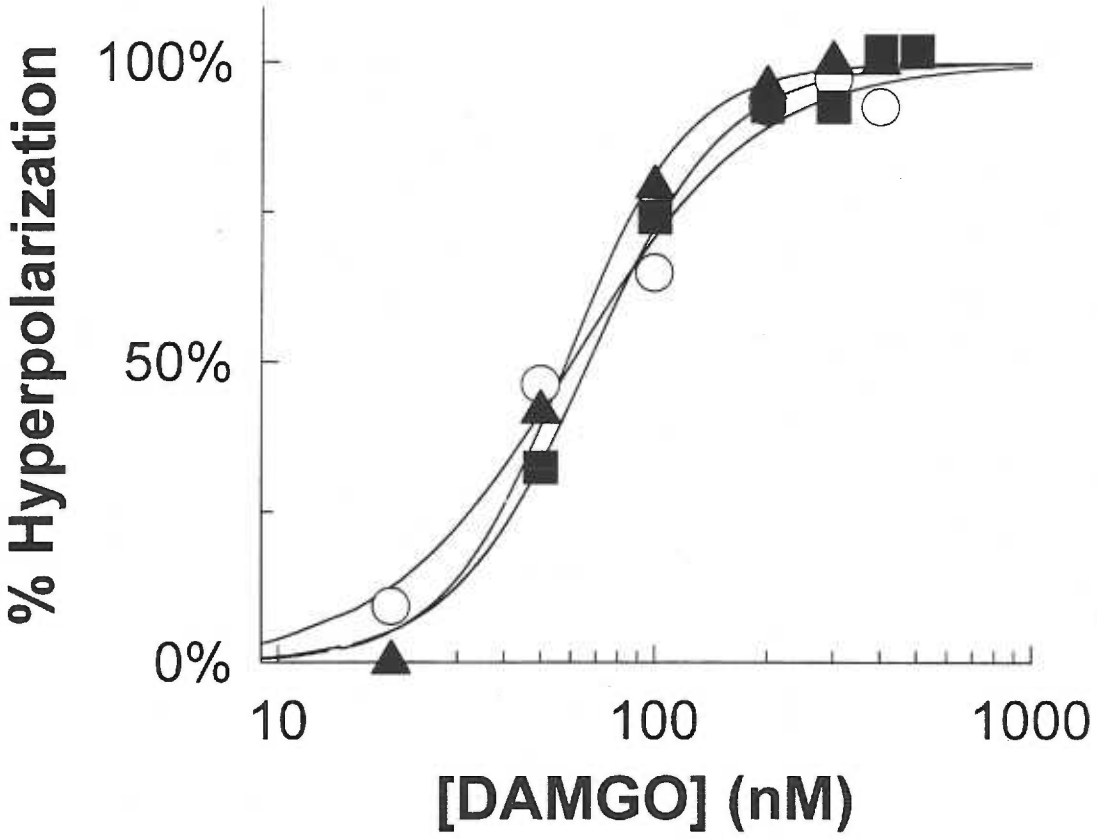
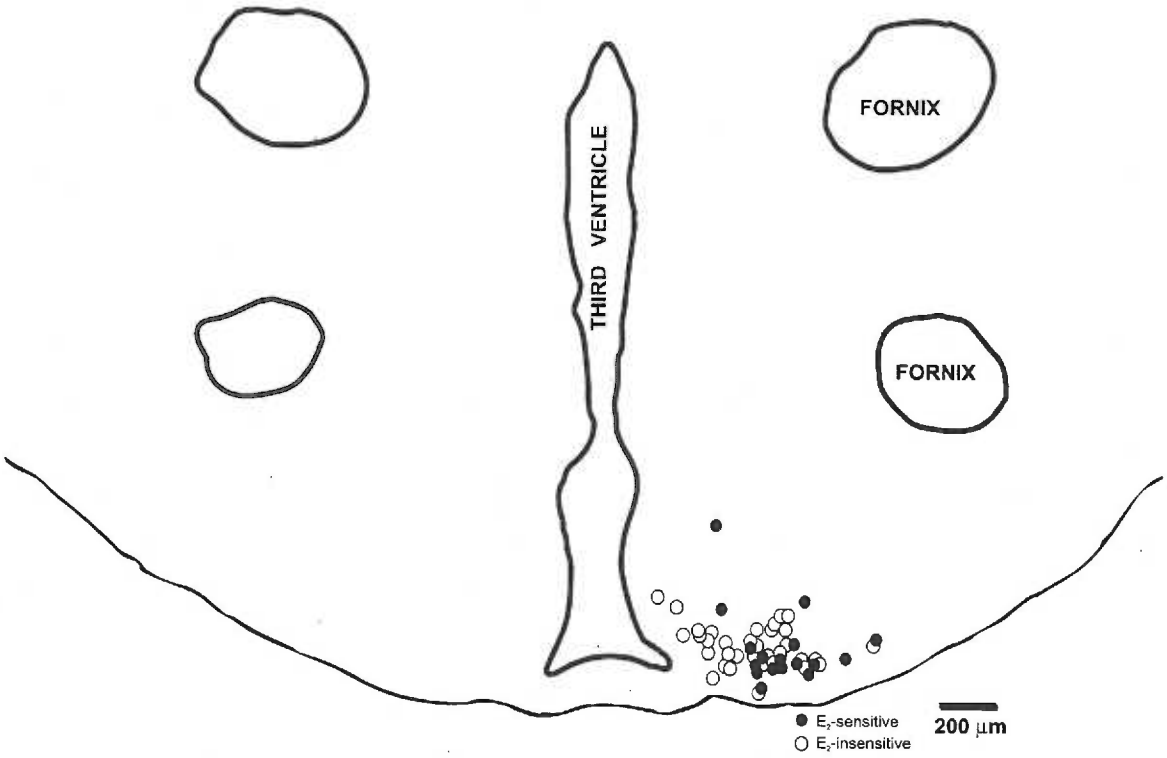
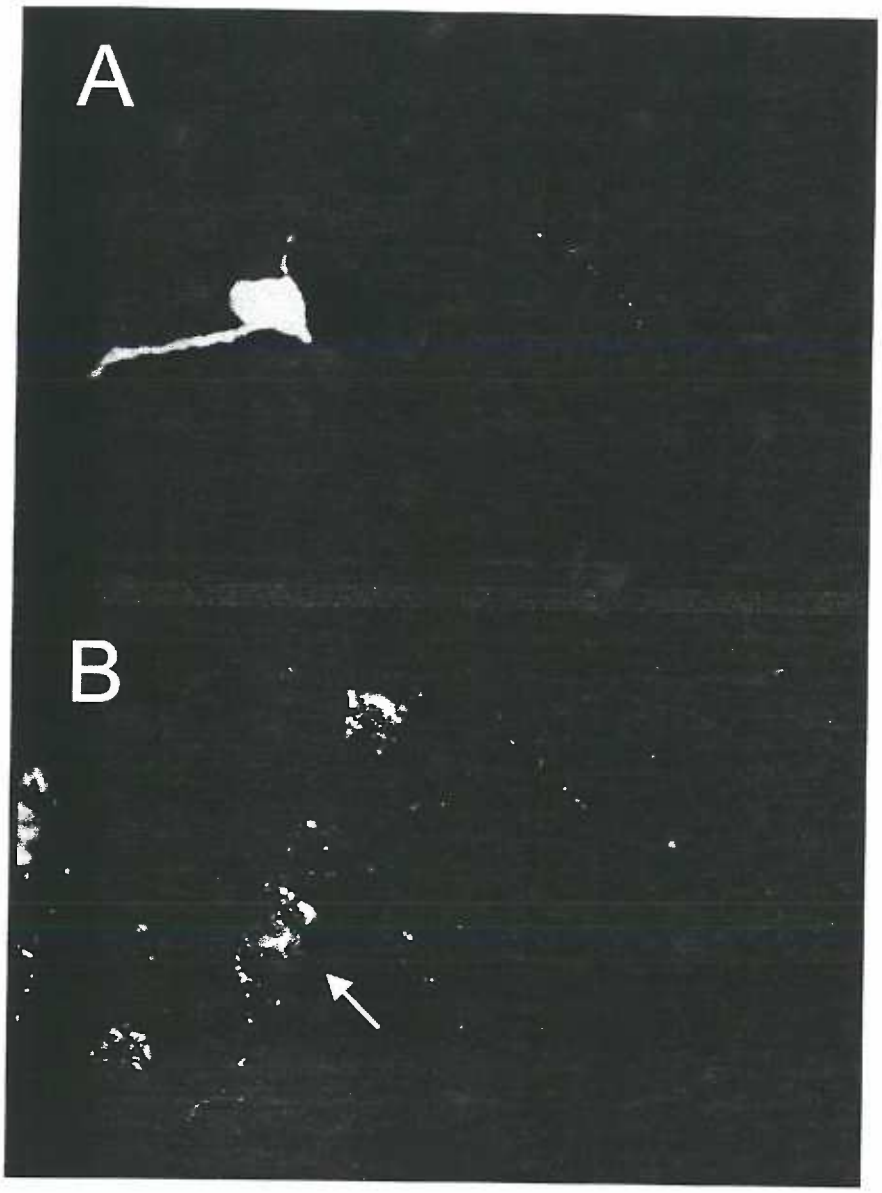

% Hyperpolarization
100%
50%
0%
10
100
1000
[DAMGO] (nM)

FIGURE 1.5: Histological distribution of E_2-sensitive neurons within the hypothalamus

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 filled circles. Coordinates were determined by measuring the distance of the biocytin-streptavidin-FITC labelled neuron from the edge of the third ventricle and ventral surface of the hypothalamus.


FORNIX
THIRD VENTRICLE
FORNIX
● E_2-sensitive
○ E_2-insensitive
200 μm


A
B

MANUSCRIPT #2

Estrogen rapidly attenuates a $GABA_B$ response in hypothalamic neurons

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ABSTRACT

GABA is a predominant neurotransmitter in the hypothalamus and an important regulator of hypothalamic function. To elucidate the cellular basis for GABAergic action in this region, we used intracellular recordings from identified hypothalamic neurons. Ninety-five percent of the mediobasal hypothalamic neurons responded to $GABA_A$ and $GABA_B$ receptor ligands, and the presence of bicuculline-sensitive synaptic potentials implied that GABA was tonically released. Stimulation of $GABA_B$ receptors hyperpolarized these cells by activating an inwardly-rectifying potassium conductance. We characterized $GABA_B$ responses by generating concentration-response curves to the $GABA_B$ agonist baclofen. There was heterogeneity in the responses to baclofen, with one-third of the cells having low baclofen potency (EC_{50} = 5.0 μM). Two-thirds of the neurons had four-fold higher potency (EC_{50} = 1.2 μM), larger somas and a more lateral distribution. Previous work has shown that hypothalamic $GABA_B$ and μ-opioid receptors open the same K^+ channels, and that the response to μ-opioid agonists is rapidly attenuated by 17β-estradiol (E_2). In order to test the hypothesis that the coupling of $GABA_B$ receptors to K^+ channels is also altered, baclofen concentration-response curves were generated before and after an E_2 challenge (100 nM, 20 min). Consistent with our hypothesis, the potency of baclofen was decreased nearly four-fold in a subset of the cells that had a high potency response to baclofen. Furthermore, decreased baclofen potency only occurred in those cells in which E_2 also altered the μ-opioid responses. Therefore, our findings suggest that a discrete subpopulation of hypothalamic neurons are sensitive to estrogen actions to alter inhibitory transmission.

We propose that the alteration of $GABA_B$ and μ-opioid input are consistent with estrogen's rapid inhibition of the reproductive axis.

INTRODUCTION

The amino acid GABA is the predominant inhibitory neurotransmitter in the hypothalamus and throughout the CNS (Decavel, Van den Pol, 1990; Krnjevic, 1974). The ubiquity of GABAergic neurons belies the intricacy with which they regulate neuronal function. These cells often contain other neurotransmitters (Kubota et al. 1994; Zhang, Eldred, 1992) and serve as both projection neurons and local interneurons (Fagg, Foster, 1983). The postsynaptic responses to GABA are mediated by multiple receptor types that are generally grouped as $GABA_A$ and $GABA_B$ receptors (Bowery et al. 1981; Alger, Nicoll, 1982). $GABA_A$ receptors inhibit neuronal firing by opening an intrinsic chloride channel. Cloning of this receptor/channel complex has revealed multiple isoforms that have binding sites for barbiturates, benzodiazepines and neurosteroids, all of which modulate channel activity (Levitan et al. 1988; Burt, Kamatchi, 1994). $GABA_B$ receptors are G-protein coupled proteins with multiple inhibitory actions, including inhibition of calcium currents and adenylate cyclase, and activation of an inwardly rectifying potassium channel ($I_{K(ir)}$) (Wojcik, Holopainen, 1992). In contrast to these "postsynaptic" effects, GABA also modulates its own release (Fassio et al. 1994) and the release of other neurotransmitters (Seabrook et al. 1990; Bonanno, Raiteri, 1993). Although baclofen is an agonist for all these

presynaptic inhibitory actions, the pharmacology of antagonists suggests that multiple receptor subtypes are involved (Bonanno, Raiteri, 1993). Despite the ubiquity of GABAergic synapses in the brain, synaptic specificity is imparted by the existence of multiple GABA cell types, postsynaptic receptors and effector systems.

The repertoire of $GABA_B$ receptor-mediated actions is further increased by interactions with other neurotransmitters. Not only do GABAergic cells contain other neurotransmitters, $GABA_B$ receptor activation modulates the postsynaptic responses to these other signals. For example, baclofen attenuates stimulation of phospholipase C by serotonin (Godfrey et al. 1988) and enhances adrenergic stimulation of adenylate cyclase (Karbon, Enna, 1985). Additionally, $GABA_B$ receptors share effector systems with a variety of receptors, including dopamine (Lacey et al. 1988), acetylcholine (Christie, North, 1988) and serotonin receptors (Andrade et al. 1986). In the hypothalamus, $GABA_B$ receptors share potassium channels with μ-opioid receptors (Loose et al. 1991). Both receptors, along with $GABA_A$ receptors, play important roles in the hypothalamic control of reproduction, with actions that depend on the steroidal milieu and phase of the cycle (McRee, Meyer, 1993). For example, estrogen alters GABAergic neuronal activity (Herbison et al. 1989) and expression of $GABA_A$ subunits (Herbison, Fénelon, 1995), and neurosteroids modulate $GABA_A$ receptor function (Mellon, 1994). In addition, hypothalamic $GABA_B$ receptor density fluctuates during the reproductive cycle (Al-Dahan et al. 1994). Additionally, μ-opioid receptors are uncoupled from their potassium channels by a brief (< 20 min) exposure to 17β-estradiol (E_2) in a subpopulation of cells (Lagrange et al. 1994). In the present paper, we sought to elucidate

estrogenic control of $GABA_B$ receptor-mediated responses, and also clarify the physiological consequences of rapid E_2 action. Therefore, we tested the ability of a brief application of E_2 to modulate the response to baclofen. Some of these findings have been presented in abstract form (Lagrange, Kelly, 1994).

RESULTS

GABA has multiple effects in the hypothalamus

Consistent with the anatomical data that nearly every hypothalamic cell receives GABAergic synapses (Decavel, Van den Pol, 1990), we found that 94% of the recorded cells responded to GABA ligands (n=144). First, there appeared to be tonic release of endogenous GABA in our preparation since 85% of the cells had postsynaptic potentials (PSPs) that were reduced (or abolished) by bicuculline (Figure 2.1A). In fact, these presumptive $GABA_A$-mediated synaptic potentials continued in the presence of 2 μM TTX (sufficient to block cell firing) in about 5% of the cells. Thus, GABA appeared to be released spontaneously, similar to what has been seen in other brain regions (Otis et al. 1991). Moreover, the vast majority of these cells were also sensitive to $GABA_B$ stimulation. Baclofen (20-40 μM) hyperpolarized 93% of the cells by 5 - 26 mV ($\bar{x}$ = 13.8 ± 0.9 mV, n=55) (Figure 2.1B) by opening an inwardly-rectifying potassium conductance (Figure 2.1C). This powerful effect increased total cell conductance nearly 30%, with baclofen inducing 0.8 ± 0.1 nS (n= 37) conductance increase over the control levels (2.9 ± 0.3 nS, n= 39). This hyperpolarizing response was used to generate cumulative concentration-response curves (Figure 2.2A).

Analysis of the baclofen EC_{50}'s in hypothalamic cells revealed that there was a bimodal distribution (Figure 2.2B). One group of cells (63% of cells) had a high baclofen potency (EC_{50} = 1.2 ± 0.1 μM, n=30), and the remaining neurons had four-fold lower baclofen potency (EC_{50} = 5.0 ± 0.4 μM, n=19). Similarly, baclofen potency has been shown to differ between two populations of hippocampal neurons (CA1 and CA3) (Beck et al. 1995). Since cells from both groups were seen in a single animal on six different occasions (12 cells), this difference was not due to variations between animals. Moreover, the histological data supported the conclusion that these different responses represent two subpopulations of neurons (Table 2.1). Cells from the high potency group were found more laterally within the arcuate nucleus, with no differences in either the ventrodorsal or rostrocaudal distributions. Furthermore, the cross-sectional area of the high potency cells were significantly larger than the low potency neurons (Figure 2.3). However, most of the cells (84%) were fusiform to pyramidal, and there were no other obvious differences in either the morphology or electrical properties between these two groups.

Estrogen rapidly uncouples $GABA_B$ receptors

$GABA_B$ receptors share K^+ channels with μ-opioid receptors and these two systems can act in concert to inhibit hypothalamic neurosecretory neurons (Loose et al. 1991). Since E_2 treatment rapidly decreases μ-opioid potency, we tested the hypothesis that E_2 also modulates the $GABA_B$ response. Application of E_2 for 20 min rapidly decreased baclofen potency in a subpopulation of neurons from an EC_{50} of 1.2 ± 0.2 μM to 4.4 ± 0.8 μM (n=9, $p < 0.005$, paired t-test) (Figure 2.4). Voltage-matched current/voltage (I/V) plots were used

to ascertain whether E_2 altered the function of the channels opened by agonists. As summarized in Table 2.2, E_2 did not affect the maximal activation of this current since neither the maximal hyperpolarization (V_{max}) nor the increased potassium conductance (Δg) induced by baclofen were altered by E_2. Furthermore, E_2 alone did not affect the I/V's (not shown), and the reversal potential of the baclofen response ($\approx E_K$) was not different before and after E_2. Therefore, estrogen uncoupled $GABA_B$ receptors from their effector systems without activating another conductance or altering the baclofen-activated conductance.

Estrogen simultaneously regulates $GABA_B$ and μ-opioid receptors

Since $GABA_B$ and μ-opioid receptors open the same K^+ channels and are both modulated by E_2, we explored the extent of this interaction by determining baclofen and DAMGO potency in the same cells. The degree of estrogenic attenuation was similar for baclofen (3.8 ± 0.5 fold increased EC_{50}, n=9) and the μ-opioid agonist DAMGO (3.5 ± 0.6 fold, n=8). More importantly, decreased agonist potency at these two receptors was always coincident. In other words, E_2 either decreased the potency of both DAMGO and baclofen (n=6) or did not affect either response (n=4) ($p < 0.005$, Fisher's Exact Probability test). We also found that E_2-sensitive cells were only found in the baclofen high potency group (pre-E_2 baclofen EC_{50} range: 0.6 - 2.2 μM), providing further evidence that estrogenic effects are seen in a discrete subpopulation of hypothalamic neurons. However, the baclofen potency of E_2-sensitive neurons after E_2 challenge was not different from low potency cells before E_2 (Table 2.3). Thus, E_2 uncoupled these two powerful inhibitory inputs in a subpopulation of hypothalamic cells.

