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Ovarian Steroids Stimulate Adenosine Triphosphate-Sensitive Potassium (K_{ATP}) Channel Subunit Gene Expression and Confer Responsiveness of the Gonadotropin-Releasing Hormone Pulse Generator to K_{ATP} Channel Modulation

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The ATP-sensitive potassium (K_{ATP}) channels couple intracellular metabolism to membrane potential. They are composed of Kir6.x and sulfonylurea receptor (SUR) subunits and are expressed in hypothalamic neurons that project to GnRH neurons. However, their roles in regulating GnRH secretion have not been determined. The present study first tested whether K_{ATP} channels regulate pulsatile GnRH secretion, as indirectly reflected by pulsatile LH secretion. Ovariectomized rats received sc capsules containing oil, 17β-estradiol (E_2), progesterone (P), or E_2+P at 24 h before blood sampling. Infusion of the K_{ATP} channel blocker tolbutamide into the third ventricle resulted in increased LH pulse frequency in animals treated with E_2+P but was without effect in all other groups. Coinfusion of tulbutamide and the K_{ATP} channel opener diazoxide blocked this effect, whereas diazoxide alone suppressed LH. Effects of steroids on Kir6.2 and SUR1 mRNA expression were then evaluated. After 24hr treatment, E_2+P produced a modest but significant increase in Kir6.2 expression in the preoptic area (POA), which was reversed by P receptor antagonism with RU486. Neither SUR1 in the POA nor both subunits in the mediobasal hypothalamus were altered by any steroid treatment. After 8 d treatment, Kir6.2 mRNA levels were again enhanced by E_2+P but to a greater extent in the POA. Our findings demonstrate that 1) blockade of preoptic/hypothalamic K_{ATP} channels produces an acceleration of the GnRH pulse generator in a steroid-dependent manner and 2) E_2+P stimulate Kir6.2 gene expression in the POA. These observations are consistent with the hypothesis that the negative feedback actions of ovarian steroids on the GnRH pulse generator are mediated, in part, by their ability to up-regulate K_{ATP} channel subunit expression in the POA. (*Endocrinology* 149: 2423–2432, 2008)

GnRH IS RELEASED IN a pulsatile manner into the hypophyseal portal vessels and conveyed to the anterior pituitary gland, where it directs synthesis and release of LH and FSH (1). In the female reproductive axis, the activity of the neural GnRH pulse-generating mechanism is subject, in turn, to regulation by the homeostatic negative feedback actions of the ovarian steroids estrogen and progesterone. Significant progress has been made in the characterization of the neural circuitries that may mediate ovarian steroid feedback, yet the molecular and cellular pathways through which they are exerted have remained ill defined. In the present studies, we have explored the hypothesis that the suppressive effects of ovarian steroids on the GnRH pulse generator are mediated by regulation of ion channels that control the excitability of hypothalamic neurons and hence the neurosecretory activity of GnRH neurons.

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Abbreviations: aCSF, Artificial cerebrospinal fluid; E_2, 17β-estradiol; ER, estrogen receptor; GABA, γ-aminobutyric acid; ID, inner diameter; icv, intracerebroventricular; K_{ATP}, ATP-sensitive potassium; MBH, mediobasal hypothalamus; NPY, neuropeptide Y; OD, outer diameter; OVX, ovariectomized; P, progesterone; PI3K, phosphoinoside 3-kinase; POA, preoptic area; POMC, proopiomelanocortin; PR, progesterone receptor; SUR1, sulfonylurea receptor-1.

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In females of several species, it has been shown that the replacement of 17β-estradiol (E_2) and P reverses ovariectomy (OVX)-induced increases in pulsatile GnRH/LH release, with E_2 exerting its primary effects upon GnRH/LH pulse amplitude (2) and P being most effective in retarding GnRH pulse frequency (3). Combined treatment with both hormones is required to fully restore LH secretion to levels found in ovary-intact animals (4). Studies of estrogen receptor (ER)-null mutant mice have revealed that the ERα isoform is probably most important in mediating E_2 negative feedback actions on LH secretion (5). Pharmacological experiments in sheep (6) and analysis of P receptor (PR) knockout mice have also provided evidence for the involvement of PR_A and/or PR_B in P's inhibitory actions (7). It has been proposed that ERα- and $PR_{A/B}$-expressing afferents to GnRH neurons (8, 9), and possibly a subset of GnRH neurons themselves (10), are the targets of these steroid hormones that mediate their negative feedback actions.

