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Acute, Nongenomic Actions of the Neuroactive Gonadal Steroid, 3α-Hydroxy-4-pregnen-20-one (3αHP), on FSH Release in Perifused Rat Anterior Pituitary Cells

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We have previously shown that the gonadal and neurosteroid, 3α-hydroxy-4-pregnen-20-one (3αHP), can selectively suppress gonadotrophin-releasing hormone (GnRH) induced follicle-stimulating hormone (FSH) release from static cultures of anterior pituitary cells during a 4-h incubation period. The actions appeared to be at the level of the gonadotroph membrane and the cell signaling pathway involving Ca^{2+} and protein kinase C (PKC). In order to investigate further if the effects of 3αHP on FSH release are generated by nongenomic mechanisms, we monitored the short-term effects of 3αHP using dispersed anterior pituitary cells in a low dead-volume perifusion system with short (≤5 min) exposures to the steroid. Pulses of GnRH (10^{-8} or 10^{-7} *M*) lasting 2–5 min resulted in marked peaks of FSH release, and the variation in FSH amounts released from the cells in a particular column were minimal if the interval between successive GnRH pulses was at least 3–4 h. A 5-min pulse of 3αHP (10^{-9} *M*) administered simultaneously with the GnRH pulse suppressed GnRH-induced FSH release. On the other hand, similar treatment with the stereoisomer 3β-hydroxy-4-pregnen-20-one (3βHP), had no effect, but progesterone and estradiol pulses augmented the GnRH-induced FSH release. Pretreatment of cells with a 5-min pulse of 3αHP, at 120, 60, or 30 min prior to a GnRH pulse suppressed the GnRH-induced FSH release. The suppression of GnRH-induced FSH release by 3αHP was only partial if the start of the 3αHP pulse occurred 0.5 or 1.0 min after the start of the GnRH pulse, and no suppression occurred if the start of the 3αHP pulse was delayed by 2–5 min. The FSH release elicited by 5-min pulses of the Ca^{2+} ionophore A23187, the Ca^{2+} agonist BAY K 8644, the PKC activator phorbol 12-myristate 13-acetate (PMA), or phospholipase C (PLC) was suppressed by simultaneous pulses of 3αHP. The suppression of FSH release by 3αHP appeared to be stereospecific, since no suppression was observed with 5α-pregnane-3,20-dione (5αP) or 3α-hydroxy-5α-pregnan-20-one (5αP3α). In separate experiments, cells were treated with pulses of BSA conjugates of 3αHP, 3βHP, or progesterone; the 3αHP-BSA, but not the 3βHP-BSA or the progesterone-BSA, suppressed the GnRH-induced release of FSH. The results of this study provide the first evidence that 3αHP exerts immediate (nongenomic) and direct effects on GnRH-induced FSH release by interacting at the level of the pituitary gonadotroph membrane and the phosphoinositol cell signaling cascade involving Ca^{2+}.

Key Words: 3α-Hydroxy-4-pregnen-20-one (3αHP); pituitary; gonadotrophs; perifusion; FSH; nongenomic steroid action.

Introduction

Hypothalamic gonadotrophin-releasing hormone (GnRH) regulates synthesis and release of the gonadotrophins, luteinizing hormone (LH), and follicle-stimulating hormone (FSH). The regulatory actions of GnRH are expressed through activation of G-protein-coupled GnRH receptors in the plasma membrane of pituitary gonadotrophs and the resulting changes in cytosolic Ca^{2+} levels (*1–3*). Much of the evidence for this cellular cascade is derived from studies involving LH release (*2,3*). The regulation of FSH release is less well understood, and the short-term role of steroids in gonadotrophin release patterns remains obscure.

It is known that the sensitivity of pituitary gonadotrophs to GnRH is influenced by extended exposure to gonadal steroids (*4–6*). Although the regulation of GnRH-induced LH synthesis involves the genomic action of steroids (*7*), recent evidence suggests that steroids may influence gonadotrophin secretion through nongenomic mechanisms (*6*).

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Our previous studies utilizing static incubation systems indicate that the gonadal and neurosteroid 3α–hydroxy-4-pregnen-20-one (3αHP) suppresses GnRH-stimulated FSH release, and possibly synthesis, through interaction with the Ca^{2+} channels and the gonadotroph plasma membrane (*8,9*). However, because the treatment period in the static culture experiments persisted for hours rather than minutes, the possibility existed that the observed effects of 3αHP on GnRH-induced FSH release could be the result of indirect actions (i.e., owing to metabolites, factors or processes triggered or mediated by 3αHP, but not necessarily to 3αHP itself). In order to determine if steroid effects on GnRH-induced FSH release are immediate (nongenomic) and direct, we utilized anterior pituitary cell perifusion systems in which the effects of short pulses (lasting only seconds or minutes) could be examined.

Perifusion models have been useful in dissecting short-term regulatory mechanisms involved in gonadotrophin release. The perifusion process removes locally produced compounds that could otherwise act as intermediates, and allows the application of GnRH and other factors in a pulsatile fashion similar to the in vivo situation (*10*). In the past, perifusion systems have been employed to study the rapid cascade of intracellular events caused by GnRH binding to its receptor (*11–13*) and occasionally to examine the effects on this response of long-term pre-exposure to steroids (*5,14–16*). The effects of short pulses of gonadal steroids on gonadotrophin release appear not to have been previously studied. In our studies, we used a perifusion system to monitor the effects of a short (5-min) pulse of 3αHP or other steroids on GnRH-induced release of FSH, as well as the rapid interaction of 3αHP with the FSH release induced by Ca^{2+} ionophores, Ca^{2+} channel agonists, and protein kinase C (PKC) activators. In addition, the membrane actions of 3αHP were investigated by treating perifused cells with a short pulse of 3αHP-BSA conjugate, which is unable to pass through a plasma membrane. The results are the first evidence of immediate (nongeno-mic) effects of gonadal steroids on GnRH-induced FSH release.