DISCUSSION

The present findings provide a cellular basis for GABAergic regulation of neuroendocrine function, as implied by previous neurochemical studies (Masotto et al. 1989; Wagner et al. 1994). Using electrophysiological recordings from hypothalamic arcuate neurons we found more than 90% of the cells responded to GABAergic ligands, and most of the cells appeared to have both $GABA_A$ and $GABA_B$ receptors. However, consistent with the heterogenous nature of the hypothalamus, the GABAergic responses varied from cell to cell. There appeared to be two populations of hypothalamic neurons based on the potency of the $GABA_B$ response, and this dichotomy was supported by the histological data. Furthermore, application of 17β-estradiol (E_2) rapidly decreased the baclofen potency in a subset of the high-potency neurons, thereby abolishing the difference between these cells and the low-potency group. A similar estrogenic modulation has been shown for the μ-opioid system, which shares potassium channels with $GABA_B$ receptors. This effect is stereospecific, concentration-dependent, requires physiological levels of E_2 and occurs within only a few minutes (Lagrange et al. 1994). By testing both $GABA_B$ and μ-opioid agonists in the same cells, we found that both receptor systems were coincidently uncoupled by E_2. Thus, estrogen simultaneously uncouples both $GABA_B$ and μ-opioid receptors in arcuate neurons, thereby rendering these cells less sensitive to powerful inhibitory inputs.

A crucial initial finding of the present studies is that there are qualitative differences in $GABA_A$ and $GABA_B$ systems that go beyond differences in receptors and effector systems. The ability of bicuculline to block PSPs implied tonic release of GABA in hypothalamic

slices. $GABA_A$ and $GABA_B$ receptors have similar affinities for GABA (Bowery, 1993). Therefore, bicuculline's block of $GABA_A$ responses should have unmasked $GABA_B$ responses to tonically released GABA. However, hyperpolarizing PSPs were not observed after bicuculline. Perhaps there were differences in GABAergic synaptic inputs (Sugita et al. 1992) and/or the spatial distribution of $GABA_A$ and $GABA_B$ receptors relative to those synaptic inputs (Bowery, 1993). Alternatively, electrophysiological data suggests that $GABA_B$-mediated events reflect strong GABA inputs whose influence becomes predominant under certain physiological conditions. These $GABA_B$-mediated events are superimposed on a background of relatively constant $GABA_A$ stimulation (Otis, Mody, 1992). This is consistent with neurochemical estimates of neuronal activity in which a tonic GABAergic inhibition of A_{12} dopamine neurons is blocked by a $GABA_A$ receptor antagonist but not by the $GABA_B$ antagonist 2-hydroxysaclofen (Wagner et al. 1994). However, baclofen inhibits basal dopamine turnover in a 2-hydroxysaclofen-reversible manner. Cellular electrophysiological studies have shown that $GABA_B$ actions occur with strong synaptic input, such as a train of electrical stimuli, while $GABA_A$ PSPs are seen after a single stimulus (Sugita et al. 1992). Thus $GABA_A$ may serve as a tonic modulator of synaptic transmission. while $GABA_B$ responses are more discrete and specific.

There was also heterogeneity among the postsynaptic $GABA_B$ responses. A subpopulation of hypothalamic neurons had a high baclofen EC_{50} (5 μM) before E_2 that appeared to was insensitive to any further modulation by this steroid. The basis for the different responses to baclofen remains undetermined. Perhaps the different baclofen

potencies reflect different receptor subtypes, as has been implied from $GABA_B$ inhibition of neurotransmitter release (Bonanno, Raiteri, 1993). Studies are currently underway to test the idea that a similar receptor heterogeneity mediates the different baclofen responses seen in the hypothalamus. Alternatively, we have found that cAMP-dependent protein kinase A (PKA) mediates the rapid modulation of μ-opioid response by E_2 (Lagrange, Kelly, 1995), and the uncoupling of $GABA_B$ receptors is presumed to involve a similar mechanism. We have found that hypothalamic brain slices have measurable PKA activity in the absence of exogenous stimulation (Lagrange, Kelly, 1995), so perhaps the low potency cells have basal PKA activity in the absence of E_2 that uncouples $GABA_B$ receptors from K^+ channels and occludes any further estrogen action. However, if the heterogeneity in the baclofen response is due to basal kinase activity, why are the μ-opioid responses relatively homogenous in these cells (Lagrange et al. 1994) ? These two neurotransmitter systems may have different subcellular localizations of the receptors/kinase (Scott, McCartney, 1994; Alger, Nicoll, 1982), or there may even be a second, $GABA_B$-specific regulatory mechanism. For example, protein kinase C activity uncouples $GABA_B$ receptors from their effector systems (Andrade et al. 1986). Thus, although μ-opioid and $GABA_B$ receptors share an effector system (i.e., $I_{K(ir)}$), these two systems may be subject to separate regulation.

E_2 modulated both μ-opioid and $GABA_B$ responses in concert, consistent with alteration of a common pathway. Evidence from other brain regions has implied that the $I_{K(ir)}$ is directly activated by G-proteins (Miyake et al. 1989). We have found that E_2 does not alter μ-opioid receptor affinity (Lagrange, Kelly, 1994) or the potassium channels that are

activated by either μ-opioid (Lagrange et al. 1994) or $GABA_B$ agonists (present findings). Therefore, although more complicated scenarios are also possible, the simplest explanation is that estrogen serves to decrease the activity of a pool of G-proteins that is shared by both receptor systems.

Nearly every neuron in the hypothalamus is the target of GABAergic synapses (Decavel, Van den Pol, 1990), including cells containing Gonadotropin Releasing Hormone (GnRH) (Leranth et al. 1985), the endogenous μ-opioid β-endorphin (Goldstein, Naidu, 1989) and even GABA itself (Horvath et al. 1992). Therefore, the consequences of modulating GABAergic responses are manifold (Mott, Lewis, 1994). For example, GABA directly inhibits GnRH neurons (Lagrange et al. 1995), and thereby decreases GnRH release (Brann et al. 1992). However, GABA can also act transynaptically to stimulate GnRH release. GABAergic inhibition of β-endorphin neurons relieves GnRH neurons from tonic opioidergic inhibition (Nikolarakis et al. 1988). When these transynaptic GABA effects are blocked with naloxone or tetrodotoxin, GABA inhibits GnRH release. Finally, GABA regulates its own release through autoreceptors (Waldmeier, Baumann, 1990) and GABA to GABA cell synapses (Horvath et al. 1992). Therefore, estrogenic changes in $GABA_B$ function may be translated into changes in other receptor systems, including $GABA_A$ receptor activation.

The coupling of μ-opioid and $GABA_B$ receptors in GnRH cells (Lagrange et al. 1995) and the synaptic connections between GABA and β-endorphin neurons (Horvath et al. 1992) provide a substrate for synergistic control of GnRH release by these two neurotransmitters. Both baclofen and μ-opiates inhibit GnRH/Luteinizing Hormone (LH) release in an E_2-

dependent manner (Akema, Kimura, 1992; Scott, Clarke, 1993; Millan, Herz, 1985). Elevated serum levels of E_2 result in tonic opioidergic inhibition of GnRH/LH that is blocked by naloxone, and the actions of naloxone are reversed by co-administration of baclofen (Brann et al. 1992; Masotto et al. 1989; Coiro et al. 1992). Furthermore, both GABA and β-endorphin neurons concentrate E_2 (Flügge et al. 1986; Morrell et al. 1985) and the rapid estrogenic modulation of μ-opioid potency is seen in a subpopulation of β-endorphin neurons (Lagrange et al. 1994) but not in GnRH cells (Lagrange et al. 1995). Since β-endorphin is an endogenous ligand for μ-opioid receptors (Goldstein, Naidu, 1989), activation of μ-opioid receptors on β-endorphin neurons represents autoinhibition, thereby reducing subsequent β-endorphin release. Thus, E_2 may rapidly release β-endorphin neurons from inhibition by $GABA_B$ and μ-opioid receptor activation. This disinhibitory effect of E_2 would increase β-endorphin release, thereby inhibiting GnRH neurons (see model in Figure 4.5). This model is supported by radioimmunoassay measurement of peptide release in which E_2 increases β-endorphin secretion but rapidly decreases secretion of GnRH (Nikolarakis et al. 1988; Sarkar, Fink, 1980).

In summary, the present study helps to elucidate the cellular actions of GABA in the hypothalamus and the modulation of $GABA_B$ actions by estrogen. Using intracellular recording from individual neurons, we found that nearly every hypothalamic cell responds to GABAergic drugs. However, the $GABA_A$ and $GABA_B$ responses vary among cells and the coupling of $GABA_B$ receptors to potassium channels is modulated by gonadal steroids. Much of the previous work in which peptide release or behavior have been measured have given conflicting results about the actions of GABA in the hypothalamus. Based on the

present results, it is easy to see how the response to GABA agonists could have different actions depending on the site of agonist application within the hypothalamus and the phase of the reproductive cycle. Thus, the present results and future studies of the cellular responses to GABA will provide a crucial substrate for understanding GABA responses and the regulation of those actions in the hypothalamus.

FIGURE 2.1: Effects of GABA on hypothalamic cells.

A, This cell received bicuculline-sensitive synaptic inputs in the absence of exogenous stimulation. Synaptic potentials were depolarizing because the membrane potential was held at -90 mV, below the chloride reversal potential. The downward deflections represent the responses to hyperpolarizing current steps used to monitor input resistance. *Between the arrows*: Time scale was compressed one-hundred fold and 5 μM bicuculline was applied. Following approximately 2.5 min of bicuculline there was a significant reduction in the synaptic input to this cell.

B, The same cell was hyperpolarized -10 mV by the $GABA_B$ agonist baclofen (8 μM) in the presence of TTX and bicuculline to block transynaptic effects.

C, Current/Voltage plots were generated before (○) and during (●) baclofen. The hyperpolarization induced by baclofen reversed at -92 mV ($\approx E_K$) and increased the cell's conductance (g) 1.7 nS, more than doubling the total conductance (pre-baclofen g = 1.0 nS).

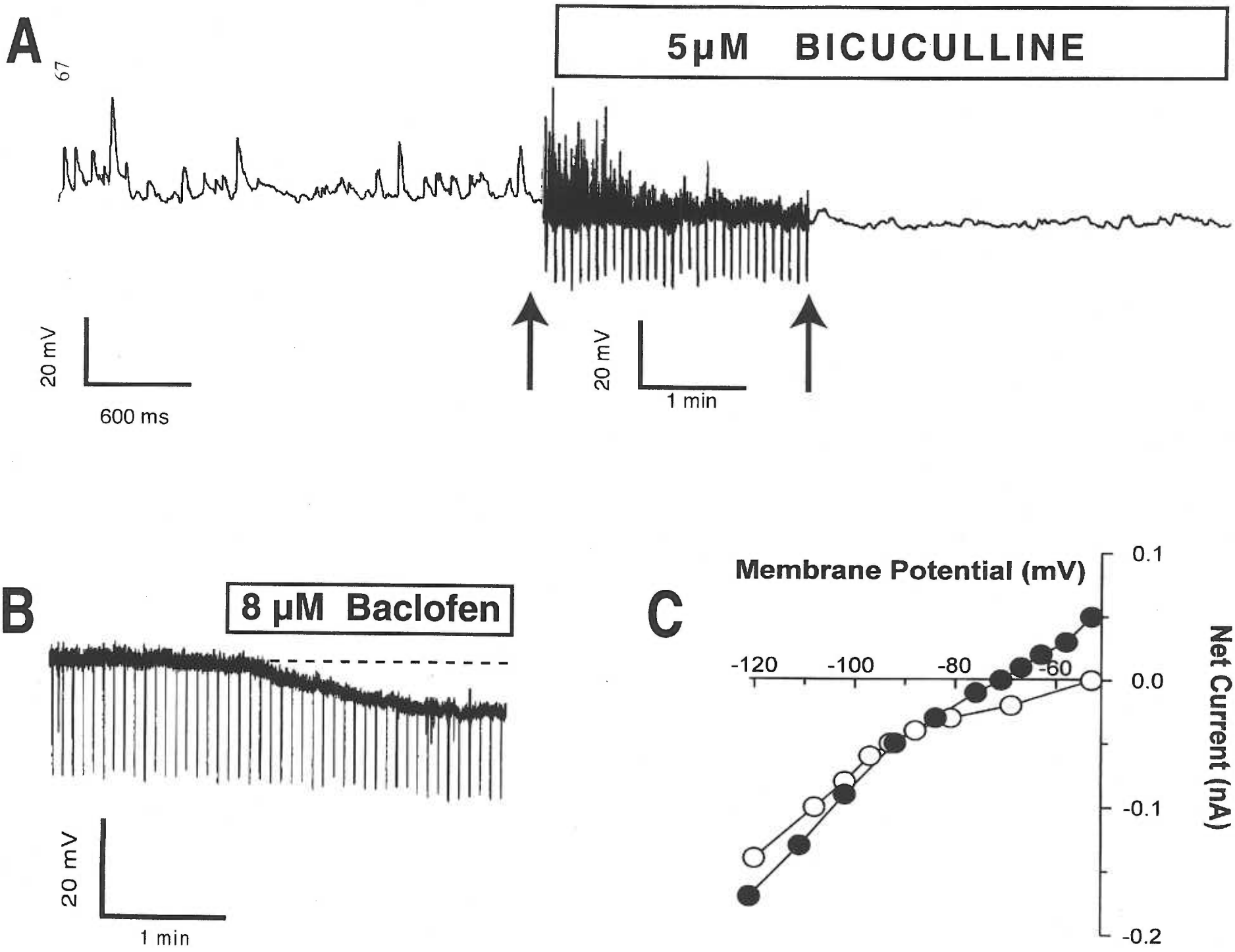
A
67
5μM BICUCULLINE
20 mV
600 ms
20 mV
1 min
B
8 μM Baclofen
20 mV
1 min
C
Membrane Potential (mV)
-120
-100
-80
-60
0.1
0.0
-0.1
-0.2
Net Current (nA)

FIGURE 2.2: Baclofen hyperpolarized hypothalamic neurons in a concentration-dependent manner.

A, Increasing baclofen concentrations (0.5, 2, 8, 20 μM) resulted in an incremental increase in membrane hyperpolarization (2, 10, 16, 16 mV, respectively) from a resting membrane potential of -54 mV. Breaks in the trace represent 30 sec segments during drug-change that were deleted to remove transient (< 400 ms) drug-change artifacts. A computer-generated fit to the logistic equation estimated a baclofen EC_{50} of 1.4 μM in this cell. Dashed lines are for reference only.

B, A frequency histogram of the baclofen EC_{50}'s had a bimodal distribution. Bars represent the number of cells in each bin, this bin width (0.5 μM) was chosen to reflect the resolution of the concentration-response curves. The curve represents a computer-generated double gaussian curve used to emphasize the difference between the two populations.

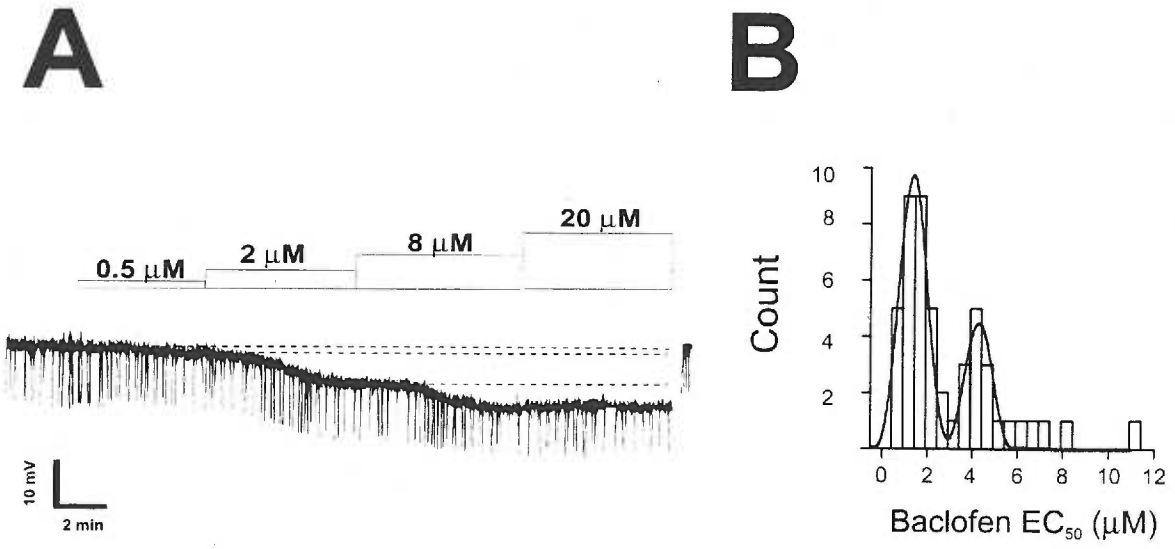
A
B
0.5 μM
2 μM
8 μM
20 μM
10 mV
2 min
Count
10
8
6
4
2
0
2
4
6
8
10
12
Baclofen EC50 (μM)

FIGURE 2.3: Histological differences between high- and low-potency baclofen cells. Photomicrographs of biocytin-filled cells from both cell groups. Recordings were made using biocytin-filled electrodes. Following fixation and processing (see Methods), cells were visualized with streptavidin-FITC and morphology data were recorded.

A, This high potency neuron (baclofen EC_{50} = 1.6 μM) was 15 μm wide by 20 μm long with three spiny fibers. Characteristic of the high potency cell group, this cell was found to be lateral within the mediobasal hypothalamus (700 μm from the edge of the third ventricle).

B, This low potency neuron (baclofen EC_{50} = 4.0 μM) was a small (11 μm diameter) cell with two beaded fibers. This cell was 350 μm from the edge of the ventricle. Bar = 18 μm.

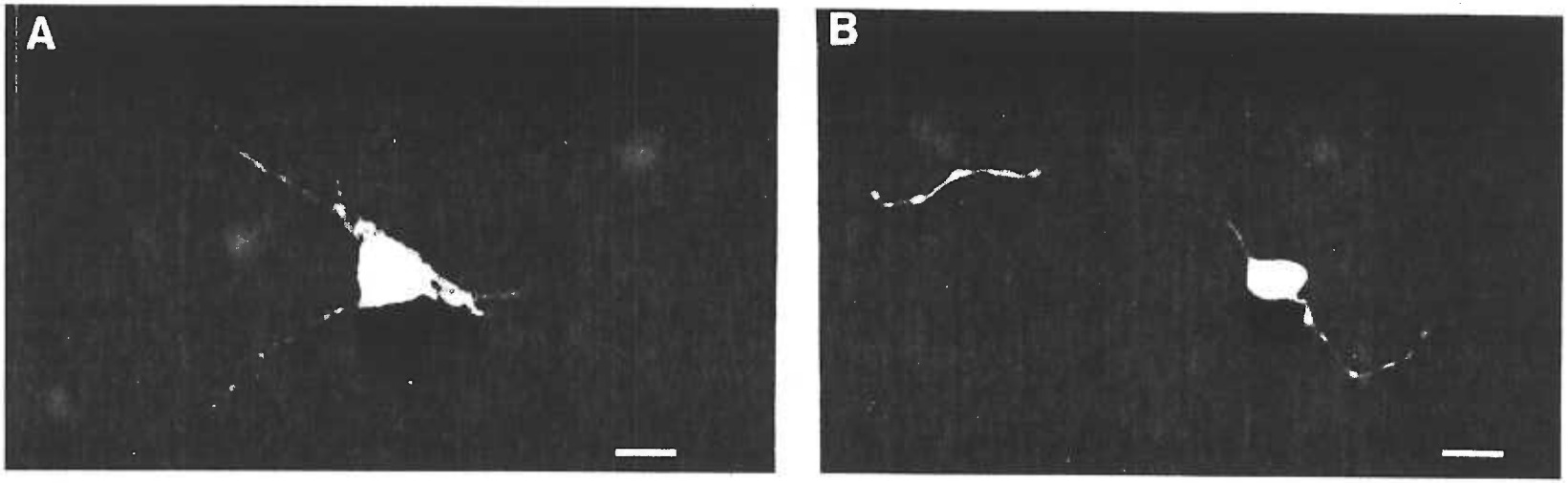
A
B

FIGURE 2.4: E_2 rapidly modulates $GABA_B$ responses.

Eighteen cells were tested before (□) and after E_2 (■, 100 nM, 20 min). This figure is a composite of the nine cells in which baclofen potency was decreased following E_2 exposure. Quantification of estrogen's actions is summarized in Table 2.3. In the remaining nine cells, E_2 had no effect. This concentration of E_2 was chosen, based on previous work, to elicit a maximal effect and is a physiological concentration for E_2 within the intact hypothalamus (Lagrange et al. 1994).