Irrespective of which neuronal populations may mediate steroid negative feedback, an elucidation of the postreceptor mechanisms mediating these processes has remained elusive. Previous studies have revealed that steroid hormones can rapidly alter the electrophysiological properties of GnRH neurons (11) as well as putative GnRH afferent neuronal populations including γ-aminobutyric acid (GABA) (12) and proopiomelanocortin (POMC) neurons (13). Among the downstream signaling mechanisms identified in these neu-

rons are the uncoupling of μ-opioid receptors from G protein-gated inwardly rectifying K^+ (GIRK) channels in POMC neurons and $GABA_B$ receptors from GIRK channels in GABA neurons (14). It is not known whether these rapid nongenomic effects are accompanied by more slowly developing genomic actions that culminate in altered ion channel gene expression. Whether these or other effects of steroids on ion channel function are relevant to *in vivo* steroid hormone feedback mechanisms also remains to be confirmed.

The present studies explore the possibility that steroid hormones can inhibit GnRH pulse generation by altering the activity of another set of inwardly rectifying potassium channels, the ATP-sensitive potassium (K_{ATP}) channels. The K_{ATP} channels function as cellular metabolic sensors, mediating glucose sensing in pancreatic β-cells and hypothalamic glucose-responsive neurons by linking intracellular metabolism with electrical excitability (15). Activation of K_{ATP} channels is also believed to mediate hypothalamic actions of insulin (16) as well as some of those of leptin (17). More broadly, K_{ATP} channels serve cellular protective roles, limiting effects of cardiac ischemia (18) and restraining hypoxia-induced seizure propagation (19). In the present studies, we have focused on the role of K_{ATP} channels in ovarian negative feedback because they are expressed in hypothalamic neurons implicated in regulating GnRH release, such as neuropeptide Y (NPY) (20), POMC (21), and GABA (20) as well as in a subset of GnRH neurons themselves (22). Moreover, gonadal steroids have been shown to exert physiological effects via activation of K_{ATP} channels in other tissues, such as the myocardium (23). We hypothesized that E_2 and P stimulate the activity and/or expression of K_{ATP} channels, thereby altering the level of excitability of GnRH neurons or the afferent neuronal groups that control GnRH neurosecretion. To assess this hypothesis, we sought to determine whether E_2 and P stimulate the expression of genes encoding the K_{ATP} channel subunit proteins and to assess whether the pharmacological closure of K_{ATP} channels produces an acceleration of GnRH pulsatility (as reflected by LH pulsatility) in steroid-replete animals.

Materials and Methods

Animals

Female Sprague Dawley rats (Charles River Laboratories, Wilmington, MA) (200–220 g) were housed in temperature-controlled facilities with a 14-h light, 10-h dark cycle (lights on 0500–1900 h). They were fed standard lab chow and had access to water *ad libitum*. All surgical and experimental procedures were used in strict accordance with protocols approved by the Institutional Animal Care and Use Committee of Northwestern University.

Experiment 1: effects of 24-h steroid treatments on pulsatile LH responses to K_{ATP} channel blockade

On d 0, rats were anesthetized with 75 mg/kg ip ketamine (Fort Dodge Laboratories, Fort Dodge, IA) and 5 mg/kg ip xylazine (Burns Veterinary Supply, Inc., Rockville Center, NY) and bilaterally OVX. At the same time, they received stereotaxic guide cannulae implants fitted with stainless steel obdurators targeted to the anterior extremity of the third ventricle (coordinates 1 mm caudal to bregma, 7.5 mm ventral to the skull, 0.2 mm lateral) (24). On d 7 after cannula implantation (1000–1200 h), animals were anesthetized by halothane inhalation, and an indwelling atrial catheter (PE-50; Becton Dickinson, Sparks, MD) was surgically implanted through the right jugular vein as previously described (25).

The animals were then divided into three treatment groups. Control (C), E_2, and E_2+P groups received sc SILASTIC capsule implants containing vehicle (sesame oil), E_2, or both E_2 and P, respectively. E_2 capsules were prepared by using a 15-mm length of SILASTIC tubing [inner diameter (ID) 0.16 cm (0.062 in.); outer diameter (OD) 0.38 cm (0.125 in.); Dow-Corning, Midland, MI] containing 100 μg E_2 (Sigma Chemical Co., St. Louis, MO)/ml sesame oil sealed off at both ends with medical-grade adhesive. P capsules consisted of one 20-mm-long capsule [ID 0.16 cm (0.062 in.); OD 0.38 cm (0.125 in); Dow-Corning] containing crystalline P (Sigma). Capsules were sealed at each end with medical-grade adhesive. They were each incubated in 0.9% saline before implantation. These treatments have been shown to sustain physiological E_2 and P levels in OVX female rats (26).