Results

Immediate Response to GnRH: Effect of GnRH Pulse Time and Interval

Treatment of anterior pituitary cells with 0.25-, 0.5-, 1.0-, or 2.0-min pulses of GnRH (10^{-8} *M*) resulted in time-related increases in FSH release. There is evidence of an increase in FSH release following a 15-s pulse of GnRH; the FSH peak is larger after a 30-s GnRH pulse, and still further increased after a 2-min GnRH pulse (Fig. 1). These results, obtained with perifusion apparatus that has zero dead volume and determined from 30-s samples, show that the response to GnRH is essentially immediate. Obtaining sufficient (measurable) amounts of FSH in the short (30-s) period required loading of each column with about 2×10^7 cells. In order to reduce the numbers of rats while still obtaining measurable amounts of FSH in the effluent samples, the remaining studies employed about $8–10 \times 10^6$ cells (4–5 pituitaries)/chamber and fractions were collected for longer (4–10 min) periods.

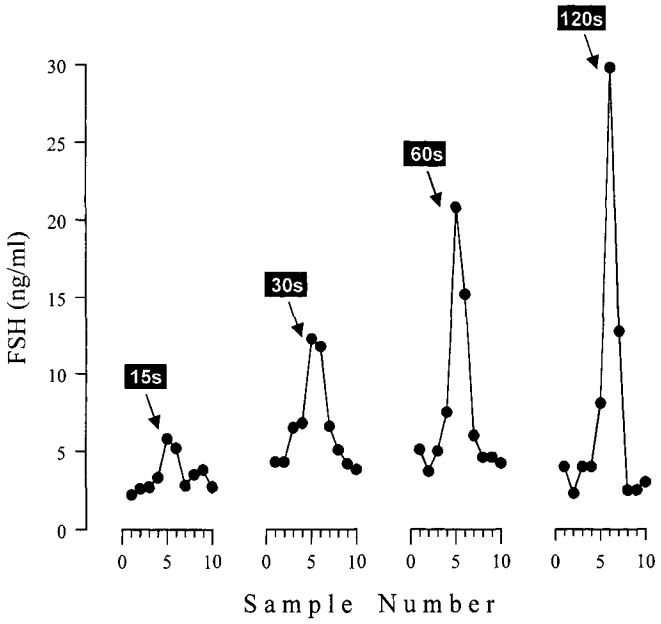

Fig. 1. Effect of GnRH pulse length on FSH release from perifused rat pituitary gonadotrophs. Cells attached to cytodex beads (10^7 cells/per column) were treated with a 15-, 30-, 60-, or 120-s pulse of GnRH (10^{-8}) and 30-s fractions were collected (flow rate = 0.1 mL/min). The time between successive pulses was 90 min. Data represent the mean FSH levels (expressed as ng of RP-2 FSH/mL) of the samples from two columns.

Numerous trials were conducted to determine the between-pulse interval that is required for consistent responses to a GnRH signal. Successive GnRH pulses separated by 45, 60, 90, and 120 min, resulted in spikes of FSH release. However, there was considerable baseline drift and inconsistent replication of FSH spike height at these intervals (data not shown). The variations were greatly diminished when the GnRH pulse interval was 3 or 4 h. Figure 2(A) shows that treatment of anterior pituitary cells with a 5-min pulse of GnRH (10^{-8} *M*) resulted in a sharp increase in FSH release; this effect was reproduced when a similar pulse was applied 4 h later.

Effect of 3αHP, 3β-hydroxy-4-pregnen-20-one (3βHP), Estradiol-17β, and Progesterone on GnRH-Induced FSH Release

The release of FSH owing to a 5-min GnRH (10^{-7} *M*) pulse (Fig. 2A) was completely suppressed when the cells were simultaneously exposed to 10^{-9} *M* 3αHP (Fig. 2B). The 3αHP effect appears to be stereospecific, since treatments with several other steroids at 10-fold higher concentrations (10^{-8} *M*) did not result in FSH suppression: a 5-min pulse of the 3β-isomer of 3αHP, 3β-hydroxy-4-pregnen-20-