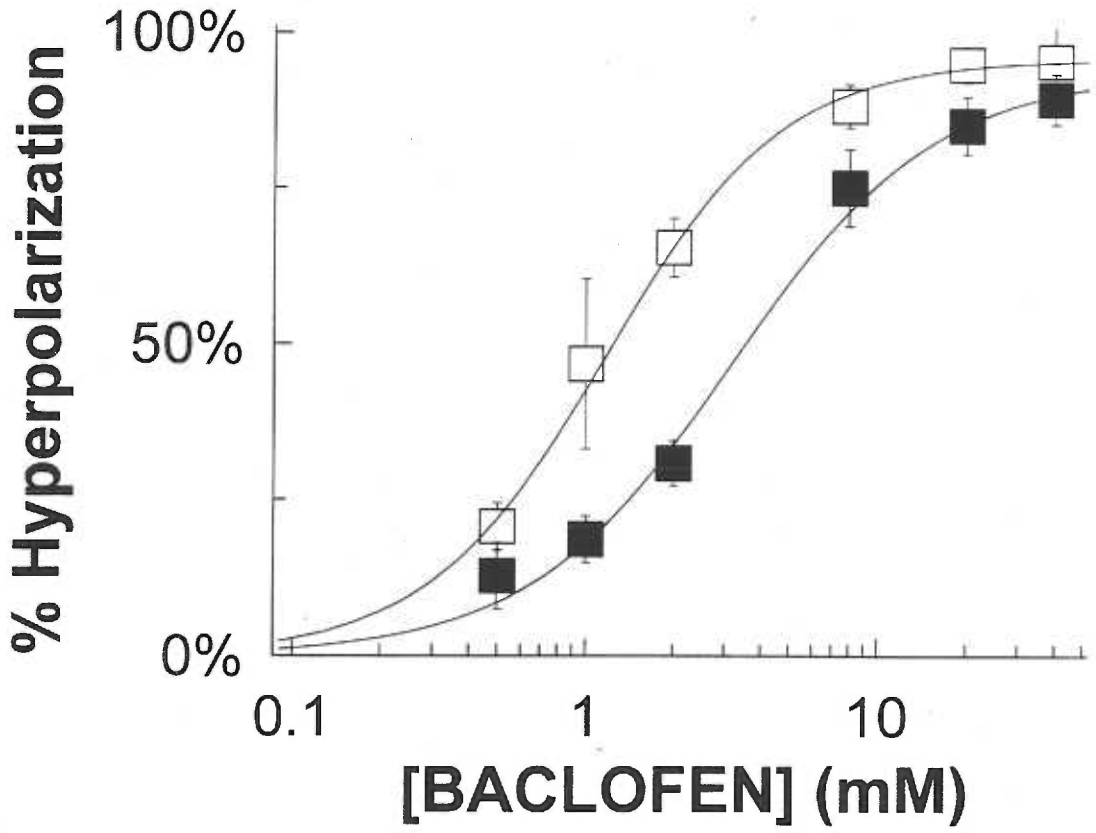
% Hyperpolarization
100%
50%
0%
0.1
1
10
[BACLOFEN] (mM)

TABLE 2.1. Hypothalamic neurons can be divided into two groups based on their responses to baclofen.

Cumulative concentration-response curves were generated to the hyperpolarizing response to baclofen and the data were fit by computer to a logistic equation. Despite a four-fold difference in baclofen EC_{50}'s, there was no difference in either the maximal hyperpolarization (14.3 ± 1.2 mV, n=30; 14.0 ± 1.7 mV, n=19) or the Hill slope (1.0 ± 0.1, n=7; 1.0 ± 0.1, n=11) between high and low potency groups, respectively. Following recording with biocytin-filled electrodes, the slice was fixed, sectioned and stained with FITC-streptavidin. Although there were differences in the mediolateral distribution, there were no differences in the ventrodorsal distribution between "high potency" (Distance from the ventral surface = 199 ± 22 μm, n = 22) and "low potency" (197 ± 22 μm, n = 13) neurons. The two groups were compared using a Welch's approximate t-test.

GROUP	EC_{50}	Distance * from IIIV	Short Axis* of Soma	Long Axis ** of Soma
High Baclofen Potency	1.2 ± 0.1 μM (n= 30)	406 ± 33 μm (n= 24)	12.9 ± 1.0 μm (n= 22)	18.8 ± 1.2 μm (n= 22)
Low Baclofen Potency	5.0 ± 0.4 μM (n= 19)	281 ± 47 μm (n= 12)	10.6 ± 0.5 μm (n= 13)	14.5 ± 0.9 μm (n= 13)

* $p \leq 0.05$ ** $p \leq 0.01$

TABLE 2.2: E_2 does not change the maximal activation of $I_{K(ir)}$ by baclofen.

Voltage-matched current-voltage relationships (I/V's) were done in current clamp before drug application and during the maximal hyperpolarization. As described in the Methods, I/V's were compared before and during baclofen to estimate the maximal difference in slope conductance (Δg) and reversal potential of the baclofen-activated conductance ($E_{Baclofen}$). The maximal hyperpolarization induced by baclofen (ΔV_{max}), Δg, and $E_{Baclofen}$ were compared before and after 100 nM E_2 with a paired t-test. E_2 induced no significant change in any of these parameters.

	Pre-E_2	Post-E_2
ΔV_{max}	16.7 ± 1.7 mV	13.3 ± 2.3 mV
	(n= 9)	(n= 9)
Δg	0.7 ± 0.2 nS	0.8 ± 0.3 nS
	(n= 6)	(n= 6)
$E_{Baclofen}$	-94 ± 4 mV	-98 ± 6 mV
	(n= 7)	(n= 5)

TABLE 2.3: E_2 rapidly modulates baclofen potency in a subpopulation of hypothalamic neurons.

E_2 treatment attenuated the baclofen response only in the high potency group. Even when both high and low potency groups were added together, there was still a significant increase in the baclofen EC_{50} after E_2 application (pre-E2 baclofen EC_{50} = 2.6 ± 0.3 μM, n=49: post-E_2 = 4.7 ± 0.6 μM, n=18, $p < 0.005$ by ANOVA, $F_{(1,65)} = 10.51$). Furthermore, in every cell in which the baclofen response was altered ("E_2-sensitive"), E_2 also attenuated the DAMGO response. Conversely, "E_2-insensitive" cells showed no change in either the baclofen or DAMGO potency following E_2.

GROUP	pre-E_2 Baclofen EC_{50} **	post-E_2 Baclofen EC_{50}	post-E_2 DAMGO EC_{50} ***
E_2-sensitive	1.2 ± 0.2 μM (n=9)	4.4 ± 0.8 μM (n=9)	211 ± 37 nM (n=6)
E_2-insensitive	4.3 ± 1.0 μM (n=9)	4.9 ± 1.0 μM (n=9)	73 ± 9 nM (n=5)

*** $p < 0.005$, ** $p \leq 0.01$, Mann-Whitney U-test, E_2-sensitive vs. insensitive

MANUSCRIPT #3

Estrogen Stimulates PKA Via an Intracellular Receptor: A Novel Mechanism to Rapidly Uncouple G Protein-Coupled Receptors

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ABSTRACT

17ß-Estradiol (E_2) rapidly (< 20 min) reduces the potency of μ-opioids to hyperpolarize hypothalamic neurons. This effect is stereospecific and concentration-dependent within a physiological range. We used intracellular recordings from hypothalamic slices to elucidate the receptor and underlying mechanism mediating E_2's rapid effects. E_2 did not compete for the μ-opioid receptor. Furthermore, the cell-impermeant and biologically-active conjugate, bovine-serum-albumin-estrogen did not mimic E_2, implying that E_2 activates an intracellular receptor. The anti-estrogen ICI 164,384 and the nonsteroidal estrogen diethylstilbestrol (DES) both blocked E_2 actions. The K_c for ICI 164,384 (0.3 nM) was similar to the classical estrogen receptor, but the rapidity of E_2 action and antagonism by DES implied the involvement of a novel estrogen receptor. We next sought to elucidate the intracellular mechanism of E_2's rapid effects and found that the PKA activators, forskolin and Sp-cAMP mimicked the actions of E_2. Furthermore, E_2 effects were blocked by selective PKA antagonists that have different chemical structures and modes of action (Rp-cAMP and KT5720). Finally, E_2 rapidly increased PKA-mediated ^{32}P-incorporation in hypothalamic slices. Thus, PKA stimulation via a distinct intracellular estrogen receptor expands the realm of E_2 action to the rapid uncoupling of G protein-coupled receptors with the potential to alter synaptic transmission throughout the brain.

INTRODUCTION

Classically, estrogen receptor (ER) actions are thought to involve increasing transcription at estrogen response elements (EREs) (O'Malley, Tsai, 1992). However, it has become clear that steroid actions are much more complex, involving heat shock proteins (Church et al. 1994), genes that are not regulated by EREs (Webb et al. 1995) and regulation by other intracellular systems (e.g., protein kinases) (Fujimoto, Katzenellenbogen, 1994; Sartorius et al. 1993), which may even activate ERs without added estrogen (Smith et al. 1993). Furthermore, there are multiple classes of estrogen receptors, some of which are in the plasma membrane (Friend et al. 1995; Towle, Sze, 1983; Orchinik et al. 1992; Ffrench-Mullen et al. 1994; Mellon, 1994; Tesarik, Mendoza, 1995). Finally, numerous rapid (< 30 min), presumably nongenomic effects of E_2 are found in the brain and other tissues (Minami et al. 1990; Becker, 1990; Aronica et al. 1994; Matsuda et al. 1993; Morley et al. 1992; Lieberherr et al. 1993); however, the pharmacology and cellular mechanisms of these effects are often poorly understood. Thus, despite this recent progress in 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 the regulation of reproduction by negative feedback on the hypothalamic-pituitary-gonadal (HPG) axis. *In vivo* and *in vitro* studies in several species have shown that E_2 rapidly (< 30 min) reduces serum Gonadotropin Releasing Hormone/ Luteinizing Hormone (GnRH/LH) levels (Yamaji et al. 1972; Condon et al. 1988; Sarkar, Fink, 1980) which has been difficult to reconcile with a genomic mode

of E_2 action. GnRH neurons do not concentrate E_2 (Shivers et al. 1983) and this estrogenic inhibition is thought to involve β-endorphin neurons that are presynaptic to GnRH cells (Thind, Goldsmith, 1988). The cellular mechanism whereby β-endorphin neurons mediate estrogen's rapid regulation of neuroendocrine secretions remains unknown. However, β-endorphin preferentially binds μ-opioid receptors (Goldstein, Naidu, 1989) and the vast majority (>90%) of hypothalamic neurons are hyperpolarized by μ-opioid activation of inwardly-rectifying potassium channels ($I_{K(ir)}$) (Kelly et al. 1990). A brief (20 min) exposure to E_2 rapidly reduces μ-opioid potency in a subpopulation of hypothalamic neurons (Lagrange et al. 1994). The EC_{50} of the μ-opioid agonist [D-Ala^2-N-Me-Phe^4-Gly^5-ol]-enkephalin (DAMGO) following E_2 is nearly four-fold greater than control values with no change in the efficacy. The actions of E_2 are stereospecific and occur at physiologically-relevant concentrations. This response is seen in approximately one-third of the hypothalamic cells with a preponderance of cells in the caudal ventrolateral mediobasal hypothalamus (MBH).

The present studies characterized the receptor and intracellular effector system(s) mediating E_2's rapid attenuation of μ-opioid response. Since cAMP-dependent protein kinase (PKA) activation uncouples purified μ-receptors from their G-proteins (Harada et al. 1990), we investigated the possibility that a protein kinase mediates estrogen's rapid actions. 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. Finally, we demonstrated that E_2 stimulates PKA activity in hypothalamic slices by

measuring ^{32}P-incorporation into a substrate peptide.

RESULTS

Estrogen rapidly attenuates the μ-opioid response in hypothalamic neurons

E_2 rapidly reduces the potency of the μ-opioid agonist DAMGO nearly four-fold in a subset of hypothalamic cells (Lagrange et al. 1994). These previous experiments used complete concentration-response curves to the hyperpolarization induced by DAMGO, and thus required 20-30 min to assess estrogenic attenuation of μ-opioid responses. In the present studies, we used an alternative technique to improve the time-resolution for measuring 17β-estradiol's effects (Figure 3.1A). Although E_2 does not reduce the maximal response to DAMGO (Lagrange et al. 1994), the response to a submaximal μ-opioid response would be attenuated by E_2 as the cell re-equilibrated to a lower potency state. The response to 100 nM DAMGO before E_2 was -9 mV (82% maximum). However, when 20 nM E_2 was added, the DAMGO response was diminished within 7 minutes, and after approximately 12 minutes the DAMGO response equilibrated to -4 mV (36 % maximum) below the resting membrane potential. In our preparation, DAMGO concentrations below 500 nM do not desensitize. In fact, there was no desensitization when this same cell was subsequently tested with higher DAMGO concentrations (up to 300 nM for 18 min, unpublished observation). Following washout of DAMGO and E_2, a complete DAMGO concentration-response curve showed the DAMGO EC_{50} (177 nM) was shifted from pre-estrogen controls (EC_{50} = 59 ± 3 nM, n = 43) (Figure 3.1B). Furthermore, current/voltage relationships generated before and during E_2 alone showed that this steroid did not directly alter ion channels (Figure 3.1C). Thus, E_2

alters μ-opioid response within a few minutes and may therefore represent a novel mechanism of E_2 action.

Estrogen acts via an intracellular binding site

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)(Schwarz, Pohl, 1994). This possibility was investigated using Schild analysis (Schild, 1947) to determine the affinity of the receptor for the opioid antagonist, naloxone (Goldstein, Naidu, 1989) before and after estrogen. Because competitive antagonists do not activate intracellular effectors, their ability to antagonize receptor activation can be used to estimate the affinity of the receptor for the antagonist (K_e). As seen in Figure 3.2, the K_e for naloxone in cells treated with 100 nM E_2 (3.2 nM, n = 6) was not different from control cells (4.0 nM, n = 4). Thus E_2 neither alters the affinity of the μ-opioid receptor for antagonist nor competitively blocks it.

The discovery of extracellular steroid receptors has been an area of much recent interest (Towle, Sze, 1983; Orchinik et al. 1992; Ffrench-Mullen et al. 1994), and the rapidity of the present phenomena implies that a similar receptor may be involved. We used an estrogen conjugate that does not cross the plasma membrane to distinguish between an intra- and extracellular E_2 binding site. By covalently linking E_2 at its C6 position to bovine serum albumin (BSA-E_2), the steroid is rendered cell-impermeant. This conjugate binds to extracellular E_2 receptors in many cell types (Tesarik, Mendoza, 1995; Pappas et al. 1995), and was able to mimic the ability of E_2 to hyperpolarize a different population of

hypothalamic neurons (Lagrange et al. 1995, Kelly et al. 1984; Minami et al. 1990) (unpublished observation). However, the BSA-E_2 conjugate (100 nM to 4 μM total E_2) had no effect on μ-opioid response, although subsequent application of free E_2 reduced DAMGO potency in the same cells (Figure 3.3). Therefore, estrogen does not seem to rapidly modulate DAMGO potency by actions at an extracellular binding site.

Pharmacology of estrogen's rapid effects

The receptor mediating E_2's rapid effects was further characterized using the antiestrogen ICI 164,384 and the nonsteroidal estrogen, diethylstilbestrol (DES). Although DES 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, n = 7). However, DES (100 nM) blocked E_2 (20 nM) actions when these two compounds were superfused together (DAMGO EC_{50} = 53 ± 4 nM, n = 9, $p < 0.0001$) (Figure 3.4). Thus, similar to its binding to the classical ER, DES acts as an estrogen receptor ligand, although it is an antagonist rather than an agonist in this system. ICI 164,384 is a well-characterized competitive estrogen antagonist (Weatherill et al. 1988). As seen in Figure 3.5A, this estrogen analog blocks E_2 action (DAMGO EC_{50} = 61 ± 4 nM, n = 7, $p < 0.0001$) and a Schild analysis (Tallarida et al. 1979) was used to estimate the affinity of the estrogen receptor for this antagonist. E_2 concentration-response curves were generated by superfusing E_2 (1 nM to 1 μM), followed by a complete concentration-response profile to DAMGO. In several cases, multiple concentrations of E_2 were applied to the same cell, each followed by a DAMGO concentration-response curve. As seen in Figure 3.5B, E_2 had a maximal effect of increasing the DAMGO EC_{50} (V_{max}) by

376 %. The EC_{50} for estrogen's actions 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 prior to and during E_2. The resulting concentration-response curve had an EC_{50} of 34.5 nM, a Hill slope of 0.5 and a V_{max} of 368%. Thus, ICI 164,384 caused a nearly five-fold parallel shift in the E_2 concentration-response curve. The affinity of the estrogen receptor for ICI 164,384 estimated by Schild analysis was 0.3 nM, which is similar to the K_i at the classical estrogen receptor (Weatherill et al. 1988).

PKA stimulators mimic the effects of E_2

The question arises, how does activation of this intracellular receptor attenuate μ-opioid response? A genomic mechanism seems unlikely because E_2 requires at least 30-60 min to alter protein synthesis (Barnea, Gorski, 1970) and probably a longer time to affect cellular physiology. In other systems, protein kinases can uncouple opioid-receptors from their effector systems (Harada et al. 1990), and work in nonneural tissues have shown that E_2 increases intracellular cAMP levels (Aronica et al. 1994). We therefore tested the hypothesis that cAMP-dependent protein kinase (PKA) may be involved in estrogen's rapid effects. Stimulation of adenylate cyclase (AC) with forskolin (1-25 μM) decreased DAMGO potency (DAMGO EC_{50} = 105-221 nM, n = 6). Furthermore, direct PKA activation by superfusion of the nonhydrolyzable cAMP analog, Sp-cAMP (Figure 3.6A) mimicked E_2 action in a concentration-dependent manner. The EC_{50} for Sp-cAMP was 84 μM, with a maximal 393% increase in DAMGO EC_{50}. 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 prior to (10 min) and during E_2 (20 min, 100 nM) (Figure 3.6B). Staurosporine blocked the effects of E_2, with a mean DAMGO EC_{50} (45 ± 6 nM, n = 11) 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 (Van Haastert et al. 1984). In contrast, KT5720 is a more selective analog of staurosporine that inhibits PKA at its catalytic site (Kase et al. 1987). Prior application of either agent blocked E_2 action. The DAMGO EC_{50} in cells treated with KT5720 + E_2 (47 ± 7 nM, n = 7) was not different from controls, but was significantly less than 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, n = 8, $p < 0.0001$). Following 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 cells (Figure 3.7). There was a slightly lower DAMGO EC_{50} in

staurosporine-treated cells than controls ($p < 0.01$), which may be due to the basal kinase activity; however none of these agents had any other effect on either the passive or DAMGO-induced electrical properties of these cells.

Estrogen stimulates cAMP-dependent protein kinase activity in hypothalamic slices

Electrophysiological recording can measure changes in μ-opioid potency within individual cells, but does not allow the direct determination of PKA activity. Since the electrophysiological data implied that PKA was both necessary and sufficient for estrogenic action, we sought to measure PKA stimulation by E_2 directly. Therefore, we used a biochemical assay to measure PKA-mediated ^{32}P incorporation into a substrate peptide in the presence and absence of E_2 (Table 3.1). Although the rapid effects of E_2 were only seen in a subset of hypothalamic cells, we were able to detect estrogenic stimulation of PKA activity in slices of the caudal mediobasal hypothalamus (MBH, see Figure 2 of (Thornton et al. 1994), 134 ± 8 % of controls, $n = 6$, $p = 0.005$) which contains the highest concentration of E_2-responsive cells (unpublished observation). In contrast, estrogen did not stimulate PKA in those slices taken 1 mm more rostrally from the middle MBH (102 ± 5%, $n = 6$). The biologically inactive isomer 17α-E_2 did not stimulate PKA activity (103 ± 5%, $n = 5$), and forskolin (10 μM), which served as a positive control, increased PKA activity to 239 ± 27% of basal level ($n = 4$, $p = 0.02$). The distribution, timecourse and steroidal sensitivity of PKA activation are consistent with previous electrophysiological measurements of estrogenic modulation of μ-opioid potency (Lagrange et al. 1994). Thus, there is both electrophysiological evidence from individual cells and biochemical data that PKA activation

is a mediator of estrogen's alteration μ-opioid potency.