Blood-sampling sessions were initiated 24 h after the sc capsule implantations, with 100 μl being withdrawn from the atrial catheters every 5 min for a total of 3 h. An equivalent volume of 0.9% saline was infused back into the catheter after each blood withdrawal. Blood samples were centrifuged at 2000 $\times$ *g* for 15 min, and plasma was collected and stored at −20 C for subsequent LH RIA.

At 90 min before the start of sampling, the obdurator was removed from the intracerebroventricular (icv) guide cannula and an infusion cannula (0.64 mm OD) was inserted. The animal was then left undisturbed throughout the remainder of the experiment. A CMA400 Microdialysis pump (CMA Microdialysis AB, North Chelmsford, MA) and a liquid switch (CMA Microdialysis) were used to deliver artificial cerebrospinal fluid (aCSF) through the cannula. Blank aCSF was infused during the first hour, followed by infusion of aCSF containing the K_{ATP} channel blocker tolbutamide (Sigma), for the second hour. Blank aCSF was again infused during the third hour of blood sampling sessions. Tolbutamide is a K_{ATP} channel blocker that is relatively specific for the sulfonylurea receptor-1 (SUR1) subunit isoforms (27), which is the predominant SUR expressed in hypothalamic and preoptic area neurons (20, 28). The dose of tolbutamide was determined on the basis of preliminary experiments in gonad-intact animals, in which a total of 6.5 μg/h into the anterior third ventricle was found to produce a significant acceleration of LH pulsatility. No grossly observable behavioral alterations, sensorimotor deficits, or neurohistological damage was produced by the tolbutamide infusions when carried out at the rate of 2 μl/min. For comparison, we note that in a previous study (29), a dose of 162 μg/h tolbutamide was similarly infused into the lateral ventricle and found to suppress counterregulatory (epinephrine and glucagon) responses to brain glucopenia or systemic hypoglycemia. For infusions, tolbutamide was first dissolved in a small volume of dimethylsulfoxide and then diluted in aCSF to 200 μM. The components of aCSF were (in mM): 124 NaCl, 5 KCl, 26 $NaHCO_3$, 2.6 NaH_2PO_4, 10 dextrose, 10 HEPES, 2 $MgSO_4$, and 2 $CaCl_2$. Final concentration of dimethylsulfoxide in aCSF was 0.1%.

Experiment 2: effects of 24-h E_2+P treatment on pulsatile LH responses to coinfusion of a K_{ATP} channel opener and tolbutamide

To demonstrate the specificity of the effects of tolbutamide, we included an E_2+P-treated group in which the K_{ATP} channel opener diazoxide (Sigma) was infused along with tolbutamide (200 μM), or by itself, during the second hour of blood sampling. Surgeries, infusions, and blood sampling were performed as described above. The concentration of diazoxide used in these experiments (8.3052 μg/h) is lower than that previously infused into the ventricles of rats by other investigators (30). As with tolbutamide, no grossly observable behavioral alterations, sensorimotor deficits, or neurohistological damage was produced by the diazoxide infusions when carried out at the rate of 2 μl/min.

Experiment 3: effects of 24-h steroid treatments on K_{ATP} channel subunits mRNA expression

Rats were OVX on d 0. At 1100 h on d 7, the animals received sc capsule implants containing oil vehicle (C), E_2, P, or E_2+P as described in the foregoing experiment. An additional E_2+P-treated group was pretreated with the PR antagonist RU486 at 0800 h on d 7 and then again

at 0800 h on d 8 (4 mg sc in 0.2 ml benzyl benzoate/sesame oil). At 1100 h on d 8, all animals were anesthetized by halothane inhalation and decapitated, brains were immediately removed, and preoptic area (POA) and mediobasal hypothalamus (MBH) tissues were dissected. The POA was defined as a 2-mm cube of tissue extending from −1.3 to 0.7 mm anteroposterior, −9.5 to −7.5 mm dorsoventral, and 1 mm bilaterally from the midline (24). The MBH was delineated rostrally by the posterior margin of optic chiasm, laterally by the hypothalamic sulci, and caudally by the mammillary body and extending 2 mm ventrally into the brain (24). These two areas were chosen to include regions including GnRH cell bodies (POA) and the soma of neuronal populations known to regulate pulsatile GnRH release (MBH), such as POMC-producing (31) and NPY-producing (32) neurons.