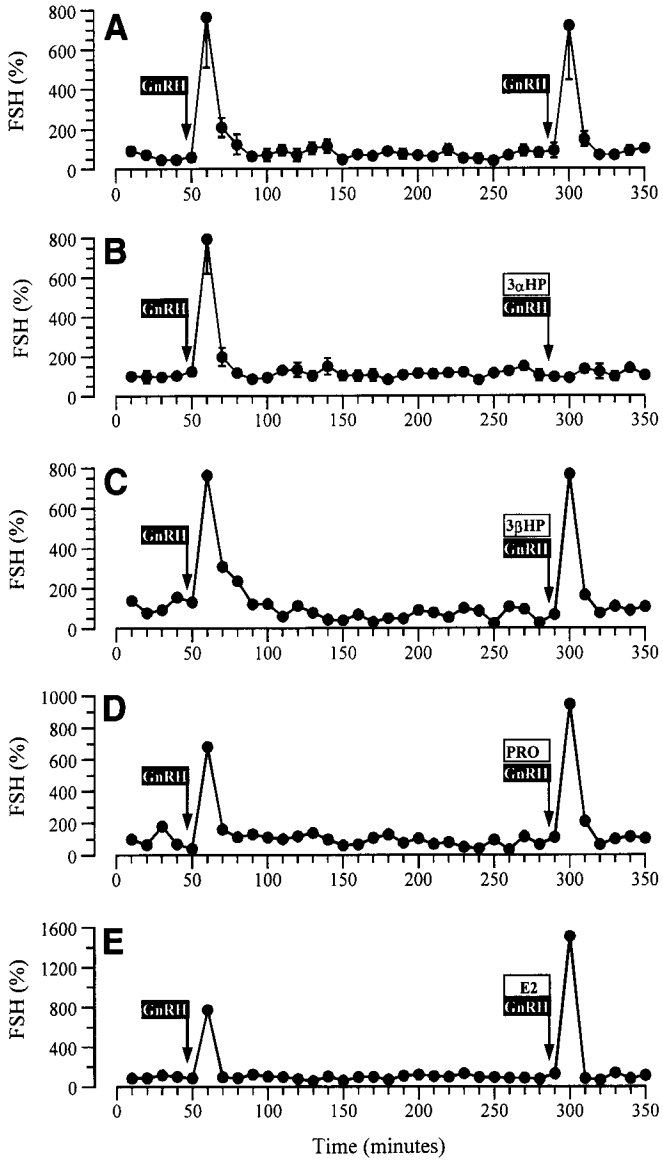

Fig. 2. Effect of steroids on GnRH-induced FSH release in perifused pituitary cells. Female rat anterior pituitary cells, attached to cytodex beads (10^7 cells/column), were perfused with M199 medium at 0.1 mL/min, and 10-min fractions were collected. After a 5 min period for basal FSH determination, a 5-min pulse of GnRH (10^{-7} *M*) served as a control for each column. Following a 4-h recovery period, a second pulse of GnRH without (**A**) or with 10^{-9} *M* 3αHP (**B**), 3βHP (**C**), progesterone (**D**), or estradiol (**E**) was applied. Results are expressed as percent of pretreatment levels of FSH (pretreatment level = 100%) and each point represents the mean ± SEM of 4 (A), 6 (B), or 2–3 (C–E) separate experiments, with 2–3 replicate columns/experiment.

one (3βHP), did not suppress the GnRH-induced release of FSH (Fig. 2C), whereas progesterone (Fig. 2D) and estradiol-17β (Fig. 2E) treatments augmented (by about 60% and 125%, respectively) the GnRH-stimulated release of FSH.

Effect of Steroid Pretreatment on GnRH-Induced FSH-Release

Pretreatment with a 5-min pulse of 3αHP (10^{-8} *M*), 240 min before a 5-min pulse of GnRH (10^{-8} *M*), had no effect

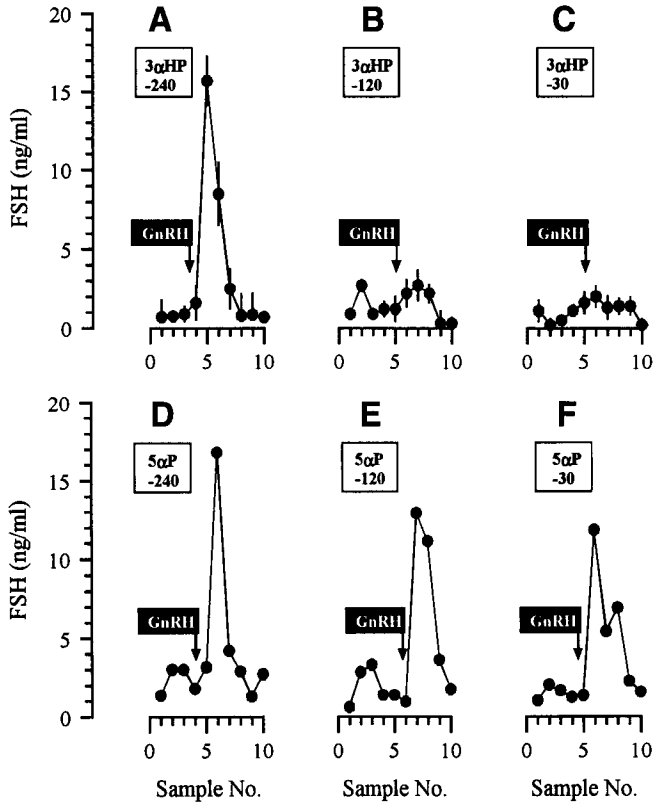

Fig. 3. Effect of steroid pretreatment on GnRH-induced FSH release. Perifused pituitary cells were pretreated with a 5-min pulse of 3αHP (10^{-8}*M*) at 240 (**A**), 120 (**B**), or 30 (**C**) min prior to a pulse of GnRH (10^{-8}*M*). One-milliliter fractions were collected, and results are presented as ng FSH/mL eluate. Each point represents the mean ± SEM of three columns. In parallel experiments, cells were pretreated with pulses of 5αP at 240 (**D**), 120 (**E**), or 30 min (**F**) prior to a GnRH pulse.

on GnRH-induced FSH release (Fig. 3A). However, pretreatment with a 5-min pulse of 3αHP at 120 (Fig. 3B), 60 (results not shown), 30 (Fig. 3C), or 5 (results not shown) min before the GnRH pulse suppressed GnRH-induced FSH release. In parallel experiments, pretreatment with 5α-pregnane-3,20-dione (5αP) at 240, 120, or 30 min prior to a GnRH pulse did not result in a significant change in total amount of GnRH-induced FSH output (Fig. 3D–F).