DISCUSSION

The present results describe a novel estrogen receptor and intracellular effector system that rapidly alters synaptic transmission. E_2 appears to act via an intracellular binding site because this steroid did not compete for μ-opioid receptors and the membrane-impermeable conjugate BSA-E_2 had no effect on DAMGO responses. This conjugate is biologically active in other systems (Tesarik, Mendoza, 1995; Lieberherr et al. 1993) and indeed mimicked another rapid action of E_2, namely hyperpolarization of hypothalamic cells (Lagrange et al. 1995, Kelly et al. 1984; Minami et al. 1990). Interestingly, there was no change in DAMGO potency in any of the ten cells hyperpolarized by E_2, suggesting that these two phenomena are expressed in different cellular populations and may be mediated by different receptors and/or effector systems.

Estrogen's rapid effects are mediated by a specific E_2 receptor. The actions of E_2 are saturable, with a physiologically-relevant concentration dependence and are not mimicked by the biologically inactive isomer, 17α-E_2 (Lagrange et al. 1994). In the present study, further pharmacological characterization revealed that the antiestrogen ICI 164,384 blocked the effects of E_2 with an estimated K_e that was very similar to the K_i for the classical estrogen receptor (Weatherill et al. 1988). Also, similar to the classical estrogen receptor, the nonsteroidal agent, DES served as an estrogenic ligand, albeit as an estrogen antagonist. However, both DES and ICI 164,384 are known to down-regulate the classical estrogen receptor (Medlock et al. 1988; Gibson et al. 1991). Although both agents blocked the effects

cAMP suggests that estrogen activates PKA via adenylate cyclase (AC), rather than direct stimulation of the kinase (Matsuda et al. 1993). Furthermore, pharmacological characterization of the estrogen binding site implies that E_2 works through a specific estrogen receptor protein, rather than directly binding AC. Finally, it remains to be determined if other intracellular effectors (e.g., PKC) are also involved in transducing estrogen's rapid effects. Nonetheless, 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 also be involved in homologous regulation of μ-opioid receptors. Chronic exposure to morphine uncouples μ-opioid receptors from their potassium channels, resulting in decreased DAMGO potency (Christie et al. 1987). Furthermore, μ-opioids inhibit AC (Schoffelmeer et al. 1992) and chronic inhibition by morphine results in a compensatory upregulation of AC and PKA (Nestler, 1994). Changes in PKA have been correlated with the development of morphine tolerance and dependence (Nestler, 1994), although oocyte expression experiments have been unable to show a PKA-induced desensitization of the μ-opioid response (Kovoor et al. 1995; Mestek et al. 1995). To our knowledge, the present study is the first report that PKA decreases μ-opioid potency in neurons. Perhaps the upregulation of PKA seen with chronic morphine causes an uncoupling of μ-receptors from their effector systems, similar to what has been shown for the β-adrenergic receptors (Pitcher et al. 1992). Since β-endorphin neurons develop tolerance to chronic morphine (Kelly et al. 1994) and are sensitive to rapid E_2 effects (Lagrange et al. 1994), it may be that acute E_2 and chronic morphine share some of the same

mechanisms (i.e., PKA activation). Studies are underway to examine the effects of PKA modulators in morphine tolerant animals.

At the level of systems physiology, the currently described phenomena provides a cellular substrate for estrogen's rapid inhibition of the HPG axis. The hypothalamus is a heterogenous nucleus with many cell types, but we have immunocytochemically identified β-endorphin and GnRH neurons as being among those hyperpolarized by μ-opioids (Lagrange et al. 1994; Lagrange et al. 1995). Because β-endorphin is the endogenous ligand at the μ-receptor, activation of μ-receptors on β-endorphin cells represents an autoinhibition that prevents further β-endorphin release. Furthermore, estrogenic modulation of μ-opioid potency is seen in some β-endorphin neurons (Lagrange et al. 1994), but not in cells expressing GnRH (Lagrange et al. 1995). We have proposed a model of E_2 negative feedback in which estrogen rapidly uncouples β-endorphin autoinhibition, resulting in increased opioid release with subsequent inhibition of GnRH neuronal activity (Lagrange et al. 1995). Furthermore, the stimulation of PKA would complement the genomic effects of E_2 since these actions are qualitatively and quantitatively different and may be active at different times in the reproductive cycle. Modulation of μ-opioid potency occurs within a few minutes, requiring nanomolar E_2 concentrations while genomic actions of E_2 require hours to days to alter cellular physiology and act with subnanomolar potency (Barnea, Gorski, 1970; Lieberman et al. 1978). At chronically low levels of E_2 genomic actions might prevail, but sudden, large changes in E_2 over the reproductive cycle would stimulate the rapid effects described here. Thus, E_2 may have opposing actions depending on the time and concentration of E_2, as has been predicted in animal models (Karsch, 1987). Finally, besides

synapsing on a number of neurosecretory neurons (GnRH, oxytocin, vasopressin, etc.) hypothalamic β-endorphin neurons send projections throughout the fore- and midbrain, including to the mesolimbic dopamine reward circuits (Koob, 1992; Di Chiara, North, 1992). Therefore, estrogenic alteration of β-endorphin neuronal activity may affect both neuroendocrine and behavioral systems.

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 an intracellular mechanism for rapid alteration of synaptic transmission that may both complement and complete the other modes of E_2 action. Thus, 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.

FIGURE 3.1: E_2 rapidly alters the response of hypothalamic neurons to the μ-opioid agonist DAMGO.

A, There was a concentration-dependent hyperpolarization to 40 nM and 100 nM DAMGO. The response to 100 nM DAMGO was reduced 50% by addition of 20 nM E_2. *Upper Dashed Line:* pre-drug membrane potential (-51 mV). *Lower Dashed Line:* maximal DAMGO-induced hyperpolarization (-62 mV). Transient downward deflections represent the response to current pulses (100 ms, 50 pA) used to monitor input resistance (R_{in}). *Arrow*: membrane potential was current-clamped to the pre-drug resting potentials to verify that DAMGO caused a decreased R_{in}.

B, Following washout of the drugs, a cumulative concentration-response curve was performed (●) and compared to pre-E_2 control responses to DAMGO (○). The line through the data represents a 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.

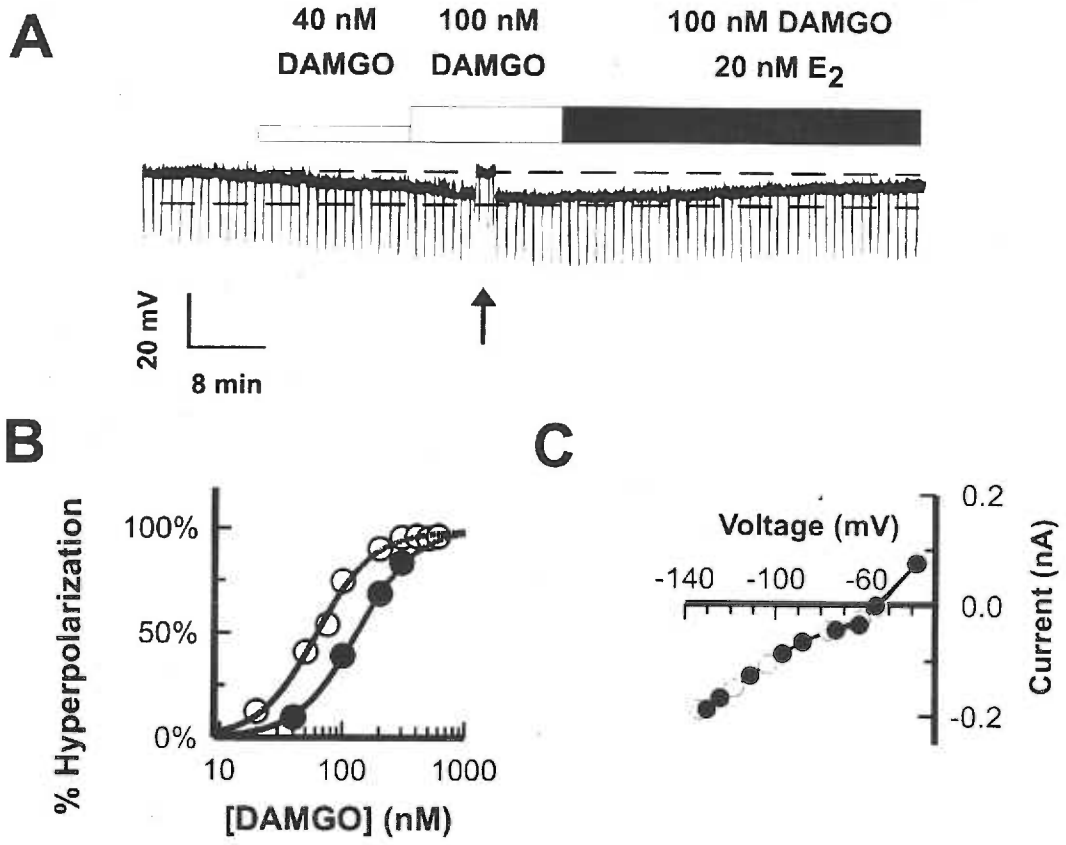
A
40 nM DAMGO
100 nM DAMGO
100 nM DAMGO
20 nM E_2
20 mV
8 min
B
% Hyperpolarization
100%
50%
0%
10
100
1000
[DAMGO] (nM)
C
Voltage (mV)
-140
-100
-60
0.2
0.0
-0.2
Current (nA)

FIGURE 3.2: Estrogen does not alter the affinity of 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 nM, 1047 nM and 9126 nM by 20 (△), 80 (◇) and 320 (□) nM naloxone, respectively.

Inset: Schild analysis was used to estimate the K_e for naloxone. The lines represent a linear regression fit of the data. The slopes of both lines are -1.0, which satisfies the criterion for Schild analysis (Schild, 1947). As seen in this figure, the K_e for naloxone before E_2 (○, 4.0 nM, n = 4) was not different from E_2-sensitive cells after E_2 (●, 3.2 nM, n =6) despite a three-fold shift of the DAMGO EC_{50} (57 ± 2 nM to 171 ± 12 nM, respectively).

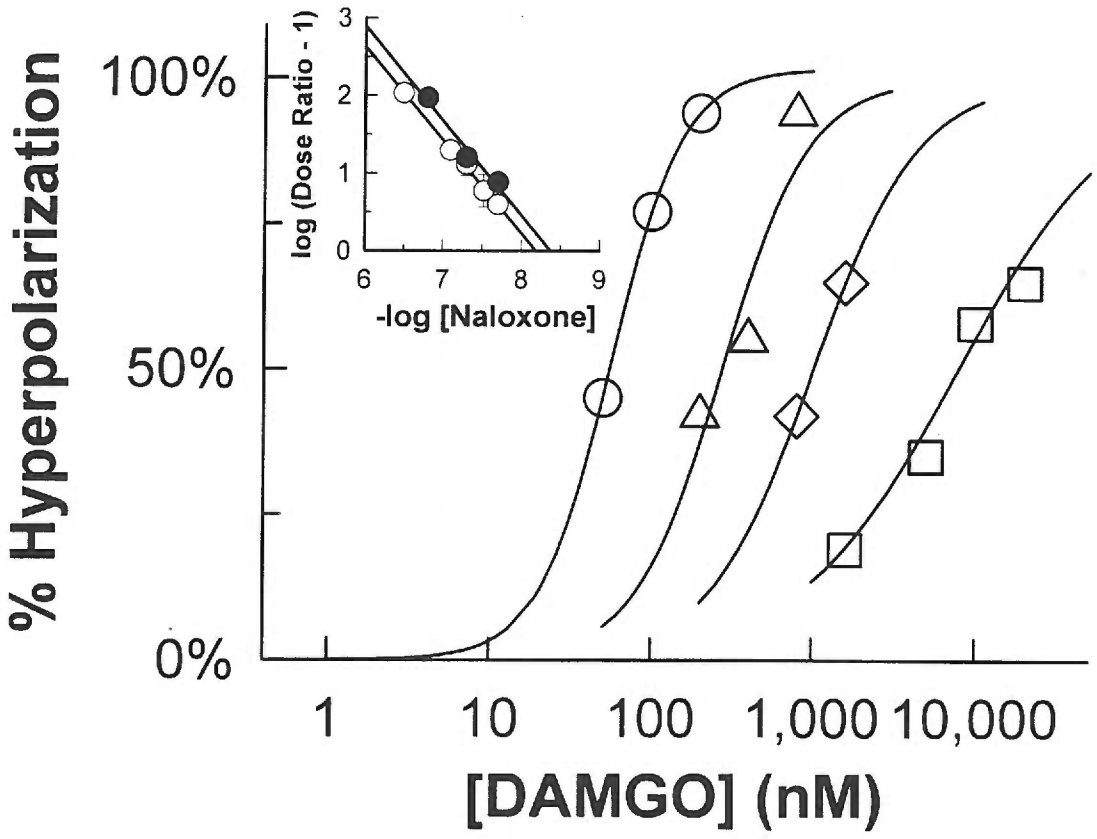
% Hyperpolarization
100%
50%
0%
1
10
100
1,000
10,000
[DAMGO] (nM)
log (Dose Ratio - 1)
3
2
1
0
6
7
8
9
-log [Naloxone]

FIGURE 3.3: Estrogen activates an intracellular receptor.

The membrane-impermeant conjugate bovine serum albumin-E_2 (BSA-E_2, 3.6 μM total E_2) did not alter the DAMGO potency (□, EC_{50} = 85 nM). However, free E_2 (100 nM) shifted the DAMGO concentration-response curve in the same cell (●, EC_{50} = 154 nM). Dashed line represents a summary of pre-E_2 DAMGO concentration-response curves.

Inset: The molecular structure of E_2 for comparison with other estrogenic ligands.

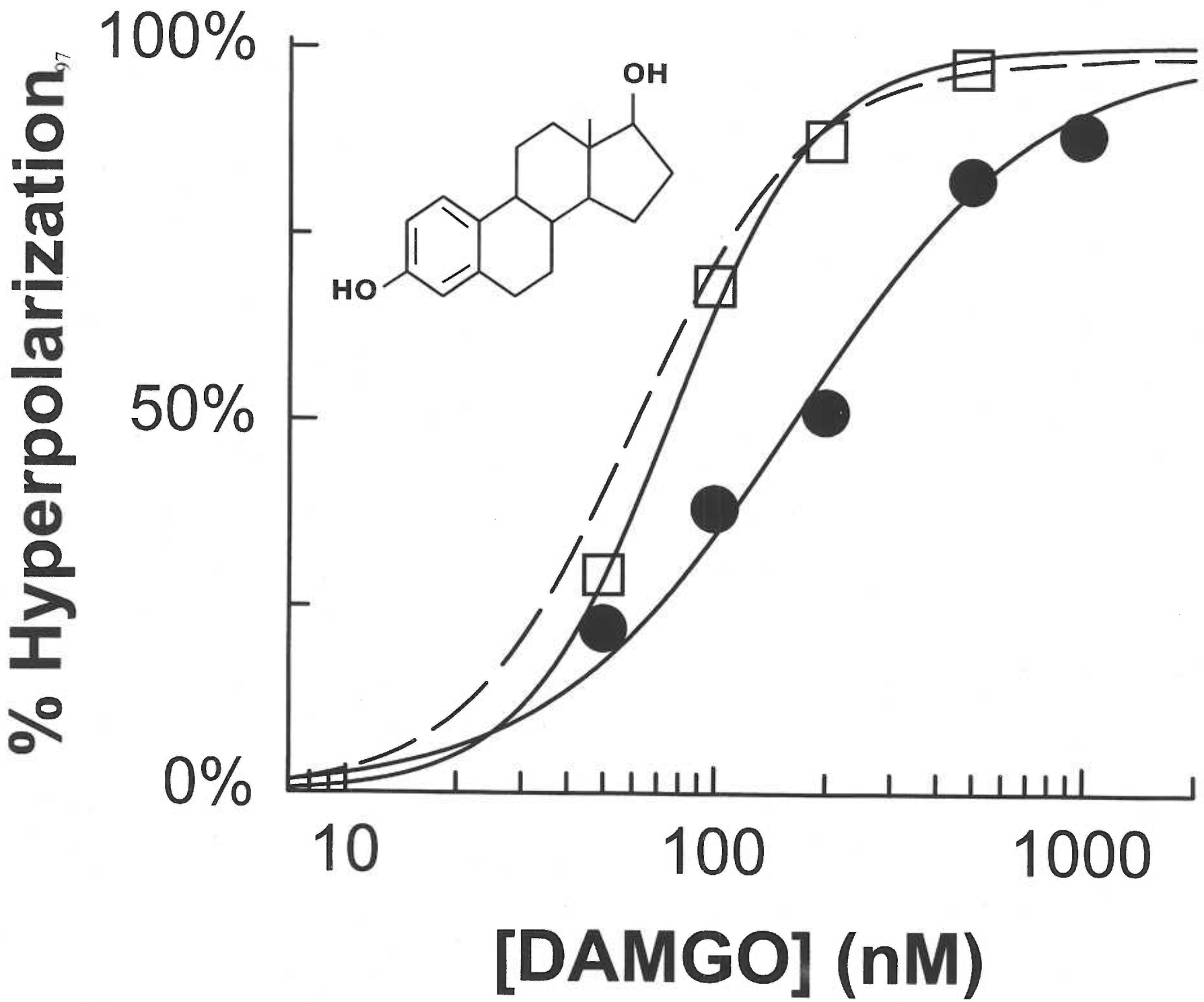
100%
50%
0%
% Hyperpolarization
OH
HO
10
100
1000
[DAMGO] (nM)

FIGURE 3.4: Diethylstilbestrol antagonized the effects of E_2.

When the nonsteroidal estrogen, DES (100 nM) 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} in this representative cell was 46 nM following DES + E_2 (▽). However, when E_2 alone was applied to the same cell, the DAMGO EC_{50} was increased to 194 nM (●). Dashed line represents a summary of pre-E_2 DAMGO concentration-response curves.

Inset: Molecular structure of DES for comparison with E_2.

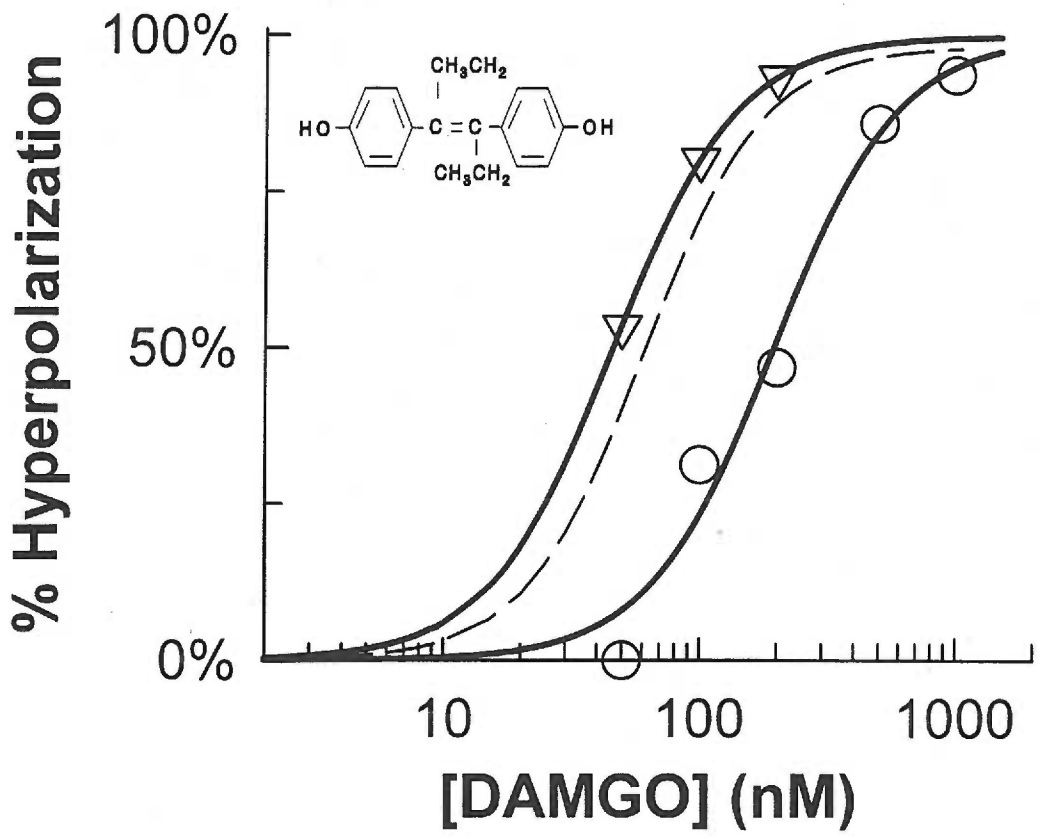
100%
50%
0%
% Hyperpolarization
10
100
1000
[DAMGO] (nM)
HO
OH
CH3CH2
CH3CH2
C = C

FIGURE 3.5: The antiestrogen ICI 164,384 blocks the rapid effects of E_2.