A semiquantitative RT-PCR method was used to assess regulation of K_{ATP} channel subunit expression as previously described (28). Specifically, total RNA from POA and MBH were extracted with TRIzol Reagent (Invitrogen, Carlsbad, CA) and treated with 10 U RNase-free DNase (Promega, Madison, WI). Total RNA (2.4 μg) was reverse transcribed in a 20-μl reaction mixture with final concentrations of each reagent as 1 mM dNTP, 110 μM MMLV, 22 μM RNasin (Promega), 25 μg/ml random hexamer (Genosys, The Woodlands, TX), 5 mM $MgCl_2$, and 1× PCR buffer (Roche, Indianapolis, IN). After first-strand synthesis, 5 μl cDNA was used for PCR amplification of either Kir6.2 or SUR1 subunit. Primers were Kir6.2 (sense, 5′-GCTGCATCTTCATGAAAACG-3′; antisense, 5′-TTGGAGTCGATGACGTGGTA-3′; 298 bp, accession no. AB043638), SUR1 (sense, 5′-TGGGGAACGGGGCATCAACT-3′; antisense, 5′GGCTCTGGGGCTTTTCTC-3′; 388 bp, accession no. L40624). The expression level of Kir6.2 or SUR1 was normalized to that of ribosomal protein L19 (RPL19) (33), which was amplified by primers (sense, 5′-CTGAAGGTCAAAGGGAATGTG-3′; antisense, 5′-GGACAGAGTCTTGATGATCTC-3′; 195 bp, accession no. XM_235216) in the same tube with each of the two subunits. All primers were obtained from Genosys. PCR was performed in a 45-μl reaction mixture containing 2 mM $MgCl_2$, 1× PCR buffer, 1.25 U *Taq* DNA polymerase (Roche, Indianapolis, IN), 300 nM each primer, 110 μM dNTP, and [^{32}P]dCTP (Amersham Biosciences Corp., Piscataway, NJ). The mixture was first incubated at 94 C for 4.5 min and then cycled through denaturing at 94 C for 30 sec, annealing at 58 C (59 C for SUR1) for 1 min, and extension at 72 C for 1 min. Finally, extension was carried out for 10 min at 72 C. The number of cycles for Kir6.2 and SUR1 amplification were 31 and 32, respectively, and 23 cycles (with Kir6.2) or 24 cycles (with SUR1) for rRPL19. The cycle number for each PCR amplification was tested to ensure that the results were obtained from the linear region of the amplification curve. After PCR, the products were separated by PAGE, and the autoradiography images were obtained by using phosphoimager (STORM 860; Molecular Dynamics, Sunnyvale, CA). The density of each band was analyzed by ImageQuant (Molecular Dynamics, Piscataway, NJ). Both RT control samples in which no MMLV-RT was added to the RT reaction mixture and PCR control sample in which Milli-Q H_2O instead of cDNA was added to the PCR mixture were included in the PCR. The expected sequences of each PCR product were confirmed by DNA sequence analysis with an ABI 3100 sequencer (Applied Biosystems, Foster City, CA).

Experiment 4: effects of 8-d steroid treatments on K_{ATP} channel subunits mRNA expression

Animals were OVX and received sc SILASTIC capsule implants containing oil vehicle (C), E_2, P, and E_2+P. The implants used in these experiments were designed after those used by Dubal *et al.* (34) and Goodman *et al.* (2), which have been shown to produce physiological E_2 and P levels over the course of at least 7 d of treatment. The E_2 capsules consisted of a 30-mm length of SILASTIC tubing [ID 0.16 cm (0.062 in.); OD 0.38 cm (0.125 in.); Dow-Corning] containing 180 μg E_2 (Sigma)/ml sesame oil. Capsules were sealed at both ends with medical-grade adhesive. The P capsules were made of a 20-mm length of SILASTIC tubing [ID 0.33 cm (0.132 in.); OD 0.46 cm (0.183 in.); Dow-Corning] containing crystalline P and sealed at each end. Vehicle capsules were capsules prepared as the above E_2 or P capsules but filled with only sesame oil. On d 8, all the animals were anesthetized and decapitated. POA and MBH were immediately dissected and stored on dry ice. Total RNA was harvested and DNase-treated, and 4.8 μg RNA was reverse transcribed. The samples were then analyzed by RT-PCR as described in experiment 2.