Immediate Interaction Between 3αHP and the GnRH Response

Simultaneous 5-min pulses of GnRH (10^{-7} *M*) and 3αHP (10^{-9}*M*) suppressed the GnRH-induced FSH release (Figs. 2B and 4A). When a 3αHP pulse was started 30 s after the start of a GnRH pulse, there appeared to be incomplete 3αHP-regulated suppression of the GnRH-induced FSH peak (Fig. 4B). Treatment with 3αHP pulses beginning at 2 or 5 min after the start of the GnRH pulse resulted in little or no suppression of GnRH-induced FSH release (Fig. 4C and D).

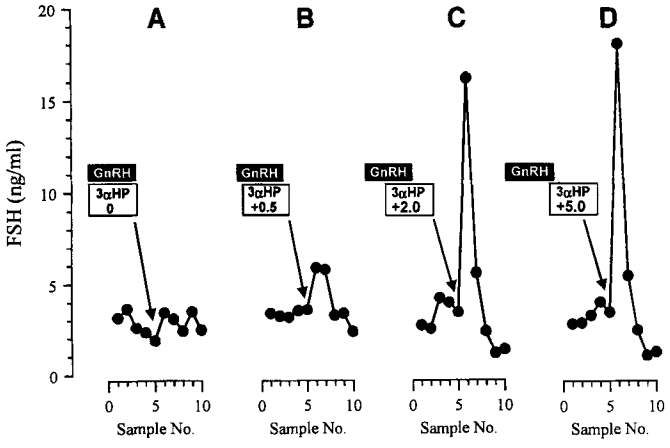

Fig. 4. Immediate interaction between 3αHP and the GnRH response. Perifused rat pituitary cells were pulsed for 5-min with GnRH (10^{-7} *M*) and 3αHP (10^{-9} *M*). The start of the 3αHP pulse occurred at the same time as the start of the GnRH pulse (**A**), or 0.5 min (**B**), 2.0 min (**C**), or 5.0 min (**D**) after the start of the GnRH pulse. Results are expressed as ng FSH/mL of eluate, and each point represents the mean of two replications.

Effect of 3αHP on FSH Release Induced by Calcium Ionophore, Calcium Channel Agonists, PKC Activation, and Phospholipase C

Five-minute pulses of the Ca^{2+} ionophore A23187 (10^{-5} *M*) increased FSH release above basal values and a simultaneous 5-min pulse of 10^{-8} *M* 3α-hydroxy-5α-pregnane-20-one (5αP3α) did not suppress the ionophore-stimulated FSH release (Fig. 5A). On the other hand, a 5-min pulse of 10^{-8} *M* 3αHP administered simultaneously with A23187 suppressed the ionophore-stimulated FSH release (Fig. 5B).

A 5-min pulse with the dihydropyridine Ca^{2+} channel agonist, Bay K 8644 (BAYK; 10^{-6} *M*) increased FSH release above basal values (Fig. 6A). Three hours later, a 5-min pulse of 3αHP (10^{-8} *M*) administered simultaneously with the BAYK pulse, suppressed the BAYK-induced increase in FSH release to basal levels (Fig. 6B). That the cells had retained their ability to respond is illustrated by the marked release of FSH following a GnRH pulse applied 6 h after the first BAYK pulse (Fig. 6C).

A 5-min pulse with the PKC activator, phorbol 12-myristate 13-acetate (PMA; 10^{-7} *M*), resulted in a peak of FSH release. The PMA-induced release of FSH was unaffected by a simultaneous pulse with 5αP3α (10^{-8} *M*) (Fig. 7A). However, treatment of perifused pituitary cells with 3αHP suppressed the PMA-induced FSH release (Fig. 7B).

A 5-min pulse with phospholipase C (PLC) resulted in a peak of FSH release (Fig. 8A). A simultaneous 5-min pulse of 3αHP (10^{-8} *M*) greatly suppressed the PLC-induced increase in FSH release (Fig. 8B). The cells were viable and had retained the ability to respond to GnRH (Fig. 8C).

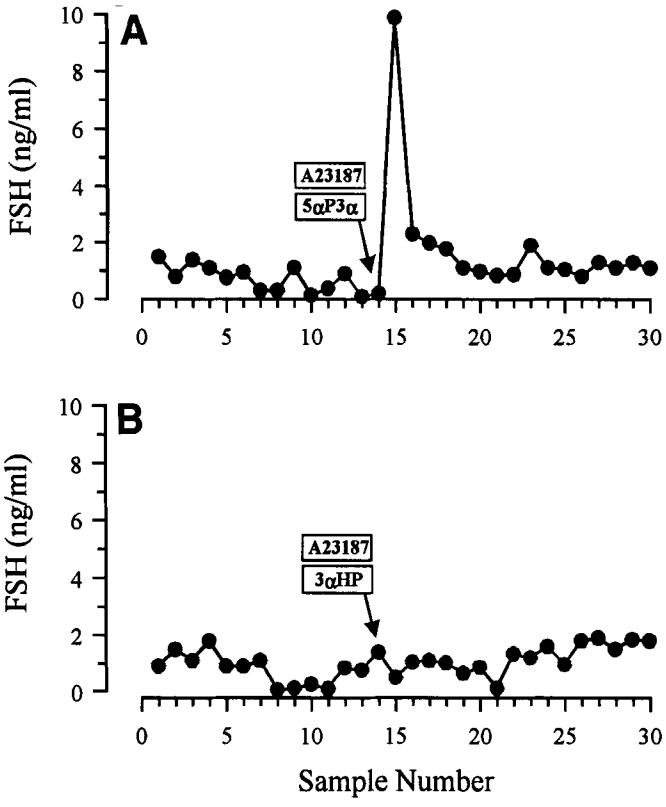

Fig. 5. Effect of 3αHP and (5αP3α) on FSH release induced by the calcium ionophore A23187. Perifused rat anterior pituitary cells were treated with a 5-min pulse (0.8 mL) of (**A**) A23187 (10^{-7} *M*) plus 5αP3α (10^{-8} *M*), or (**B**) A23187 plus 3αHP (10^{-8} *M*). Five-minute fractions were collected, and FSH levels (expressed as ng of FSH /mg protein) are represented as the mean of two replications.