A, ICI 164,384 (100 nM) was applied for 10-15 min prior to E_2, and then co-superfused with E_2 (20 nM, 20 min). As represented by this cell, the resulting DAMGO EC_{50} was not different from controls. When E_2 was superfused, the DAMGO EC_{50} shifted from 50 nM (ICI 164,384 + E_2, ◊) to 166 nM (E_2 alone, ●). Dashed line represents a summary of pre-E_2 DAMGO concentration-response curves.

Inset: Molecular structure of ICI 164,384 for comparison with E_2.

B, Schild analysis of ICI 164,384's antagonism of E_2. DAMGO concentration-response curves were performed before and after various concentrations of E_2 (1 nM - 1 μM). The data were used to generate an E_2 concentration-response curve (●). Addition of 1 nM ICI 164,384 (◆), shifted E_2 concentration-response curve to the right. A modified Schild analysis estimated the affinity of this receptor for ICI 164,384 to be 0.3 nM.

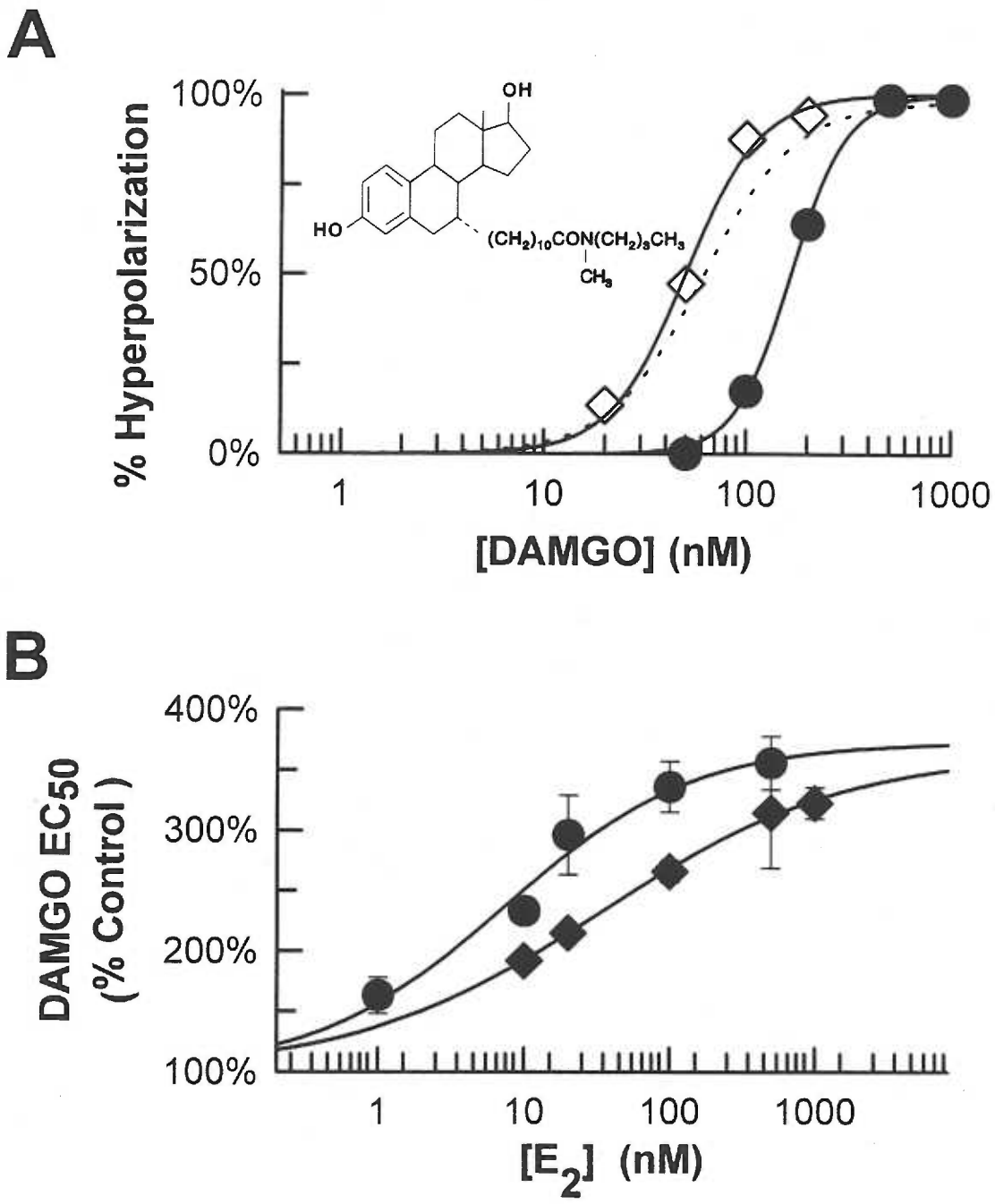
A
100%
50%
0%
% Hyperpolarization
OH
HO
(CH2)10CON(CH2)3CH3
CH3
1
10
100
1000
[DAMGO] (nM)
B
400%
300%
200%
100%
DAMGO EC50
(% Control)
1
10
100
1000
[E2] (nM)

FIGURE 3.6: PKA is necessary and sufficient to alter μ-opioid potency

A, The effects of E_2 are mimicked by the PKA activator Sp-cAMP. DAMGO concentration-response curves were generated in this cell following superfusion of the slice with 50 μM (▲, DAMGO EC_{50} = 104 nM) and then subsequently with 150 μM Sp-cAMP(◆, DAMGO EC_{50} = 175 nM). Open circles with the dashed line represent a summary of pre-E_2 DAMGO concentration-response curves. A similar effect was seen in 3 of 10 cells.

B, Estrogen's rapid attenuation of DAMGO potency is blocked by PKA inhibitors. When E_2-sensitive and insensitive cells were combined, the DAMGO EC_{50} was significantly higher than controls. Staurosporine (100 nM) and the more selective PKA inhibitors KT5720 (60 nM) and Rp-cAMP (100 μM) all blocked the effects of E_2 (100 nM, 20 min) when these agents were superfused for 10 min prior to and during E_2. Following each of these kinase inhibitors, E_2 was applied alone and shown to reduce DAMGO potency, confirming the E_2-sensitivity of these cells. Staurosporine caused a small but significant reduction in the DAMGO EC_{50} compared to 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 to controls

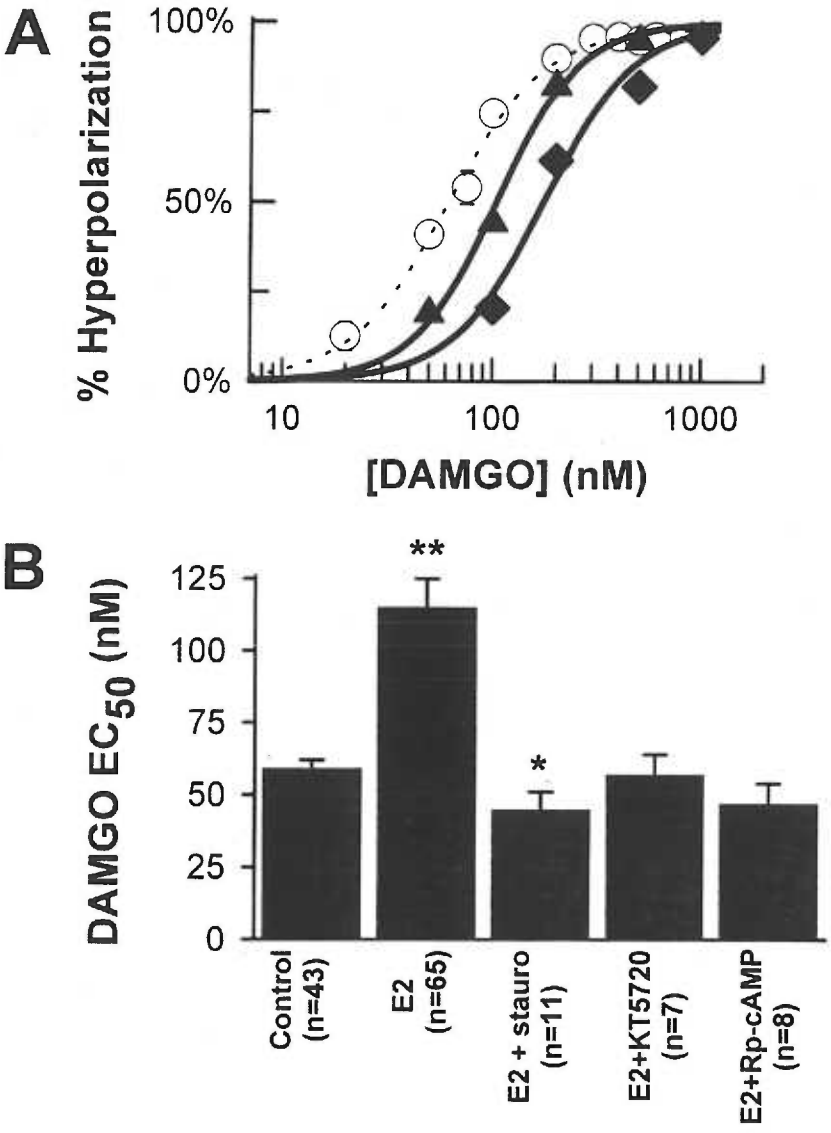
A
% Hyperpolarization
100%
50%
0%
10
100
1000
[DAMGO] (nM)
B
DAMGO EC50 (nM)
125
100
75
50
25
0
**
*
Control (n=43)
E2 (n=65)
E2 + stauro (n=11)
E2+KT5720 (n=7)
E2+Rp-cAMP (n=8)

FIGURE 3.7: 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) .

C, The inhibition of PKA by Rp-cAMP was overcome by Sp-cAMP (◆ , 200 μM, 15 min; DAMGO EC_{50} =256 nM). All of these studies were done within the same cell over a five hour period, during which time there was < 10% rundown of the DAMGO response.

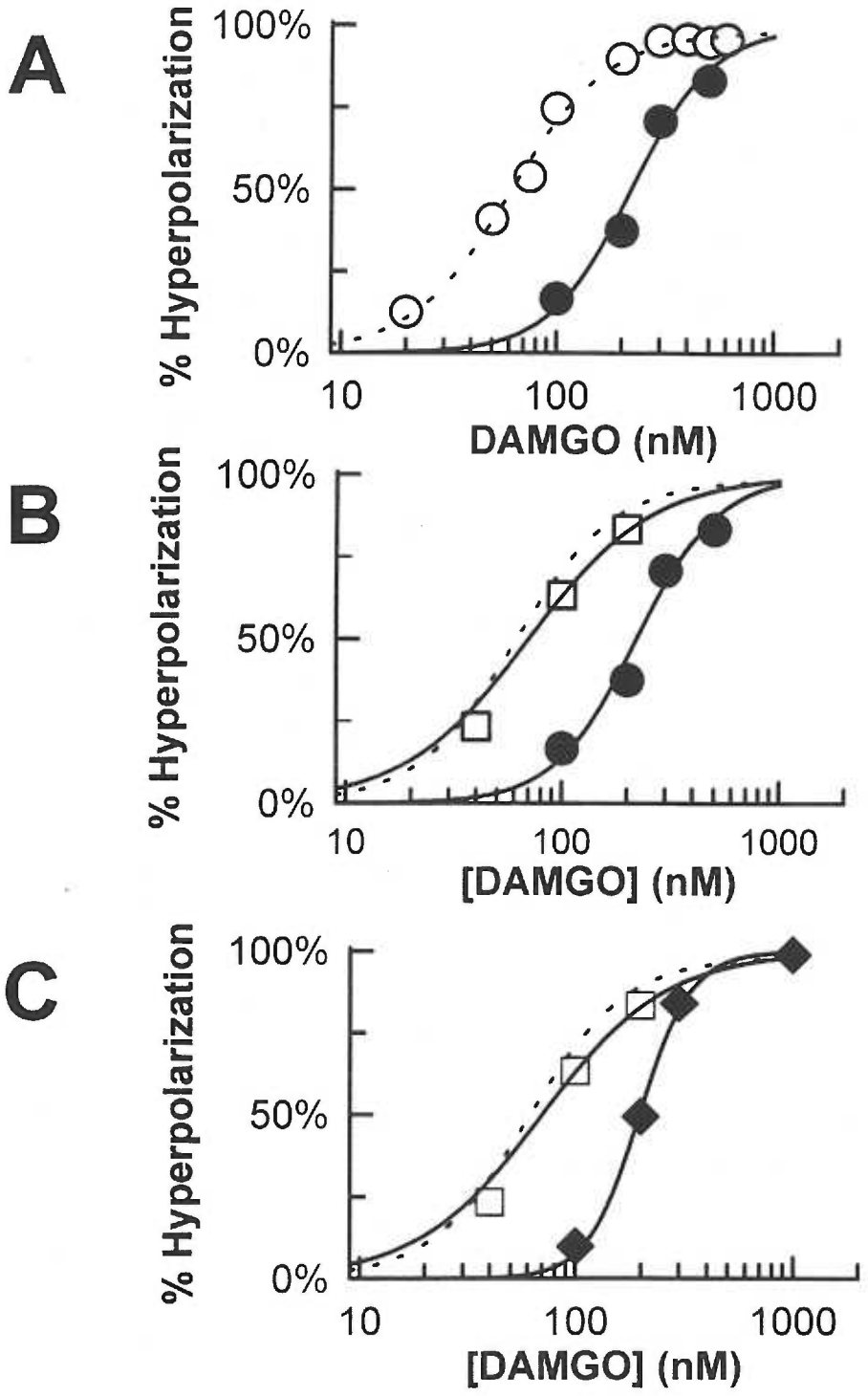
A
100%
50%
0%
% Hyperpolarization
10
100
1000
DAMGO (nM)
B
100%
50%
0%
% Hyperpolarization
10
100
1000
[DAMGO] (nM)
C
100%
50%
0%
% Hyperpolarization
10
100
1000
[DAMGO] (nM)

FIGURE 3.8: Model of estrogen's rapid modulation of μ-opioid response.

A, During low levels of E_2, stimulation of μ-opioid receptors by DAMGO activate potassium channels, thus hyperpolarizing the cell.

B, Following elevated serum levels of 17β-estradiol, the coupling between μ-opioid receptors and potassium channels is rapidly attenuated, thus reducing the potency of DAMGO. The present evidence suggests that E_2 binds to an intracellular receptor that activates cAMP-dependent protein kinase (PKA), probably through stimulation of adenylate cyclase. PKA then "uncouples" μ-opioid receptors from their ion channels, thereby altering the ability of μ-opioids to alter synaptic transmission.

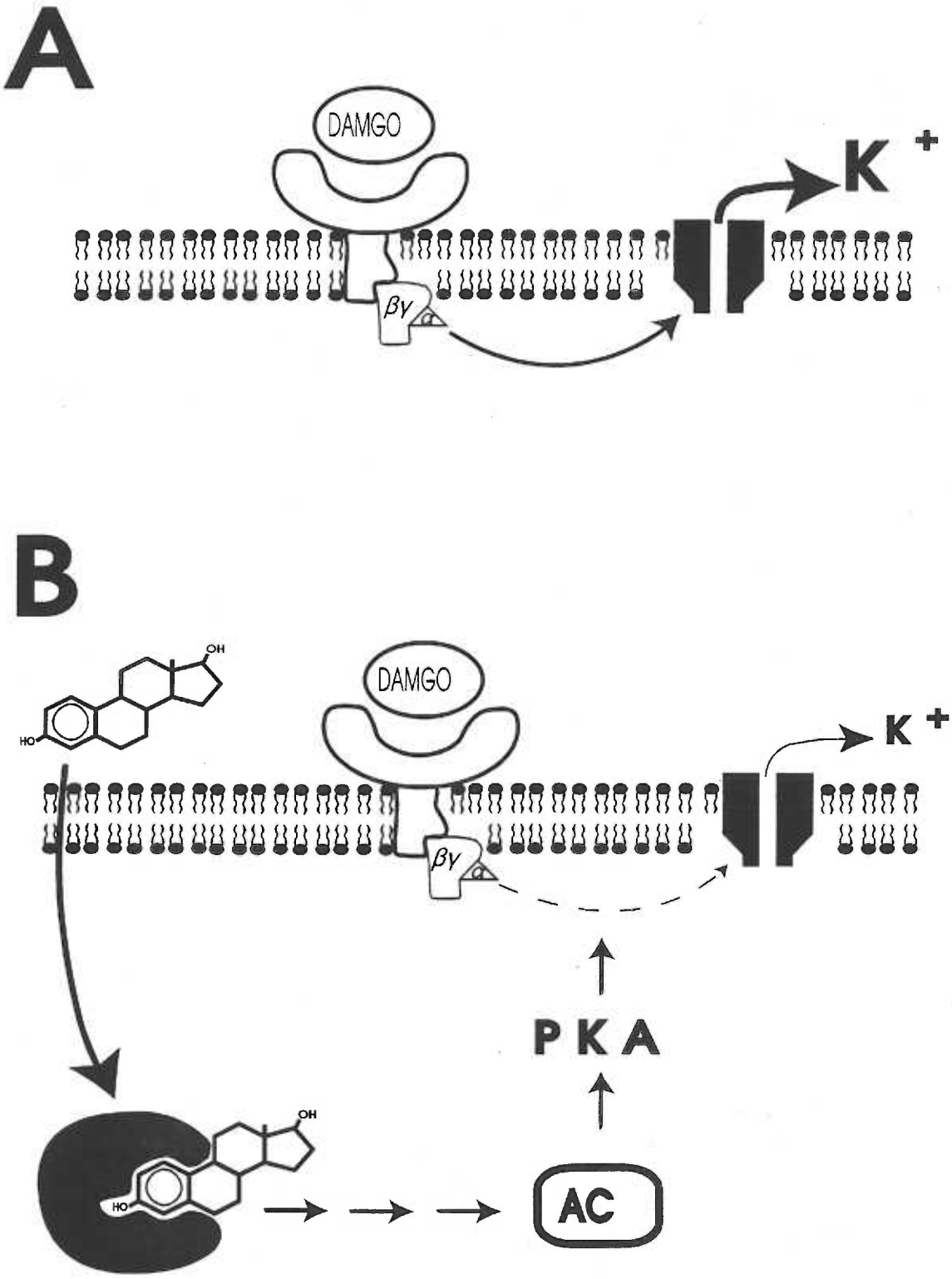
A
DAMGO
K +
βγ
B
OH
HO
DAMGO
K +
βγ
PKA
OH
HO
AC

TABLE 3.1. E_2 stimulates PKA activity in hypothalamic slices.

Treatment		**Location**	**^{32} P incorporation (% of Controls)**
17β-E_2	(n = 6)	Caudal MBH	134 ± 8 %**
17β-E_2	(n = 6)	Middle MBH	102 ± 5%
17α-E_2	(n = 5)	Caudal MBH	103 ± 5%
Forskolin	(n = 4)	Middle + Caudal MBH	239 ± 27%*

E_2 increased PKA activity as measured by incorporation of ^{32}P into a substrate peptide. Slices were perfused with 100 nM E_2 for 15 min, immediately homogenized and assayed. The biologically-inactive isomer 17α-E_2 served as the negative control, while forskolin (13 μM) served as the positive control.