RIAs

Plasma LH levels were determined by using RIA reagents obtained from the National Institute of Diabetes and Digestive and Kidney Diseases (National Institutes of Health, Bethesda, MD), including LH reference (RP-3) and antirat LH antibody (S-11). The assay had a lower limit of detection of 0.2 ng/ml. Intraassay and interassay coefficients of variance were 8.28 and 9.66%, respectively. Steroid hormone RIAs were performed using kits purchased from Diagnostic Products Corp., Los Angeles, CA (E_2 RIA) and MP Biomedicals, Costa Mesa, CA (P RIA). The estradiol assay had a lower limit of detection of 2 pg/ml, and the intraassay and interassay coefficients of variance were 2.7 and 2.85%, respectively. The P RIA had a lower limit of detection of 0.15 ng/ml, and the intraassay and interassay coefficients of variance were 12.0 and 16.5%, respectively.

Statistical analysis

All the reported values were presented as mean ± SEM. Plasma LH pulses were analyzed using ULTRAGUIDE pulse analysis software (35). In each animal group, LH pulse frequency, pulse amplitude, and mean LH level before and after icv tolbutamide infusion was compared with values observed during the infusion by using one-way ANOVA with repeated measures, followed by Bonferroni's *post hoc* test. Comparison among treatment groups of Kir6.2 and SUR1 mRNA expression were made by one-way ANOVA, followed by Bonferroni's *post hoc* test. For all statistical analyses, differences were considered statistically significant if $P < 0.05$.

Results

Experiment 1: effects of 24-h steroid treatments on pulsatile LH responses to K_{ATP} channel blockade

The effects of 24-h vehicle, E_2, or E_2+P treatments on pulsatile LH responses to icv tolbutamide infusions are depicted in Figs. 1, 2, and 3. In OVX, vehicle-treated rats, LH pulse frequency, pulse amplitude, and mean level were not significantly altered by icv infusion of tolbutamide (Fig. 1). Similarly, no significant alterations of those parameters were observed in OVX, E_2-treated rats (Fig. 2). In contrast, after the animals were treated with E_2+P for 24 h, LH pulse frequency was significantly increased by icv tolbutamide infusion (1.80 ± 0.37, 3.60 ± 0.24, and 1.40 ± 0.40 pulses/h before, during, and after infusion, respectively; $P < 0.05$; Fig. 3). The LH pulse amplitude and LH mean level were not significantly affected by icv tolbutamide infusions (Fig. 3).

Experiment 2: effects of 24-h E_2+P treatment on pulsatile LH responses to coinfusion of a K_{ATP} channel opener and tolbutamide

The increase in LH pulse frequency in response to tolbutamide was completely blocked by coadministration of the K_{ATP} channel opener diazoxide (Fig. 4). In addition, there was a significant reduction in mean LH levels before *vs.* after drug infusions (Fig. 4D). Infusion of diazoxide alone in E_2+P-treated animals resulted in a significant suppression of mean LH pulse amplitude (Fig. 5C) and mean LH levels (Fig. 5D).

Experiment 3: effects of 24-h steroid treatments on K_{ATP} channel subunit mRNA expression

The expression levels of Kir6.2 and SUR1 mRNA in the POA and MBH after 24 h treatment with vehicle, E_2, P, or E_2+P are depicted in Fig. 6. The E_2+P treatment significantly up-regulated Kir6.2 mRNA expression in the POA by 25% compared with vehicle (1.72 ± 0.09 in vehicle