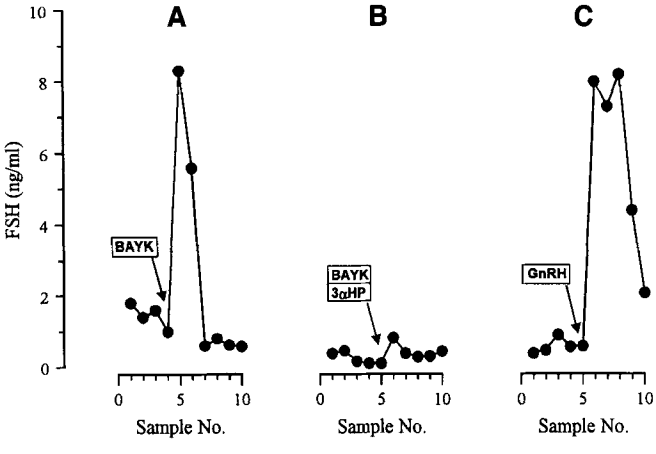

Fig. 6. Effect of 3αHP on dihydropyridine Ca^{2+} channel agonist, (BAYK) induced FSH release from perifused rat anterior pituitary cells. Cells were initially treated with a 4-min pulse of (**A**) BAYK (10^{-6} *M*), (**B**) 3 h later with BAYK and 3αHP (10^{-8} *M*), and (**C**) 6 h later with GnRH (10^{-7} *M*). Results are expressed as ng FSH/mL protein, and each point represents the mean of two replications.

Effect of 3αHP-BSA Conjugate on GnRH-Stimulated FSH Release

Treatment of perifused pituitary cells with a pulse of 3αHP-BSA conjugate (equivalent to 10^{-9} *M* 3αHP) resul-

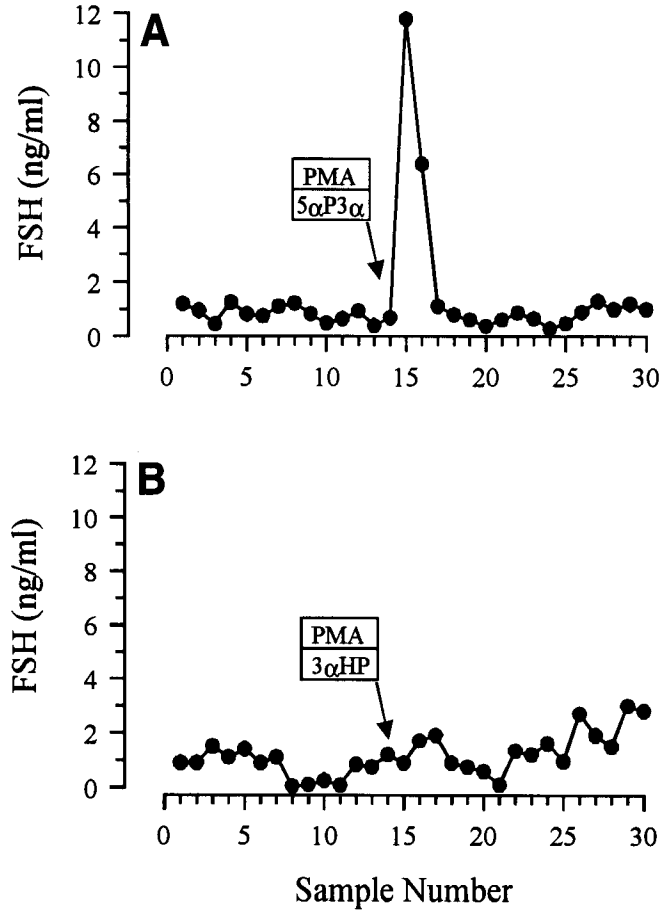

Fig. 7. Effect of 3αHP and 5αP3α on PKC activator PMA-induced FSH release. Perifused rat anterior pituitary cells were treated with a 5-min pulse of **(A)** PMA plus 5αP3α (10^{-8} *M*), or **(B)** PMA plus 3αHP (10^{-8} *M*). Results are expressed as ng FSH/mL eluate, and each point represents the mean of two replications.

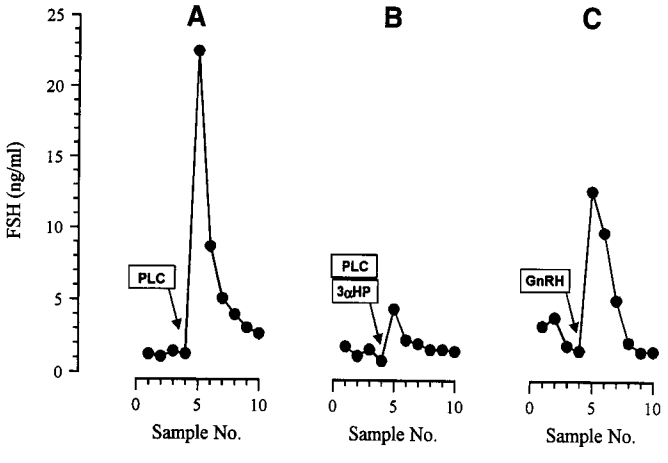

Fig. 8. Effect of 3αHP on PLC-induced FSH release from perifused rat anterior pituitary cells. Cells were treated with a 5-min pulse of **(A)** 0.5 U PLC, **(B)** 0.5 U PLC plus 3αHP (10^{-8} *M*), and **(C)** GnRH (10^{-7} *M*). Treatments (A), (B), and (C) were separated by 3 h of perifusion with medium only. Results are expressed as ng FSH/mL eluate, and each point represents the mean of two replications.