* $p \leq 0.02$ ** $p \leq 0.005$

MANUSCRIPT #4

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 a 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 (Lagrange et al. 1994), 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 (Barnea, Gorski, 1970). However, studies in a number of ovariectomized animal models have shown that E_2 injections can quickly reduce serum LH levels (<30 min) (Yamaji et al. 1972; Negro-Vilar et al. 1973; Condon et al. 1988) and portal blood GnRH (Sarkar, Fink, 1980). 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 (Shivers et al. 1983), thereby suggesting that GnRH release is controlled by E_2-sensitive neurons that are presynaptic to GnRH neurons (see (Kalra, Kalra, 1984)). During the menstrual cycle, negative feedback control of LH secretion by E_2 appears to involve the hypothalamic opioid systems (Ferin et al. 1984). Morphine attenuates the LH surge and prevents ovulation in the rat (Ieiri et al. 1980). Conversely, the opioid antagonist, naloxone potentiates the duration and magnitude of the LH surge (Ieiri et al. 1980; Kalra, 1981), increases GnRH levels in portal blood in monkeys (Ferin et al. 1982) and advances ovulation in women (Rossmanith et al. 1988; Genazzani et al. 1993). Although direct synaptic contacts between β-endorphin and GnRH neurons have been found in monkeys (Thind, Goldsmith, 1988) and rat (Chen et al. 1989), 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 (Lagrange et al. 1994). Since β-endorphin binds to the μ-opioid receptor with high affinity (Bunzow et al. 1995), 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.

RESULTS

Electrophysiological recordings were obtained from eight immunocytochemically identified GnRH neurons (Figure 4.1). The morphology of the GnRH neurons was similar to previously published descriptions (Kelly et al. 1984). 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 (10 x 12 μm). The passive membrane properties of GnRH neurons which did not differ significantly from other arcuate neuronal types (Kelly et al. 1990; Loose et al. 1990), were the following (n=8): Resting Membrane Potential (RMP)=-55 ± 3 mV; $\tau = 23 \pm 5$ ms; $R_{in} = 483 \pm 65$ MΩ. In addition, GnRH neurons expressed several conductances that are pertinent to bursting in parvocellular neurosecretory neurons (Kelly, Rønnekleiv, 1994), 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 ($\Delta g_{DAMGO} = 0.30 \pm .04$ nS) that resulted in a mean hyperpolarization of 9 ± 3 mV. As previously reported for other hypothalamic cells (Loose, Kelly, 1990) the reversal potential of the DAMGO-induced conductance (E_{DAMGO}) in GnRH cells was close to E_K (-95 ± 4 mV, n=5). Figure 4.2 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 (Leranth et al. 1985), 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 (Loose et al. 1991). 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 function, cells were perfused with E_2 (100 nM, 20 min) *in vitro*. As reported previously (Kelly et al. 1984), E_2 hyperpolarized GnRH neurons by opening K^+ channels (Figure 4.3). 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 (Lagrange et al. 1994), but this effect was not seen in

GnRH neurons (Figure 4.4). Although both the hyperpolarization and decreased opioid potency can be seen with 1 nM E_2 (Kelly et al. 1984; Lagrange et al. 1994; Nabekura et al. 1986), 100 nM was chosen to elicit the maximal effect to compare with the DAMGO maximal effect.

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 (Kalra, Kalra, 1984)). 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 (Maggi et al. 1994). Furthermore, we have previously shown that the μ-opioid receptor and $GABA_B$ receptor are coupled to the same K^+ channel (Loose et al. 1991). 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 would be inhibited by the increased opioidergic tone, in addition to being directly hyperpolarized by E_2 (Figure 4.5). 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 (Grosser et al. 1993), and why naloxone stimulation of GnRH release is not attenuated following ovariectomy in rats (Karahalios, Levine, 1988).

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 (Orchinik et al. 1992; Matsuda et al. 1993) or an intracellular receptor? Perhaps E_2 is exerting a nongenomic action of the classical estrogen receptor (Castoria et al. 1993). 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 (Condon et al. 1989) that may be mediated by an increase in α_1-noradrenergic receptors (Etgen et al. 1992). 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 4.1: Double-labelling of GnRH neurons

A, Streptavidin-FITC staining of a biocytin-filled cell following electrophysiological recording.

B, Immunocytochemical identification of this cell (at the arrow) using and anti-GnRH primary antibody (EL-14) and a Texas Red labelled secondary antibody. Bar = 20 μm.

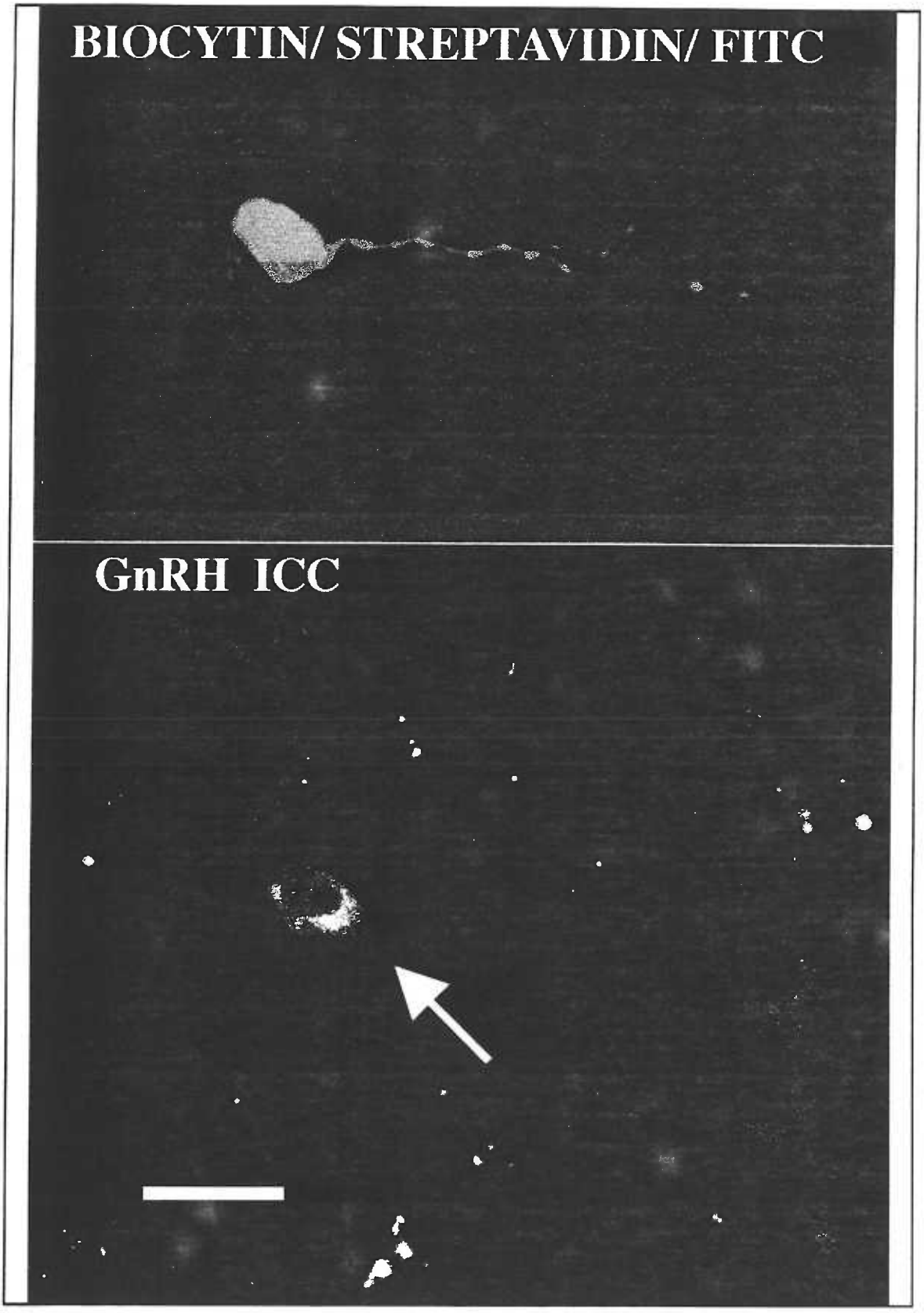

BIOCYTIN/ STREPTAVIDIN/ FITC
GnRH ICC

FIGURE 4.2: DAMGO hyperpolarizes GnRH neurons.

A, 200 nM DAMGO hyperpolarized this cell by 7 mV. The RMP of this cell was -55 mV (dotted line). Break in recording represents the generation of I/V plots followed by washout.

B, Current-voltage (I/V) relationship 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 recording represent the generation of I/V plots followed by drug equilibration

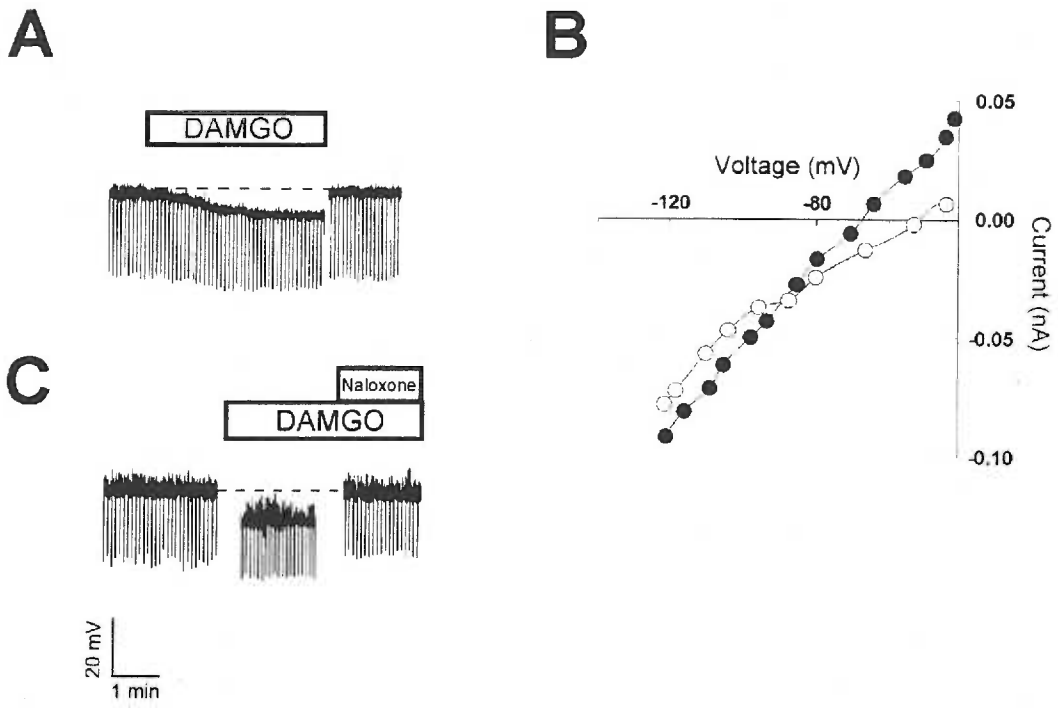
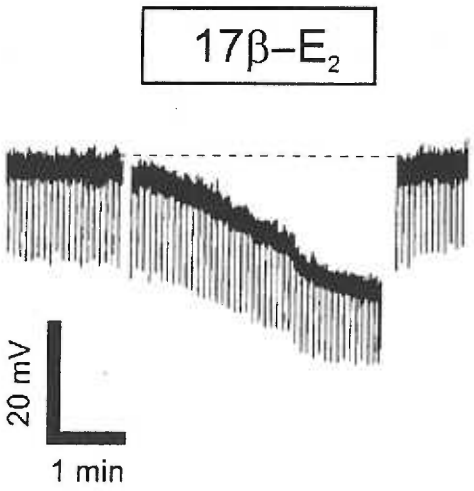

A
DAMGO
B
Voltage (mV)
-120
-80
0.05
0.00
-0.05
-0.10
Current (nA)
C
Naloxone
DAMGO
20 mV
1 min

FIGURE 4.3: 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 (Δ) E_2. E_2 caused a 2.0 nS conductance increase with a reversal potential near E_K (-100 mV).


17β–E2
20 mV
1 min

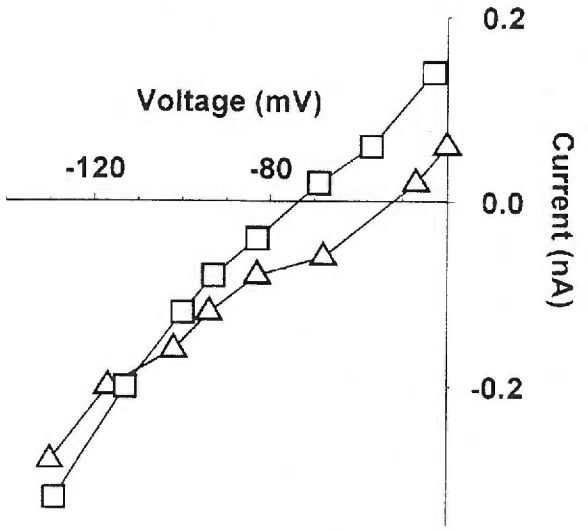

Voltage (mV)
-120
-80
0.2
0.0
-0.2
Current (nA)

FIGURE 4.4: Potency of DAMGO is not altered by E_2 in GnRH neurons

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, ○).

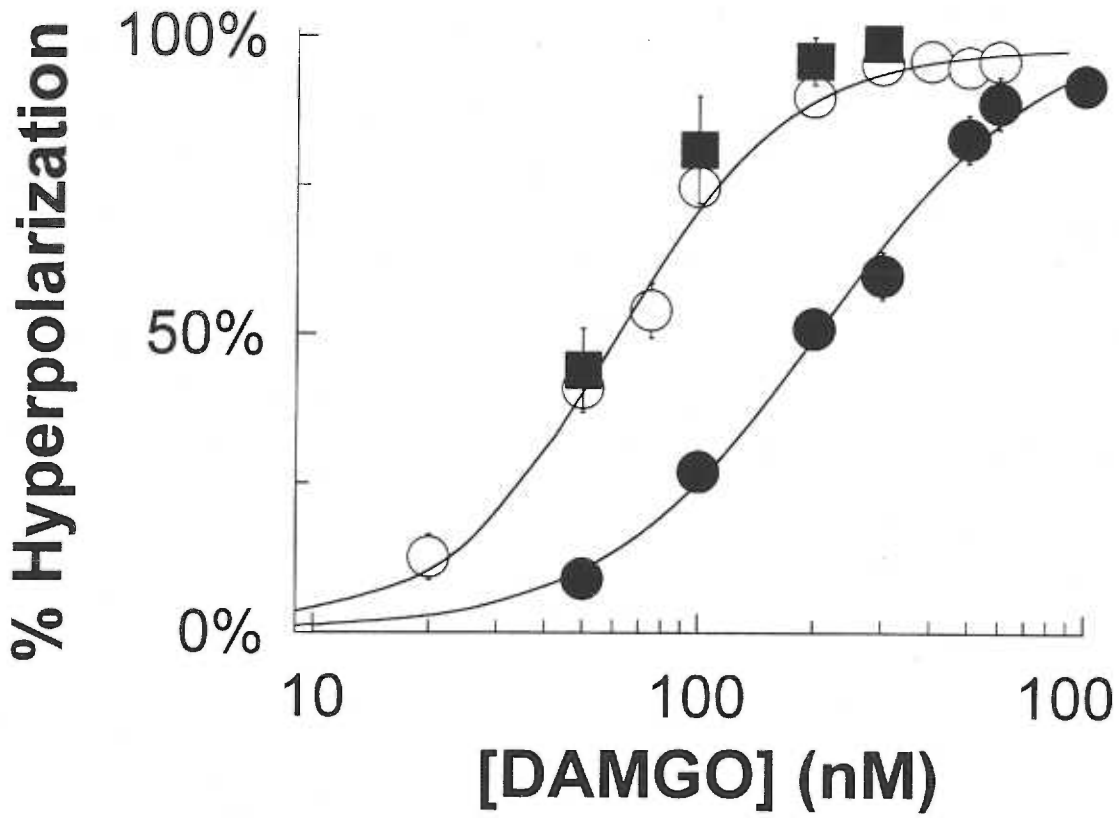
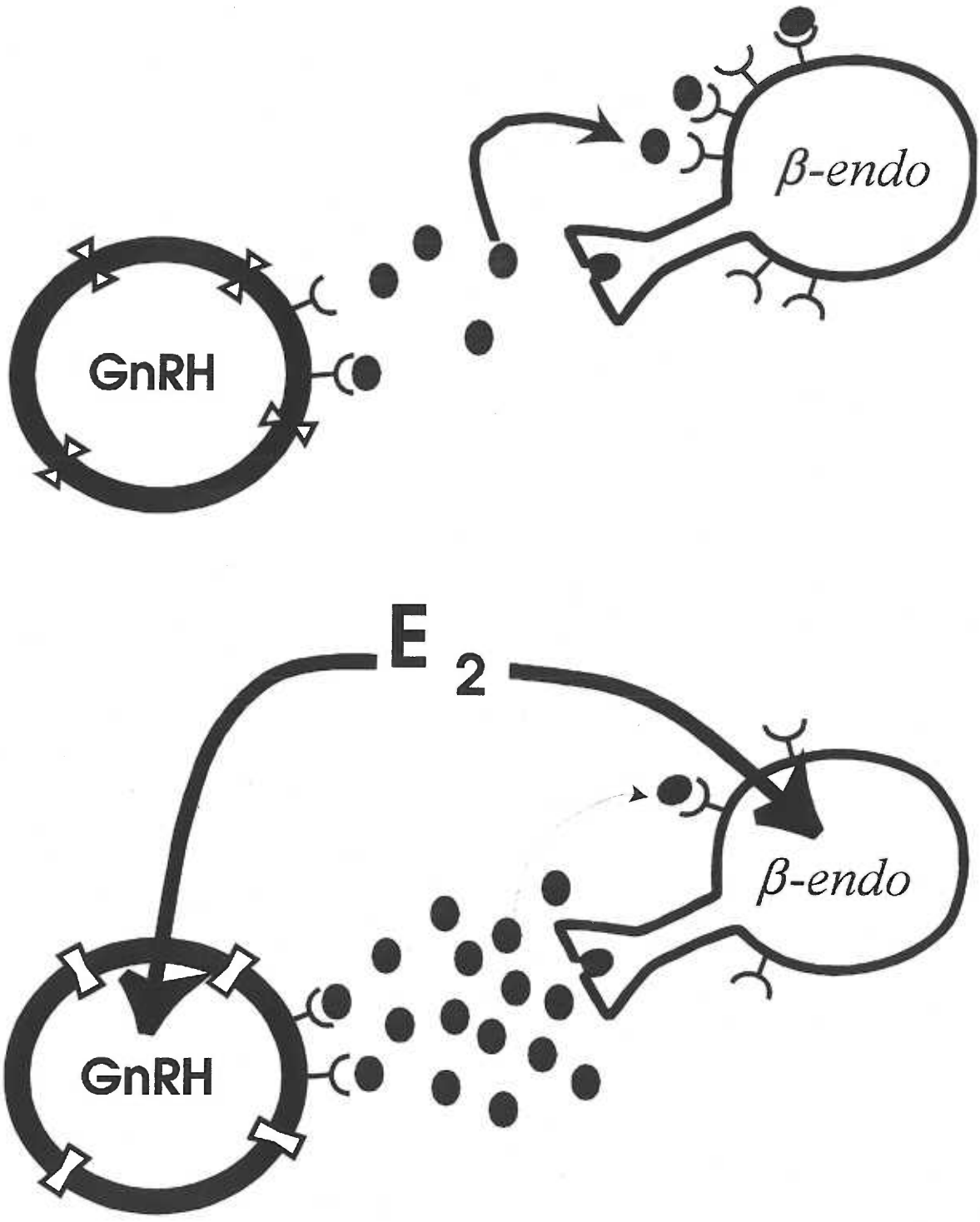

% Hyperpolarization
100%
50%
0%
10
100
100
[DAMGO] (nM)

FIGURE 4.5: 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.