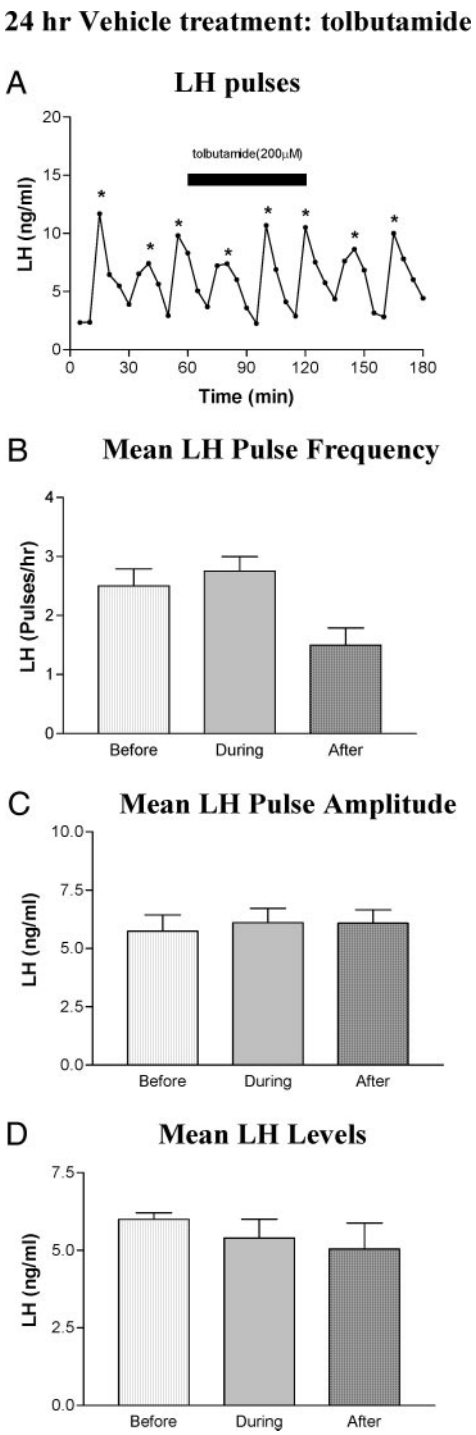

FIG. 1. Representative profile of pulsatile LH release (A) in OVX, 24-h vehicle-treated rats and comparison of mean LH pulse frequency (B), mean LH pulse amplitude (C), and mean LH level (D) before, during, and after icv infusion of 200 μM tolbutamide (n = 4). *Asterisks* denote pulses determined by ULTRAGUIDE pulse analysis software. The *black bar* indicates the time when tolbutamide was infused into the third ventricle.

group *vs.* 2.15 ± 0.04 in E_2+P group; *, $P < 0.05$; Fig. 6A), whereas E_2 or P alone had no effect on Kir6.2 expression. The stimulatory effect of E_2+P on Kir6.2 mRNA expression in the POA was abolished by pretreatment with RU486 (Fig. 6A). SUR1 mRNA expression levels in the POA were not significantly altered by any of the steroid treatments (Fig. 6B). Expression levels for both subunits in

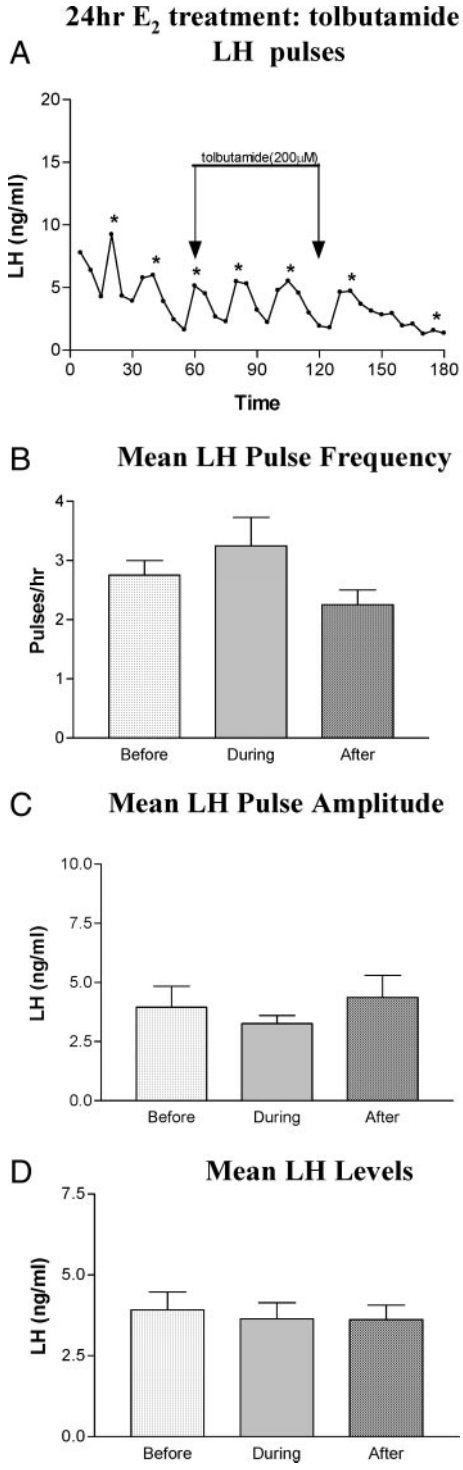

FIG. 2. Representative profile of pulsatile LH release (A) in OVX, 24-h E_2-treated rats and comparison of mean LH pulse frequency (B), mean LH pulse amplitude (C), and mean LH level (C) before, during, and after icv infusion of 200 μM tolbutamide (n = 5). *Asterisks* denote pulses determined by ULTRAGUIDE pulse analysis software. The *black bar* indicates the time when tolbutamide was infused into the third ventricle.