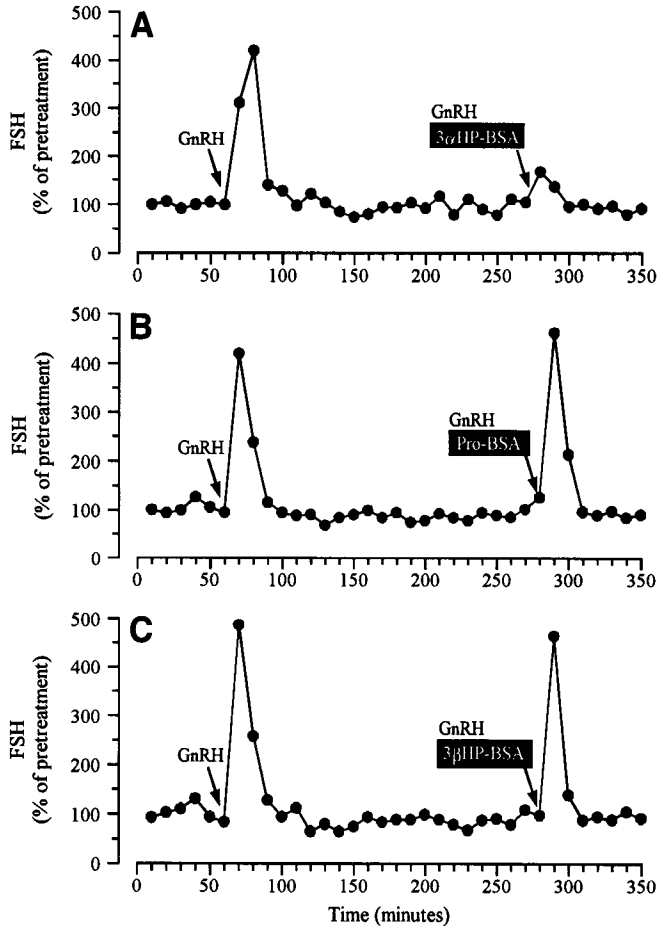

Fig. 9. Suppression of GnRH-induced FSH release by BSA-bound 3αHP. Perifused anterior pituitary cells were treated with a 5-min pulse of GnRH (10^{-7} *M*), and then 4 h later with GnRH plus BSA conjugated to **(A)** 3αHP, **(B)** progesterone, and **(C)** 3βHP. Results are expressed as percent of pretreatment levels of FSH (pretreatment level = 100%), and each point represents the mean of two replications.

ted in a marked suppression of GnRH-stimulated FSH release (Fig 9A), whereas a pulse with an equivalent concentration of progesterone-BSA (Fig. 9B) or 3βHP-BSA (Fig. 9C) conjugate resulted in no apparent effect on the GnRH-induced FSH release.

Discussion

In agreement with results from our previous studies using static cultures of rat anterior pituitary cells, the present study shows that the steroid 3αHP suppresses GnRH-induced FSH release from perifused rat anterior pituitary cells, whereas other steroids examined either have no effect on FSH release or augment FSH release. The results also confirm earlier findings suggesting that FSH suppression by 3αHP occurs at the level of the gonadotroph membrane, and involves interactions with the Ca^{2+} and PKC component of the GnRH signal transduction cascade. More importantly, the studies provide the first evidence that gonadal steroids may be involved in regulating

moment-to-moment variation in GnRH-induced FSH release. Because the treatment period for the static cultures (*8,9,17*) was 4 h (rather than seconds or minutes), it was not possible to discern between an immediate, direct effect of 3αHP and a delayed, indirect effect, which could have resulted from other factors or metabolites induced by the 3αHP. The anterior pituitary cell perifusion system, employing short pulses lasting only seconds or minutes, demonstrated that the 3αHP effects on FSH release appear to be immediate and direct.

The levels of 3αHP employed in these studies (10^{-9} and 10^{-8} *M*) were intended to be in the physiological range. Serum levels of 3αHP in cycling female rats have been determined to vary from about 3.2×10^{-8} *M* to 6.6×10^{-8} *M* between estrus and diestrus (*18*) and to drop from about 4.3×10^{-8} to 1.7×10^{-8} *M* on the day of proestrus (unpublished results); in both cases, the serum FSH levels varied inversely to the 3αHP levels. In addition, the synthesis of 3αHP by pituitary and hypothalamus has been demonstrated (*19*) and pituitary levels of 3αHP oscillate between about 9 and 18 ng per gland on the day of proestrus.

Previous studies have shown that steroids act on pituitary cells to influence pituitary gonadotrophin release both in static cultures (*20–23*), and in perifusion systems (*5,14,15,24*). However, those investigations followed experimental procedures, whereby cells were preincubated with steroids for 24–72 h (*5,20,21,25*), and the effects, therefore, were attributed to the genomic actions of steroids. The results of studies in this article provide the first evidence of an immediate effect of gonadal steroids on pituitary FSH release.