β-endo
GnRH
E 2
β-endo
GnRH

DISCUSSION

Summary

17β-estradiol rapidly attenuates the ability of G-protein coupled receptors (μ-opioid and $GABA_B$) to hyperpolarize a subset of hypothalamic neurons. Estrogen's rapid effects are mediated by a specific intracellular receptor because they are not mimicked by the biologically inactive isomer 17α-E_2 or the membrane-impermeable BSA-17βE_2, and they are blocked by the antiestrogen ICI 164,384. Although the pharmacology of this effect is very similar to the classical estrogen receptor, there are enough differences that an isoform of the classical ER is likely to be involved. Estrogen's uncoupling of G-protein coupled receptors appears to involve activation of cAMP-dependent protein kinase. Although still very tentative, the most like site of receptor uncoupling is the interaction between the potassium channels and the G-proteins since $GABA_B$ receptors are simultaneously uncoupled by E_2 (see below). These rapid effects occur with a biologically-relevant concentration dependence, and so may have many physiological consequences. For example, β-endorphin neurons are subject to μ-opioid uncoupling by E_2. This would disrupt opioid autoinhibition and cause more β-endorphin to be released. Moreover, the μ-receptors on GnRH neurons are not rapidly uncoupled by estrogen. Therefore, consistent with *in vivo* studies, estrogenic modulation of endogenous opioids would rapidly inhibit GnRH neuronal activity.

Novel E_2 receptor or novel action of the classical estrogen receptor?

The present studies describe a novel effect of 17β-estradiol to alter the electrical activity of a subset of hypothalamic neurons. In less than 10 minutes, E_2 uncouples G-protein coupled receptors, such as μ-opioid and $GABA_B$ receptors, from their potassium channels. This effect is specific as it is not mimicked by the biologically-inactive isomer 17α-E_2, but is blocked by an antiestrogen (ICI 164,384). The effects of E_2 are concentration-dependent and more importantly, occur with physiologically-relevant E_2 concentrations. Because estrogen's regulation of protein synthesis occurs at lower potencies with a longer latency (Barnea, Gorski, 1970; Lieberman et al. 1978), the involvement of the classical estrogen receptor seems less likely. Furthermore, diethylstilbestrol (DES) antagonized the currently described phenomena and there was no apparent downregulation even after several hours of estrogen exposure. The most parsimonious interpretation of the data is that these actions are mediated by a novel intracellular receptor or isoform of the classical ER. However, we are unable to exclude the possibility that PKA activation represents a novel action for the classical ER.

There is growing evidence that the classical ER is a multifunctional protein with several functional domains. The AF-1 and AF-2 regions of this receptor are involved in ligand binding and receptor activation (Tzukerman et al. 1994). The AF-2 region is at the C-terminus and is most important in mediating estrogen's regulation of transcription at EREs (Tzukerman et al. 1994). The nonsteroidal estrogenic ligand tamoxifen antagonizes estrogen action via the AF-2 (Tzukerman et al. 1994). The functions of the AF-1 domain at the N-terminus of the ER are less well-understood. Both tamoxifen and estrogen are agonists for

the AF-1 mediated actions of the ER, including expression at AP-1 sites (Webb et al. 1995). These two domains may act either in concert or independently, depending on the ligand and conditions (Tzukerman et al. 1994). For example, tamoxifen is widely used to prevent growth of E_2-sensitive breast cancers because it antagonizes E_2 in the breast but acts as an agonist in uterus and bone, thereby precluding some of the negative side effects of more "pure" antiestrogens (Webb et al. 1995). Because the pharmacology and transduction mechanism of the classical ER is still somewhat unclear, it is impossible to distinguish the classical ER from a novel estrogen receptor with certainty.

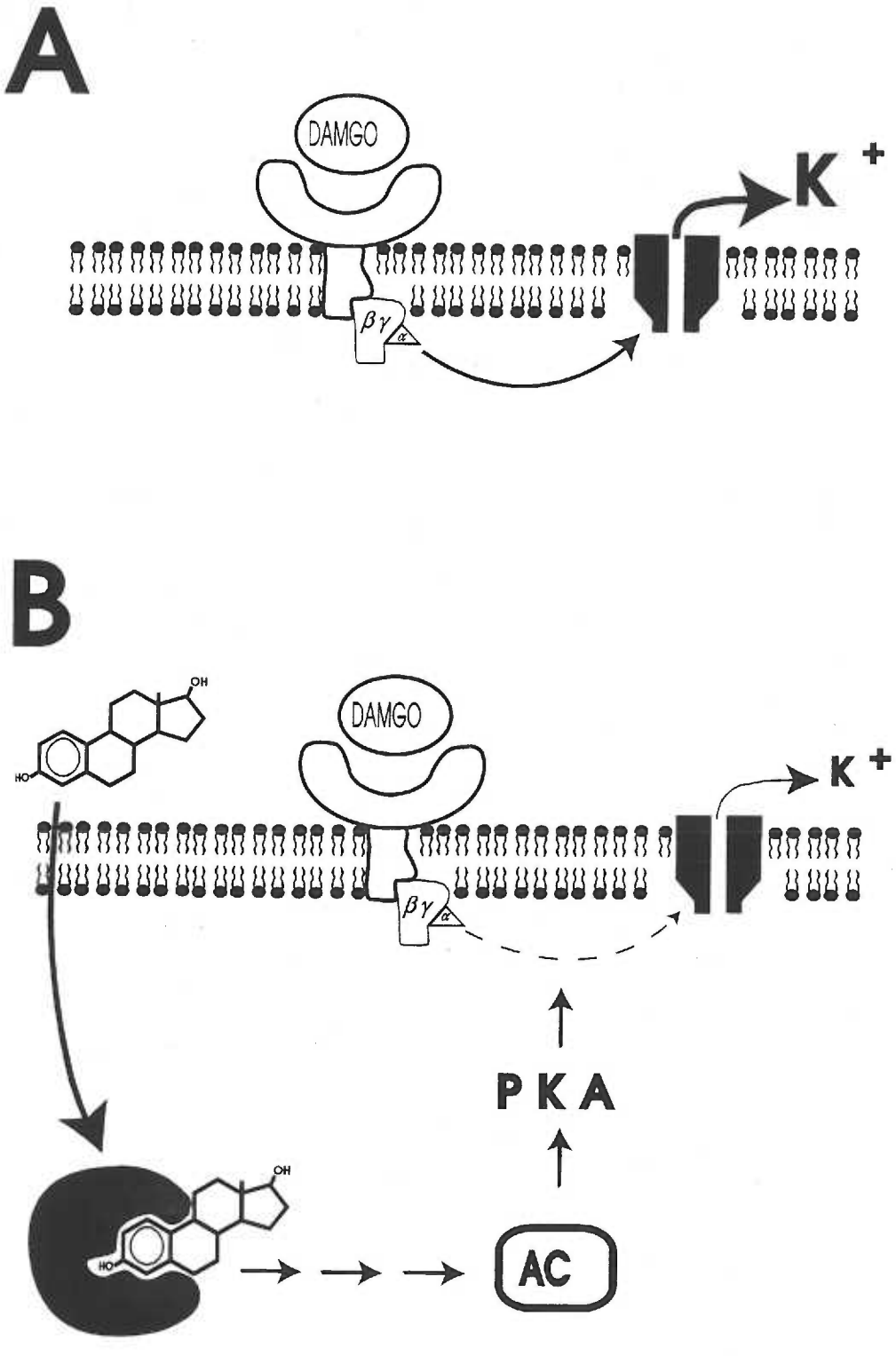


FIGURE 5.1: Subcellular model of estrogenic modulation of G-protein coupled receptors

Mechanism of E_2 action

Although it is clear that PKA mediates estrogen's rapid actions, the steps from estrogen binding a receptor to the activation of PKA remain unknown. Estrogen may directly stimulate the protein kinase, as has been shown for a mutant tyrosine kinase found in breast cancer cells (Matsuda et al. 1993). However, the ability of Rp-cAMP to reverse the effects of E_2 implies that increased cAMP levels are mediating the stimulation of PKA. A similar effect of E_2 to stimulate intracellular levels of cAMP is seen in the uterus (Aronica et al. 1994). Although those studies showed an estrogenic stimulation of adenylate cyclase, one must be cautious in drawing parallels because there are substantial differences between estrogen actions in the uterus and hypothalamus. Although the ability of E_2 to increase cAMP in primary uterine cell culture is insensitive to protein or RNA synthesis inhibitors, it requires a longer time course (30 - 60 min) than PKA activation in the hypothalamus (10 min). Furthermore, estrogen has a higher potency in the uterus (EC50 = 10 pM) and is mimicked by both ICI 164,384 and DES. These discrepancies may reflect differences in the cellular regulation of the estrogen receptor (i.e., as seen for tamoxifen agonism/antagonism), but they may also suggest that activation of PKA by E_2 is mediated by distinct receptors with different mechanisms of action. For example, although uterine effects are mediated by stimulation of adenylate cyclase, the actions in the hypothalamus may involve inhibition of phosphodiesterase. Moreover, although estrogen may interact directly with adenylate cyclase or phosphodiesterase, other enzyme systems may also mediate estrogenic stimulation of PKA. In fact, preliminary studies have shown that stimulation of PKC with 1 μM PDBu can partially mimic the actions of E_2 and the effects of E_2 can be blocked by the PKC

inhibitor Calphostin C (See Appendix A). Thus the capacity of E_2 to stimulate cAMP may be one component of a complex web of intracellular enzymes. However, much work needs to be done to confirm a role for PKC and other enzymes in mediating E_2 actions. After more than 30 years, the mechanisms of estrogen's genomic actions are still being defined, and there is no reason to believe the other actions of this steroid are any less complex.

The estrogen receptor is one component of a complex intracellular machinery

It is becoming clear that estrogen functions as only one node in a complex web of intracellular systems. The numerous interactions with other effector systems serve to greatly expand the diversity of estrogen action. For example, besides actions at their own response elements (e.g., CREs) (Hagiwara et al. 1993), protein kinases substantially alter the genomic functions of steroid hormone receptors (Le Goff et al. 1994). The estrogen receptor is a phosphoprotein whose transcriptional activity is greatly attenuated by mutating a few key serines to alanines (Le Goff et al. 1994). Stimulation of PKA results in phosphorylation of the ER that is probably mediated by a proline-directed- or DNA-dependent protein kinase (Arnold et al. 1995; Le Goff et al. 1994). Furthermore, PKA activation alters the function of the ER in such a way that the antagonist tamoxifen becomes a partial agonist that is able to stimulate transcription at EREs. At the same time, the ability of ICI 164,384 to inhibit E_2 action is unchanged (Fujimoto, Katzenellenbogen, 1994; Beck et al. 1993; Sartorius et al. 1993). Furthermore, this effect is cell-type and promoter-specific, implying the involvement of other intracellular systems. On a physiological level, although many breast cancer patients may initially respond well to tamoxifen suppression. However, after a few months

to years this drug not only loses its effectiveness, it actually stimulates growth of the cancerous cells (Horowitz, 1995). Thus, regulation of ER function by intracellular cascades may play an important part in the diverse effects of estrogen under different conditions in various tissues. There is even evidence that protein kinase regulation of steroid receptors can activate those proteins in the absence of their steroid ligands (Smith et al. 1993; Denner et al. 1990; Power et al. 1991). For example, dopamine can cause increased expression at ERE-driven genes. This effect apparently involves activation of the ER because it is abolished by either addition of ICI 164,384 or mutation of a single amino acid in the ER from a glycine to a valine (Smith et al. 1993). There is similarly, extensive interaction between dopaminergic systems and progesterone receptors. Dopamine agonists can stimulate progesterone-dependent behaviors without added steroid, but are nonetheless readily inhibited with progesterone antagonists (Mani et al. 1994). Thus protein kinases may modulate steroid hormone actions, and may even use the steroid effector system in the absence of steroid. The converse idea that steroids can alter protein kinase function is currently being explored in several laboratories. Although phosphorylation of the ER is stimulated by ligand binding (Lahooti et al. 1994), the possibility that protein kinases may mediate the genomic actions of E_2 remains unproven. Both estrogen and estrogen antagonists (ICI 164,384 and tamoxifen) stimulate ER phosphorylation, and it is unclear whether this binding activates kinase activity or places the receptor in a conformation that is more easily phosphorylated. There is, however, recent evidence that steroids can alter the ability of protein kinases to affect other systems. By altering transcription of protein kinases and other effector systems, steroid may help regulate other intracellular systems (Maeda,

Lloyd, 1993). Alternatively, E_2 causes a cAMP-mediated increased expression at CRE-promoters (Aronica et al. 1994) that is not blocked with protein synthesis inhibitors. Thus, the ability of the classical estrogen receptor to interact with protein kinases greatly expands the activity of both systems.

Site of μ-opioid uncoupling?

It remains to be determined how the intracellular actions of E_2 and PKA regulate membrane-delimited G-protein coupled receptors. Although this dissertation did not specifically address this issue, preliminary data suggests that the uncoupling of G-protein receptors may involve alteration of the coupling of the G-proteins to the potassium channels. We have shown that E_2 does not alter the affinity of the μ-opioid receptor for antagonists. Therefore, E_2 does not appear to be competing with DAMGO for the μ-opioid receptor or altering the ability of μ-receptors to bind ligands. We have not tested the possibility that E_2 may alter the number of spare receptors, but the fact that the Hill slope is unchanged by E_2 would argue against a reduction in spare receptors (Taylor, Insel, 1990). To further elucidate the site of receptor uncoupling, we made use of the fact that the $GABA_B$ and μ-opioid receptors are linked to the same potassium channel (Loose et al. 1991). We found that the $GABA_B$ receptors were similarly modulated by estrogen, and in fact, both μ-opioid and $GABA_B$ receptors were attenuated simultaneously and to the same degree in the same cells. Although it is certainly conceivable that E_2 acts at multiple sites, the most parsimonious explanation is that some common effector is altered. Even though the two receptors appear to activate the same population of potassium channels, E_2 does not seem to directly alter

membrane conductances. The degree of rectification was not altered, neither were the ΔV_{max} or Δg_{max} induced by DAMGO or baclofen. Although controversial, it has been postulated that activation of an antagonistic conductance could reduce the apparent sensitivity the μ-opioid receptor (Alreja, Aghajanian, 1993). This does not seem to be happening in this system as the reversal potential for both μ-opioid and $GABA_B$-mediated hyperpolarization is unchanged by E_2. Therefore, it appears that E_2 may alter the activation of the I_K by G-proteins either by actions at the channel or by affecting a common pool of G-proteins. To help exclude the possibility that E_2 is down-regulating μ-opioid receptors, Furchgott analysis with β-FNA to antagonize DAMGO actions in control and E_2-treated cells should be done (Furchgott, Bursztyn, 1967). Further elucidation of the exact site of E_2 action will require more artificial systems in which G-proteins may be introduced and the structure of the potassium channel may be manipulated. For example, the μ-opioid receptor, brain potassium inward-rectifier, and isoforms of the estrogen receptor could be expressed in oocytes (Chen, Yu, 1994; Henry et al. 1994). This would allow both introduction of various proteins (such as various isoforms of G_i or PKI), as well as mutagenesis of the potassium channel to pinpoint the site of E_2 action. Although the present work provides some insight into the actions of steroids and protein kinases to modulate G-protein coupled receptors, further work is needed to pinpoint the site of receptor/K^+ channel uncoupling.

Physiological significance of rapid and genomic E_2 action

The rapid effects of E_2 complement what is known about the genomic actions of this steroid and serve to expand the repertoire of estrogen action. For example, a brief (20 min)

exposure to E_2 uncouples μ-opioid receptors in a subset of β-endorphin neurons, but not in GnRH cells. However, *in vivo* estrogen treatment for 24 hours attenuates the μ-opioid response in all arcuate hypothalamic neurons. It remains to be determined whether the effects at 24 hours are a consequence of the rapid E_2 actions (e.g., desensitization secondary to increased β-endorphin release) or a qualitatively different mechanism. One way to differentiate these possibilities would be to treat *in vivo* for 24 hours with DES. This compound is a potent estrogen agonist for the genomic effects of that steroid, but antagonizes the current phenomena. Therefore, if DES mimics the 24 hour actions of E_2 , this would imply that E_2 actions at 24 hours and 20 minutes are via a different mechanisms. The qualitatively different actions of E_2 may be interdependent, as well as complementary. In fact, long-term E_2 treatment substantially alters synaptic connectivity in the hypothalamus (Naftolin et al. 1993) and some steroid effects are lost in long-term ovariectomized animals and only regained following prolonged EB treatment (Resko et al. 1977).

These multifaceted alterations of μ-opioid response are consistent with the previously described ability of estrogen to regulate the reproductive axis in a complex, opioid-dependent fashion (Ferin et al. 1984). When E_2 is injected into ovariectomized monkeys, there is a rapid reduction in GnRH release, followed approximately 36 hours later by a synchronized release of GnRH (Levine et al. 1985). In intact animals and humans, E_2 levels rise quickly to very high levels the day before the LH surge and if naloxone is given at this time, the LH surge is advanced. Therefore, the rapid effects of estrogen may be involved in the preovulatory inhibition of LH release which may restrain the LH surge until the rest of the HPG axis is ready for ovulation. By disrupting an ultrashort loop negative feedback on β-

endorphin cells, E_2 increases β-endorphin release (Frautschy, Sarkar, 1995) and inhibits GnRH cells. However, a day later the ability of β-endorphin to inhibit GnRH release is also reduced (Kelly et al. 1992), allowing stimulatory inputs to cause an LH surge.

The significance of estrogenic uncoupling of $GABA_B$ receptors is less clear because very little is known about the role of GABA in the reproduction axes of either the guinea pig or primate. However, this amino acid is the predominant inhibitory neurotransmitter in the hypothalamus and throughout the CNS (Decavel, Van den Pol, 1990; Krnjevic, 1974). Nearly every hypothalamic neuron receives GABAergic synapses (Decavel, Van den Pol, 1990), including cells containing GnRH (Leranth et al. 1985), β-endorphin (Horvath et al. 1992) and even GABA itself (Horvath et al. 1992). Therefore, estrogenic modulation of GABAergic inhibition has the potential for broad repercussions that merit further investigation.

Modulation of β-endorphin neurons may have widespread consequences

The present work focused on the ability of E_2 to alter μ-opioid potency in β-endorphin neurons, but other cell types may be similarly regulated. Moreover, since estrogenic control of μ-opioid potency may alter β-endorphin release (Frautschy, Sarkar, 1995), even those cells that are not directly sensitive to E_2 may be affected. For example, prolactin release from the anterior pituitary is subject to tonic inhibition by dopamine released into the portal blood by hypothalamic arcuate neurons (Neill, 1988). These dopaminergic cells are inhibited by μ-opioid receptor activation (Loose et al. 1990), and the release of prolactin is sensitive to naloxone in an estrogen-dependent fashion (Melis et al. 1985; Cetel et al. 1985).

Hypophyseal dopamine levels are low at the time of the E_2-induced rise in prolactin (de la Escalera, Weiner, 1992), and estrogen rapidly decreases the activity of dopaminergic neurons (Pasqualini et al. 1991).

Since hypothalamic β-endorphin neurons send projections throughout the brain to regulate a variety of functions (Bloom et al. 1978), alteration of the μ-opioid response might not be limited to hypothalamic control of pituitary function. For example, projections to the ventral tegmental area (VTA) are thought to modulate reward circuits and may therefore be involved in regulating motivation. As expected from the addictive nature of opiate drugs like morphine, intracerebroventricular injection of β-endorphin or injection of more selective μ-opioid agonists into the VTA are strongly rewarding in conditioned place preference paradigms (Devine, Wise, 1994; Amalric et al. 1987). The opioid antagonist naloxone is aversive in those same paradigms (Mucha, Herz, 1985). Furthermore, destruction of endogenous opioid input by MBH lesions abolishes the aversive properties of naloxone. without altering the rewarding properties of morphine (Mucha et al. 1985). Thus, the endogenous opioid pathways may modulate the reward pathways that help determine behavioral motivation (Koob, 1992). Endogenous opioids also alter pain transmission from the midbrain to the spinal cord (Koob, 1992). Intracerebral injection of opioid peptides raises the threshold for nociception without altering other sensations, such as touch and temperature sensitivity (Loh et al. 1976; Koob, 1992). Since nearly all of the β-endorphin in the fore- and midbrain is derived from projections from hypothalamic neurons (Bloom et

al. 1978), estrogenic regulation of these cells may have far-reaching implications on a variety of neuronal systems.