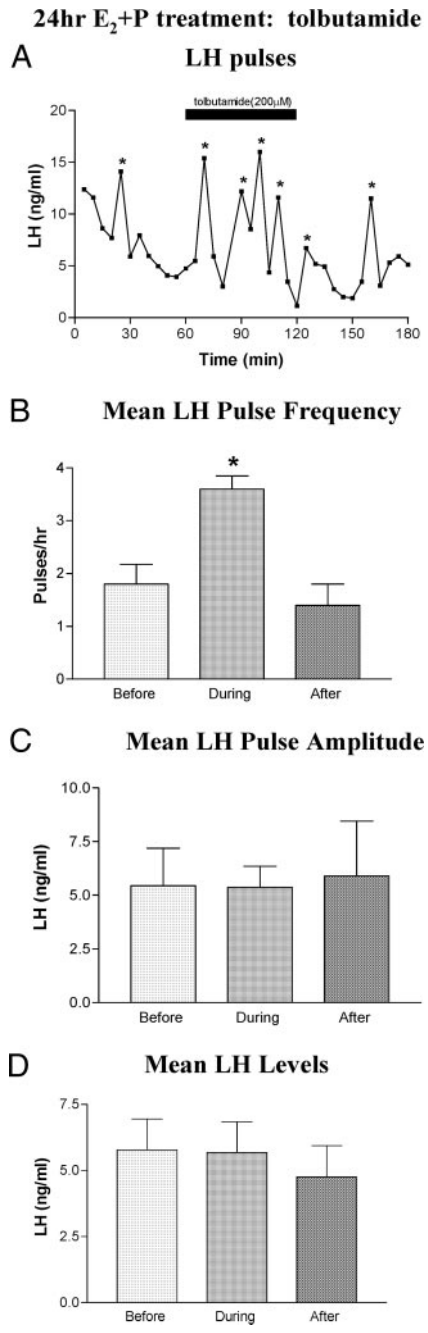

FIG. 3. Representative profile of pulsatile LH release (A) in OVX, 24-h E_2+P-treated rats and comparison of mean LH pulse frequency (B), mean LH pulse amplitude (C), and mean LH level (D) before, during, and after icv infusion of 200 μM tolbutamide (n = 5). *Asterisks* denote pulses determined by ULTRAGUIDE pulse analysis software. The *black bar* indicates the time when tolbutamide was infused into the third ventricle. *, $P < 0.05$ *vs.* before and after.