The rapid actions of 3αHP are demonstrated by the immediate suppression of FSH release that occurs when 3αHP is added at the start of the GnRH pulse. The evidence presented (Fig. 4A) indicates that for complete suppression of GnRH-induced FSH release, 3αHP must be applied no later than the beginning of the GnRH pulse. When 3αHP pulses are started 30 s after the start of a GnRH pulse, FSH suppression by 3αHP is incomplete (Fig. 4B). The rapid actions of 3αHP have also been shown in rat cerebral cortical synaptoneurosomes, where 5-s incubations with 3αHP significantly enhanced muscimol-stimulated Cl-uptake (*26*). Other steroids have also been shown to exert rapid actions. Relatively short-term (3 h) progesterone treatments shift subthreshold $[Ca^{2+}]_i$ responses to oscillatory responses, and oscillatory to biphasic responses in GnRH-stimulated gonadotrophs (*27*). More recently, it has been shown that 60–120 s applications of the neuroactive steroid allopregnanolone (5αP3α) can stimulate peak levels of $[Ca^{2+}]_i$ within 2–3 s after application in central nervous system neurons (*28*). The present results suggest that at least some of the actions of gonadal steroids on gonadotrophin release are also of a very rapid, nongenomic nature.

The rapid effects of steroids involved in the modulation of gonadotrophin release may be owing to steroid–pituitary plasma membrane interactions (*29*). A BSA-3αHP conjugate, which is unable to pass through the plasma membrane (*29*), was shown to be effective in suppressing GnRH-induced FSH release in both static cultures (*8*) and in the perifusion systems reported here. One interpretation of the 3αHP-BSA-induced suppression of FSH release is that 3αHP may be interacting with specific membrane acceptor/receptor sites for 3αHP, an interaction that then leads to regulatory effects by way of the intracellular cell signaling pathways.

In support of this hypothesis are results of studies in progress, wherein saturation analyses of [1,2,6,7-^{3}H]-3αHP uptake by isolated pituitary cell membranes indicate binding sites with high affinity. Specific binding sites on membranes of anterior pituitary cells (*30,31*) and of various other cell types (*29,32–35*) have been documented for other steroids.

There is also evidence that 3αHP binds to the $GABA_A$ receptor complex on neuronal membranes in rat cerebral cortex and exerts analgesic effects that are measurable within 15 min (*36*). Whether 3αHP interacts with GABA receptors in pituitary gonadotrophs has not been examined, although steroid-mediated activity of the $GABA_A$ receptor complex at the pituitary level has been demonstrated (*37*). At the present time, the evidence from studies with the free as well as the conjugated forms of 3αHP, suggest that at least one immediate site of action of this steroid may be receptors in the plasma membrane. The result of such 3αHP-receptor binding could potentially affect the GnRH-receptor binding or other membrane protein actions involved in FSH release (*38*). Other possible nongenomic sites of action of 3αHP have been proposed (*38*), and include interactions with the cell signaling pathway, calcium and other ion channels, and the membrane lipid bilayer.

Although it had been suggested that cultured pituitary cells possess the ability to remain responsive to steroids that have been present in, and then removed from, the extracellular environment (*39*), a steroid pretreatment perifusion study using only a short pulse of steroid application, followed by a long, steroid-free incubation period, had not been investigated previously. Results of the present study show that GnRH-induced FSH release from pituitary gonadotrophs is suppressed by short pulses of 3αHP applied as much as 120 min before a GnRH pulse in a time-dependent fashion. The selective nature of FSH suppression by 3αHP is shown by the lack of response to other steroids. The results suggest an abiding ("memory") effect of a steroid on the gonadotrophs, which persists for some time after the steroid is depleted. These in vitro results may be related to the in vivo effects of presurge elevated estradiol, which is thought to cause, in part, the elevated GnRH responsiveness of the anterior pituitary on the day of proestrus (*40,41*).

Estradiol may influence LH and FSH release by facilitating the synthesis of a protein or proteins that interact with

the Ca^{2+}, and/or diacylglycerol, and cAMP signal transduction pathways associated with gonadotrophin release (*21,42*). The immediate increase in GnRH-induced FSH release resulting from a short pulse of estradiol (Fig. 2E) suggests that a rapid steroid effect on hormone release is not limited to 3αHP. The results are of interest, because estradiol stimulates FSH release, but 3αHP suppresses FSH release. Whether the onset, amplitude, and frequency of oscillations of FSH release during the follicular phase are determined by the interplay between 3αHP and steroids, such as estradiol, remains to be investigated. Steroids have also been found to modulate PKC activity in pituitary gonadotrophs (*43,44*), and activators of PKC are known to induce release of gonadotrophins from pituitary cells (*45*). 3αHP, but not 5αP3α, treatments in our perifusion system suppressed PKC activator (PMA)-induced FSH release, suggesting that 3αHP may interact with gonadotrophs at the level of the PKC component of the GnRH cell signaling pathway.

Because the signal transduction cascade whereby GnRH regulates the synthesis and release of gonadotrophins involves Ca^{2+} (*2*), we used several pharmacological tools known to affect cytosolic Ca^{2+} levels in our perifusion system. The Ca^{2+} ionophore, A23187, is a pharmacological agent known to increase cytosolic Ca^{2+} levels (*46*), and such agents are known to provoke gonadotrophin release (*2*). The role of voltage-sensitive Ca^{2+} channels in gonadotrophin release has also been explored (*47*). In static cultures, the dihydropyridine-sensitive Ca^{2+} agonist Bay K 8644-increased FSH release in unstimulated cells, and BAYK-stimulated FSH release was suppressed by 3αHP (*8*). Results of the present study indicate that 3αHP may suppress the rise in intracellular Ca^{2+} required for GnRH-induced gonadotrophin release, and that 3αHP may be acting at least in part on the dihydropyridine-sensitive Ca^{2+} channel to suppress FSH secretion. Experiments using PLC antagonists, neomycin and U73122, showed a suppression in GnRH-induced LH release, and indicate the importance of PLC in pituitary gonadotrophin secretion (*3*). Our results indicate that 3αHP may also interact with PLC to suppress FSH release. Since the perifused pituitary cell treatments examined did not extend beyond 5 min, the interactions of 3αHP may be considered direct nongenomic effects on the FSH secretory mechanism regulated by the cytosolic Ca^{2+} concentrations and the related cell signaling mechanisms.