Estrogenic control of G-protein coupled receptors and the role of PKA

Both μ-opioid and $GABA_B$ receptors are uncoupled by E_2, therefore other G-protein coupled receptors may be similarly regulated. For example, dopamine D_2 receptors are coupled to many of the same effector systems as μ-opioid and $GABA_B$ receptors (inhibition of adenylate cyclase, activation of potassium channels) (Albert et al. 1990; Lacey et al. 1988). Chronic estrogen reduces the D_2-receptor mediated inhibition of adenylate cyclase, without altering the expression of dopamine D_2 receptors, G-proteins or adenylate cyclase (Maus et al. 1990). Instead, the ability of pertussis toxin to ribosylate $G_{i,o}$ is increased, implying that E_2 somehow stabilizes the trimeric, unstimulated form of these proteins (Maus et al. 1990). Estrogen also alters the response of hypothalamic cells to norepinephrine (NE). In the ovariectomized state (low E_2), β-adrenergic receptor function predominates and serves to inhibit the reproductive axis (Etgen et al. 1992). Following two days of E_2 treatment, β-adrenergic receptors are uncoupled (Ungar et al. 1993), and the effects of NE are mediated primarily by α-adrenergic receptors which stimulate GnRH release (Etgen, Petitti, 1987). Similar actions are seen at the cellular level in which the β-adrenergic agonist isoproterenol inhibits and the α-adrenergic agonist methoxamine excites hypothalamic neuronal firing (Condon et al. 1989). The balance of these two receptors is shifted to a stimulatory/α-adrenergic effect following 36 hours of estrogen treatment (Condon et al. 1989). Thus in both cellular and *in vivo* studies, estrogen changes NE from being a predominantly inhibitory

transmitter to an excitatory neurotransmitter (Etgen et al. 1992). These effects require 24 - 48 hours to become manifested, consistent with a stimulatory role in the E_2-induced LH surge. Perhaps estrogen also produces a rapid estrogenic modulation of adrenergic responses that complements the slower actions on this system. Alternatively, perhaps the time-dependent estrogenic control of the hypothalamus involves the selective uncoupling of different receptors at different times. For example, μ-opioid receptors inhibit adenylate cyclase (Carter, Medzihradsky, 1993), while hypothalamic β-adrenergic receptors stimulate the same enzyme in an E_2-sensitive manner (Etgen et al. 1992). Perhaps acute estrogen uncouples opioidergic inhibition of adenylate cyclase, leaving adrenergic stimulation of this enzyme unchanged. Several hours later, a different mechanism of estrogen action could uncouple adrenergic stimulation. This would result in an acute stimulation of PKA that after several hours would return to baseline or even be inhibited.

Uncoupling is a ubiquitous mechanism of control for G-protein receptors

Modulation of receptor potency is a widespread mechanism for regulating receptor function, allowing the cell to adapt its response to the availability of ligand (Dohlman et al. 1991). This process maintains homeostasis when receptors are excessively stimulated, whether this is due to exogenous drugs (e.g., tolerance to chronic morphine) (Andrade et al. 1983) or endogenous overproduction (e.g., adrenergic desensitization in pheochromocytoma) (Hamada et al. 1995). There are many components to this phenomena including internalization and down-regulation of the receptors. These slower effects are complemented by a more rapid desensitization in which the receptor is uncoupled from its effector systems

(Dohlman et al. 1991). For example, the μ-opioid coupling to its potassium channel has been shown to rapidly desensitize following exposure to high levels of an agonist (Harris, Williams, 1991). The mechanism of this desensitization remains unclear (Harris, Williams, 1991; Kovoor et al. 1995; Mestek et al. 1995), but work on the β-adrenergic receptor has implied that multiple protein kinases may be involved (Pitcher et al. 1992). Furthermore, modulation of receptor function may alter multiple proteins within that receptor's intracellular messenger system(s). For example, some receptors may interact with multiple effector systems via numerous G-protein subtypes and these responses may be differentially regulated during prolonged agonist exposure. Stably transfected A_3 adenosine receptors couple to both endogenous $G_{i\alpha\text{-}2}$ and $G_{i\alpha\text{-}3}$, resulting in inhibition of adenylate cyclase. Prolonged agonist exposure decreases agonist potency to inhibit adenylate cyclase. Western blot analysis using specific antibodies show that although $G_{i\alpha\text{-}3}$ and G_{β} proteins are down-regulated, there is no change in the $G_{i\alpha\text{-}2}$ levels (Palmer et al. 1995). Alternatively, in systems where two receptors are coupled to the same effector system, chronic stimulation of one receptor may result in heterologous desensitization, causing the response of the other receptor to be attenuated as well (Kalso et al. 1993; Nah et al. 1993). These interactions can become even more complex when considering the fact that different receptors can share multiple effector systems and that a given agonist may activate multiple receptors. For example, neuronal activity in the locus coeruleus is regulated by both μ-opioid and adrenergic receptors. Both μ-opioid and α_2-adrenergic receptors activate $I_{K(ir)}$ (North, Williams, 1985, Aghajanian, Wang, 1987) and chronic treatment with the μ-opiate, morphine, causes tolerance to the μ-opioid induced hyperpolarization, without altering the

α_2 response (Christie et al. 1987). However, these cells also contain β-adrenergic receptors that stimulate adenylate cyclase (Nestler, 1992), while μ-opioid receptors inhibit that enzyme (Carter, Medzihradsky, 1993). The response of locus coeruleus neurons to chronic morphine is to upregulate the expression of adenylate cyclase and PKA, thereby maintaining some of the response of PKA to β-adrenergic stimulation despite inhibition by morphine (Nestler, 1992).

Estrogenic regulation of μ-opioid receptors may be seen as yet another level of receptor modulation in which a third, unrelated signal causes uncoupling. $GABA_B$ and $5HT_{1A}$ receptors are coupled to the same potassium channels in CA1 neurons (Andrade et al. 1986), and chronic cortisol treatment causes a selective attenuation in the serotinergic response (Joëls et al. 1991). In contrast, the present studies show that both components (i.e., μ-opioid and $GABA_B$) of a shared system can be attenuated simultaneously. Moreover, analogous to the situation with chronic morphine, steroid actions may reorganize the intracellular effectors being regulated by these receptors. Besides opening potassium channels, both μ-opioid and $GABA_B$ receptors are known to be coupled to calcium channels and adenylate cyclase. It is conceivable that E_2 might selectively uncouple these receptors from one effector (i.e., I_K) but not the others. Such a scenario might allow E_2 to decrease the ability of μ-opioid to affect electrical activity without altering opioidergic/GABAergic regulation of genomic expression at CREs.

PKA may be a site of synergistic actions between steroids and neurotransmitters

Since many G-protein coupled receptors regulate PKA activity, these receptors may uncouple <u>other</u> G-protein coupled receptors. For example, catecholaminergic fibers have

been shown to synapse on GnRH (Hoffman et al. 1982), GABA (Leranth et al. 1988) and β-endorphin cells (Horvath et al. 1992) and so could potentially alter the physiology of these cells. Furthermore, since both norepinephrine (Etgen et al 1992) and estrogen stimulate PKA, there may be cross-talk between these two systems. Although estrogen rapidly stimulates PKA activity, levels of this hormone in the hypothalamus are not thought to fluctuate widely from minute to minute (Fritz et al. 1992). Furthermore, estrogen concentrations within the hypothalamus are estimated to be 5-10 nM (Eisenfeld, Axelrod, 1965), well below the concentrations needed for maximal stimulation of PKA. Therefore, while estrogen may provide tonic modulation of PKA activity, transient adrenergic input from the brainstem may allow for a more rapid and transient regulation of PKA activity, with subsequent attenuation of μ-opioid response.

Other rapid steroid actions

Although the genomic model of steroid action still predominates, it has recently been complemented with advances in our understanding of some of the other mechanisms of steroid action (McEwen, 1991). For example, estrogen has been shown to directly alter potassium conductances in both hypothalamic and amygdala neurons (Kelly et al. 1977; Nabekura et al. 1986). In addition to steroids synthesized in the gonads and adrenal glands, steroids are synthesized within the CNS *de novo* from cholesterol, mostly by glial cells (Corpéchot et al. 1993). The predominant species of these "neurosteroids" are derivatives of pregnenolone, progesterone, dihydroepiandrostenedione (DHEA) and corticosterone. The levels of neurosteroids can reach 100 nM in the brain (Corpéchot et al. 1993). One well-

characterized action of these progesterone derivatives is the modulation of $GABA_A$ channel activity, similarly to barbiturates and benzodiazapines (Majewska, 1991). Thus, steroids produced in the brain may be anxiolytic and function as a "natural valium". For example, alphaxolone (5α-pregnane-3α-hydroxy-11,20 dione) is a progesterone derivative that was used as a general anesthetic for years in Great Britain (Child et al. 1971). This compound appears to mimic many actions of barbiturates to alter $GABA_A$ channel function. Both alphaxolone and pentobarbital increase muscimol binding and the potency and efficacy of muscimol-induced chloride flux. Conversely, both compounds inhibit the binding of the $GABA_A$ pore-blocker TBPS. However because the effects of these two compounds are additive on both muscimol binding and activity, they appear to be working at different sites on the $GABA_A$ receptor protein (Turner et al. 1989). Clinically, alphaxolone has the advantage over benzodiazepines and barbiturates of having a more rapid onset and recovery (Hogskilde et al. 1987). Conversely, some naturally occurring neurosteroids seem to act as $GABA_A$ receptor antagonists. Pregnenolone sulfate and DHEA-sulfate both act to inhibit $GABA_A$ channel function, albeit with a lower, micromolar potency (Majewska, 1991). More recently, it has become clear that neurosteroids may have other means to modulate neuronal activity, specifically inhibition of Ca^{++} currents (Ffrench-Mullen et al. 1994) and potentiation of the NMDA responses (Bowlby, 1993). In CA1 hippocampal neurons both N- and L-type Ca^{++} currents are inhibited more than 50% by pregnenolone sulfate. This effect is thought to involve an extracellular receptor because it is not seen when pregnenolone sulfate is applied intracellularly via the recording pipette. Furthermore, this receptor may be G-protein coupled because this effect is blocked by pertussis toxin treatment or addition of GTPβS to

pipette. Finally, the effects of pregnenolone sulfate are reduced by PKC inhibitory peptide and bisindolylmaleimide, but not by PKA inhibitors thus implying that these receptors may activate PKC. Potentiation of the NMDA current is not pertussis toxin-sensitive and requires micromolar concentration of pregnenolone sulfate. Therefore, steroids in the brain may work through different receptors and have diverse actions to alter neuronal excitability.

Although initially a novel finding, the rapid effects of E_2 in the hypothalamus are becoming one component of a rapidly developing field of nonclassical steroid effects (McEwen, 1991). These actions include non-ERE mediated actions of the classical estrogen receptor, interactions with other intracellular effector systems, and even the discovery of new receptor proteins. However, our understanding of physiological significance of these actions has been limited by the relative paucity of studies investigating the pharmacology and intracellular mechanisms of these effects. Thus, these recent discoveries have greatly expanded our view of steroid action: but at the same time it has become less clear how these diverse actions work together to regulate cellular physiology. The presently described estrogenic uncoupling of G-protein receptors via PKA activation is consistent with the known physiology of E_2 to alter the reproductive axis. Furthermore, alteration of both opioid and nonopioid-systems in the hypothalamus and throughout the CNS may have diverse repercussions. However, much work remains to be done to define the mechanism and physiological significance of the rapid actions of estrogen and other steroids.

Future Experiments

Although the currently described work elucidates many aspects of rapid E_2 action, it

leaves many questions.

1) Are the rapid actions of estrogen truly nongenomic?

Although estrogen can induce mRNA synthesis within two minutes (Maurer, 1982), it seems highly unlikely that a protein could be transcribed, post-translationally modified and correctly trafficked within the timecourse observed here (10 - 20 minutes). However, this possibility should be excluded using a protein synthesis inhibitor like cycloheximide (Karst, Joëls, 1991). These experiments would entail pre-treating the slices with an appropriate concentration of cycloheximide, followed by a DAMGO dose-response curve, and then another DAMGO dose-response curve following E_2 + cycloheximide.

2) What is the nature of the E_2 receptor?

a) The BSA-E_2 conjugate mimics E_2 action at multiple extracellular receptors (Tesarik, Mendoza, 1995; Pappas et al. 1995), however it is conceivable that it is not biologically active at this particular receptor. This problem could be addressed by including BSA-E_2 in the pipette. These experiments were not done previously because a negative result would have been difficult to interpret. The BSA-E_2 conjugate may unable to reach the estrogen either because of the subcellular localization of the receptor (e.g., the nucleus) or even the inability of this large conjugate to diffuse out of the pipette. Another possibility would be to repeat the studies using estrone-sulfate both extracellularly and within the pipette.

b) Immunocytochemistry or *in situ* hybridization could be used to learn whether the classical estrogen receptor or some isoform of it is expressed in E_2-sensitive

neurons. Although these studies would be correlative, they would allow us to detect whether these cells have the intracellular machinery for a classic ER-mediated action. Unfortunately, identification of E_2-sensitive neurons requires exposure of the slices to E_2 that down-regulates the ER (Meredith et al. 1994; Gibson et al. 1991). Thus, our initial attempts to stain for the ER were unsuccessful. Although ER stain was robust in control slices, there was no stain in the E_2-treated tissue. Alternatively, we are currently developing *in situ* techniques that may be able to selectively detect either the classical ER or some isoform thereof, even after E_2 (Skipper et al. 1993; Friend et al. 1995).

c) Compare the actions and potency of 17β-estradiol with other estrogens (e.g., estriol and estrone) and other steroids (progesterone, dihydrotestosterone and cortisol).

d) Determine whether the nonsteroidal antagonist/partial agonist tamoxifen mimics or blocks the effects of E_2. These experiments were not done previously because this compound interacts with two components of our system, a protein kinase and an ion channel (Hardy, Valverde, 1994; Zerr, Feltz, 1994; Eyster, Clark, 1989).

2) What are the other components of the rapid E_2-stimulated intracellular cascade?

a) We need to further explore the possible involvement of PKC. The first experiment to do would be to see in the biologically inactive phorbol ester 4α Phorbol, 12, 13 Didecanoate mimics the effects of PDBu. Subsequently, we would need to inhibit the effects of E_2 with a selective PKC antagonist that is structurally and mechanistically dissimilar from Calphostin C.

b) The involvement of other serine kinases (e.g. CaM kinase, MAP kinase) should

also be investigated using appropriate inhibitors.

c) Other possible cascades tyrosine kinases, which we could try to inhibit with some broad spectrum tyrosine kinase inhibitor, such as Tyrphostin B46 or B 48. Nitric oxide may be involved, so E_2 could be superfused along with the nitric oxide synthetase inhibitor, N-methyl arginine. Finally, indomethacin could be used to block prostaglandin production.

3) What is the role of these physiological E_2 actions in modulating hypothalamic output?

We have proposed a model for rapid estrogenic regulation of opioid and GnRH release based on the present data. This model should be tested by measuring the release of GnRH and β-endorphin from *in vitro* hypothalamic slices. Estrogen would be expected to rapidly increase β-endorphin release and inhibit GnRH release in a naloxone-reversible manner. Furthermore, unlike other estrogenic effects, DES should antagonize these actions.

4) What other cell types are also rapidly modulated by E_2?

The hypothalamus contains many different cell types with a variety of functions. Identification of the other cell types that are E_2-sensitive would help us gain a better understanding of the physiological significance of estrogenic actions.

a) GABAergic neurons contact nearly every cell in the hypothalamus (Decavel, Van den Pol, 1990) and E_2 rapidly uncouples $GABA_B$ receptors. Therefore, identification of GABAergic neurons among E_2 sensitive cells would imply a disruption of GABAergic autoinhibition, analogously to the β-endorphin cells.

b) Control of the reproductive axis includes complex interactions between progesterone and estrogen (Knobil, 1974) and nearly every expressing the PR also contains the ER (Blaustein, Turcotte, 1989). Therefore, by staining for the progesterone receptor (PR), we may help elucidate this interaction. In preliminary studies, we have found that 4 out of 10 cells that stained for the progesterone receptor were E_2-sensitive.

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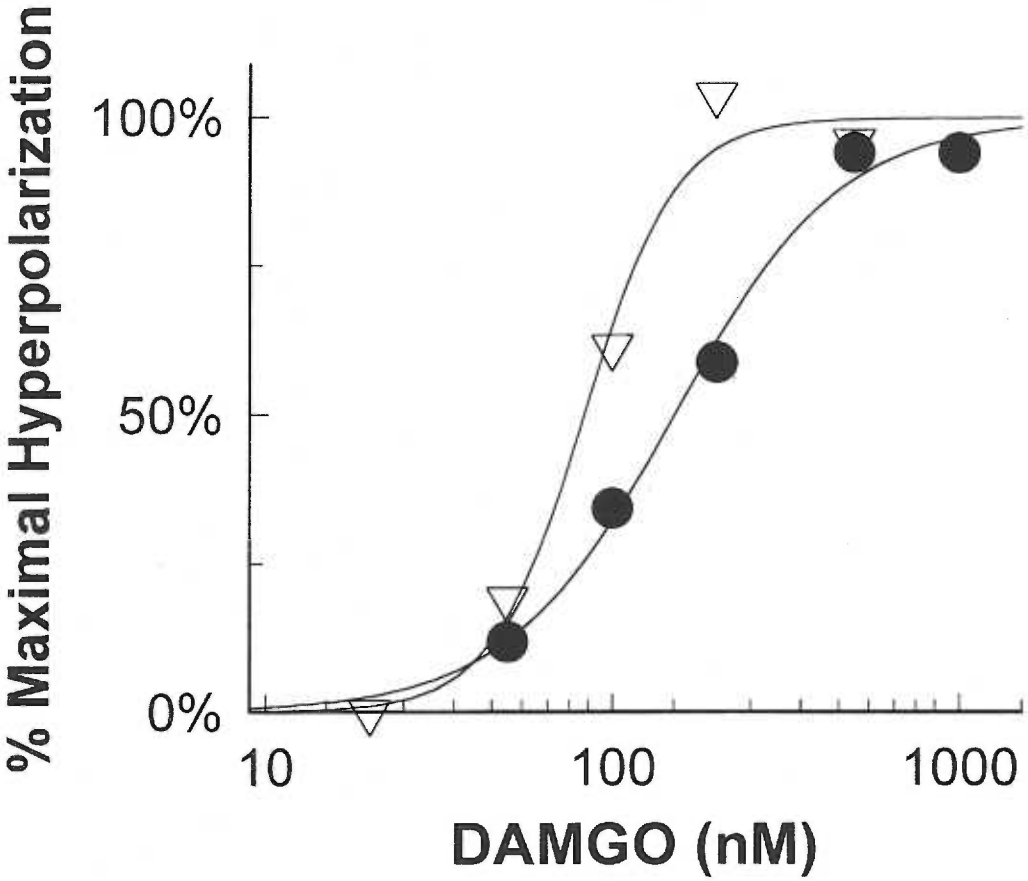
APPENDIX A

Preliminary evidence for a role for PKC in E_2's rapid effects

FIGURE 6.1: The selective protein kinase C (PKC) inhibitor blocks estrogen action.

Calphostin C was superfused under room light at 50 nM for 12 minutes, followed by Calphostin C + 100 nM E_2 for 18 minutes. This concentration was chosen to approximate the K_i of Calphostin C for PKC (Kobayashi et al. 1989). The resulting DAMGO concentration-response curve (DAMGO EC_{50} = 53 ± 7 nM, n=8) was not different from untreated controls. The DAMGO EC_{50} in this representative cell was 83 nM following Calphostin C + E_2 (▽). This cell was subsequently shown to be an E_2-sensitive neuron by superfuing E_2 (100 nM) alone. Following this treatment, the DAMGO EC_{50} was increased to 151 nM (●). The maximal hyperpolarization induced by DAMGO after Calphostin C + E_2 and after E_2 alone were -10 and -9 mV, respectively.

Conversely, the PKC stimulator PDBu (1 μM) mimicked the effects of estrogen (DAMGO EC50 = 135 nM, n =2 or 3 cells tested).


% Maximal Hyperpolarization
100%
50%
0%
10
100
1000
DAMGO (nM)