In conclusion, the present study provides evidence that 3αHP suppresses GnRH or Ca^{2+} -induced FSH release in perifused rat anterior pituitary cells. The suppression appears to involve a rapid, nongenomic mechanism, which may encompass an interaction with the gonadotroph membrane and/or Ca^{2+} channels. This suppressive effect can be exerted even after removal of 3αHP from the extracellular environment for a period of time before a GnRH pulse is applied. Since 3αHP is present in the circulation as well as the hypothalamus and pituitary, it may be involved in vivo in regulating rapid changes in GnRH–induced FSH secretion.

Materials and Methods

Animals

Prepubertal (23–28 d) female Sprague-Dawley rats (100–150 g) were purchased from Charles River Laboratories (St. Constant, QC). Animals were maintained according to approved laboratory conditions (2–4 per cage) under a 14-h light, 10-h dark photoperiod at 20–22°C, and were fed Prolab RMH 3000 chow (Agway Country Foods, Syracuse, NY) and water ad libitum.

Reagents and Hormones

Rat FSH (FSH, RP-2), hormones for iodination, and antibodies for the RIA of FSH were kindly provided by the Hormone Distribution Office, National Pituitary Agency, NIDDK, NIH (Bethesda, MD). Whatman glass microfiber filters (GF/C) were purchased from VWR (Mississauga, Ontario). Supplemented calf serum (SCS) was purchased from Gibco BRL (Grand Island, NY) and was charcoal-stripped before use. PMA was purchased from Research Biochemicals International (Natick, MA). The ^{125}I (in NaOH) solution was purchased from Amersham Life Science Canada (Oakville, Ontario). Bovine serum albumin (BSA), phenol red-free Medium 199, *N*-[2-hydroxyethyl]piperazine-N'-[2-ethane-sulfonic acid] (HEPES), gentamicin solution, ovalbumin, cytodex 3 microcarrier beads for cell culture, hyaluronidase (type II), and collagenase (type I) were obtained from Sigma Chemical Co. (St. Louis, MO). The treatment reagents, Bay K 8644, estradiol 17β, progesterone, 5α-P3α, and 5αP (all obtained from Sigma) were dissolved in ethanol and then diluted (to a final concentration of ethanol at 0.1%) in medium. PLC (phosphatidylcholine cholinephosphohydrolase, Sigma) was dissolved in 1% ovalbumin in PBS, and diluted in perifusion medium. A23187 (Sigma) was dissolved in dimethyl sulfoxide (DMSO) and then diluted in perifusion medium. The final concentrations (0.1%) of ethanol or DMSO were shown not to affect GnRH-induced release of FSH. GnRH (acetate salt; Sigma) was dissolved in perifusion medium. The steroids, 3αHP and 3βHP, were synthesized in our laboratory as described previously (*48*). Estradiol-17β, and progesterone were purified by recrystallization, and ethanol was glass-distilled before use.

Anterior Pituitary Cell Culture

Rats were sacrificed by decapitation and pituitaries were quickly excised and collected in ice-cold perifusion medium (Medium 199 containing 0.1% BSA, 10 m*M* HEPES, and 20 μg/mL gentamicin). The procedure for isolating the pituitary cells has been described (*8*). In a number

of experiments, we followed the procedure of allowing cells to attach to cytodex beads; the dispersed cells were plated at a density of 1.5–2 × 10^7 cells/dish with medium containing serum and cytodex III microcarrier beads. After 5–6 d, these cultures (approx 1 × 10^7 cells adhering to cytodex beads) were transferred to each column. Later, we discovered that the step involving 5–6 d of attachment to beads was not necessary, and that similar results were obtained if cells were placed directly into the chambers and used the next day. In this paradigm, presoaked cytodex beads were layered to a depth of about 3–4 mm at the bottom of each chamber and perifused with medium for 2 h prior to the addition of 500 µL of freshly isolated pituitary cells in suspension (approx 1 × 10^7 cells per chamber). The cells were perifused for about 15 min, then the peristaltic pump was stopped, and the cells were incubated overnight. In both scenarios, fraction collection began only after an initial 60-min stabilization period.

Perifusion

A four-chambered Plexiglas perifusion apparatus, designed in our laboratory, with zero dead-volume connections was used for the perifusion experiments. Whatman glass microfiber filters suspended on polypropylene disks formed the base of each chamber (1.0-mL volume). A peristaltic pump (Gilson, Minipuls 2) was used to provide each chamber with sterile-filtered perifusion medium (M199/BSA/HEPES + gentamicin) at defined flow rates (0.1–0.25mL/min). The perifusion medium, peristaltic pump, and perifusion apparatus were housed in an incubator at 37°C. After the initial 60-min stabilization period (see under Anterior Pituitary Cell Culture), the effluent was collected in a fraction collector (at room temperature) during successive timed (4-, 5-, or 10-min) intervals. Cells were generally subjected to 5-min pulses of treatment solution unless otherwise stated. Steroids that served as controls for 3αHP included progesterone, 3βHP, 5αP, 5αP3α. Fractions were stored at –20°C within 15 min of sample collection until FSH levels were estimated by RIA (1–4 d after freezing).

RIAs

FSH levels were determined as described previously (*8,17*) on duplicate 40-µL aliquots.

Presentation of Results

Because of variation between experiments, in cell numbers and levels of FSH released, some data were transformed to percent of pretreatment values, i.e., pretreatment (basal) amount of FSH was designated as 100%.

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