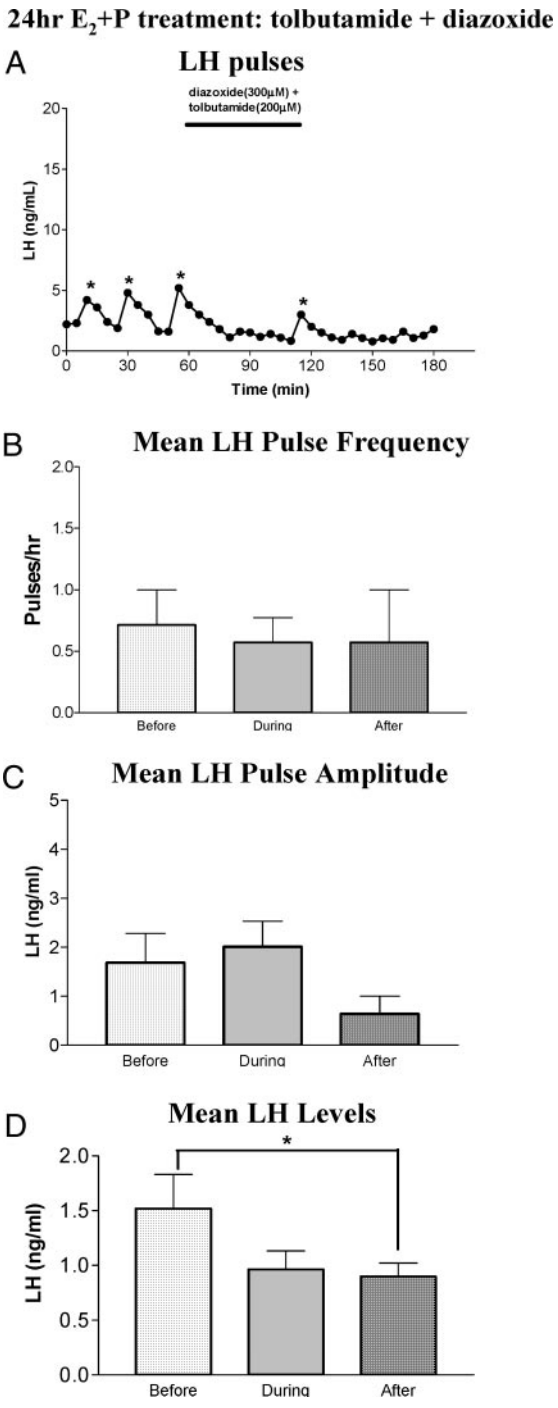

FIG. 4. Representative profile of pulsatile LH release (A) in OVX, 24-h E_2+P-treated rats and comparison of LH pulse frequency (B), pulse amplitude (C), and mean LH level (D) before, during, and after icv infusion of 300 μM diazoxide plus 200 μM tolbutamide (n = 7). *Asterisks* denote pulses determined by ULTRAGUIDE pulse analysis software. The *black bar* indicates the time when the drugs were infused into the third ventricle. *, $P < 0.05$ before *vs.* after.

the MBH tissues were not affected by any steroid hormone treatment (Fig. 6, C and D).

Experiment 4: effects of 8-d steroid treatments on K_{ATP} channel subunit mRNA expression

The expression levels of Kir6.2 and SUR1 mRNA in the POA and MBH after 8 d treatment with different ovarian steroids are depicted in Fig. 7. In the POA, Kir6.2, but not SUR1, subunit expression was significantly up-regulated by 78% after E_2+P treatment (4.94 ± 0.51 in vehicle group *vs.* 8.81 ± 1.30 in E_2+P group; $P < 0.05$; Fig. 7, A and B). In the MBH, SUR1 instead of Kir6.2 mRNA expression was signif-

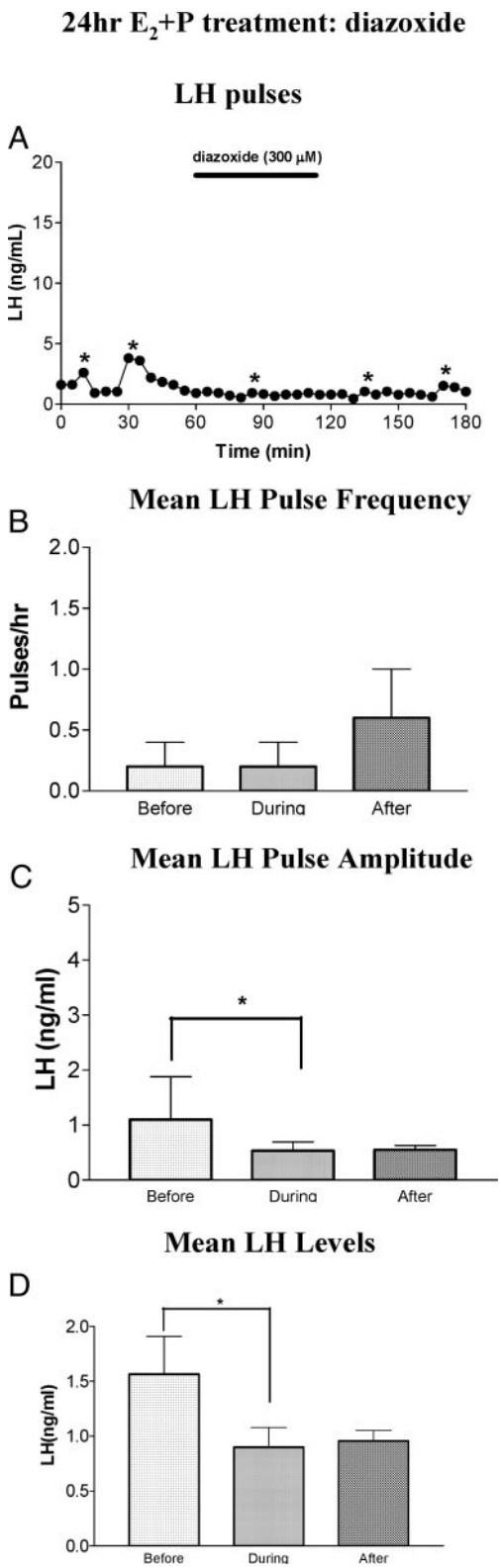

FIG. 5. Representative profile of pulsatile LH release (A) in OVX, 24-h E_2+P-treated rats and comparison of LH pulse frequency (B), pulse amplitude (C), and mean LH level (D) before, during, and after icv infusion of 300 μM diazoxide (n = 6). *Asterisks* denote pulses determined by ULTRAGUIDE pulse analysis software. The *black bar* indicates the time when diazoxide was infused into the third ventricle. *, $P < 0.05$ before *vs.* during.

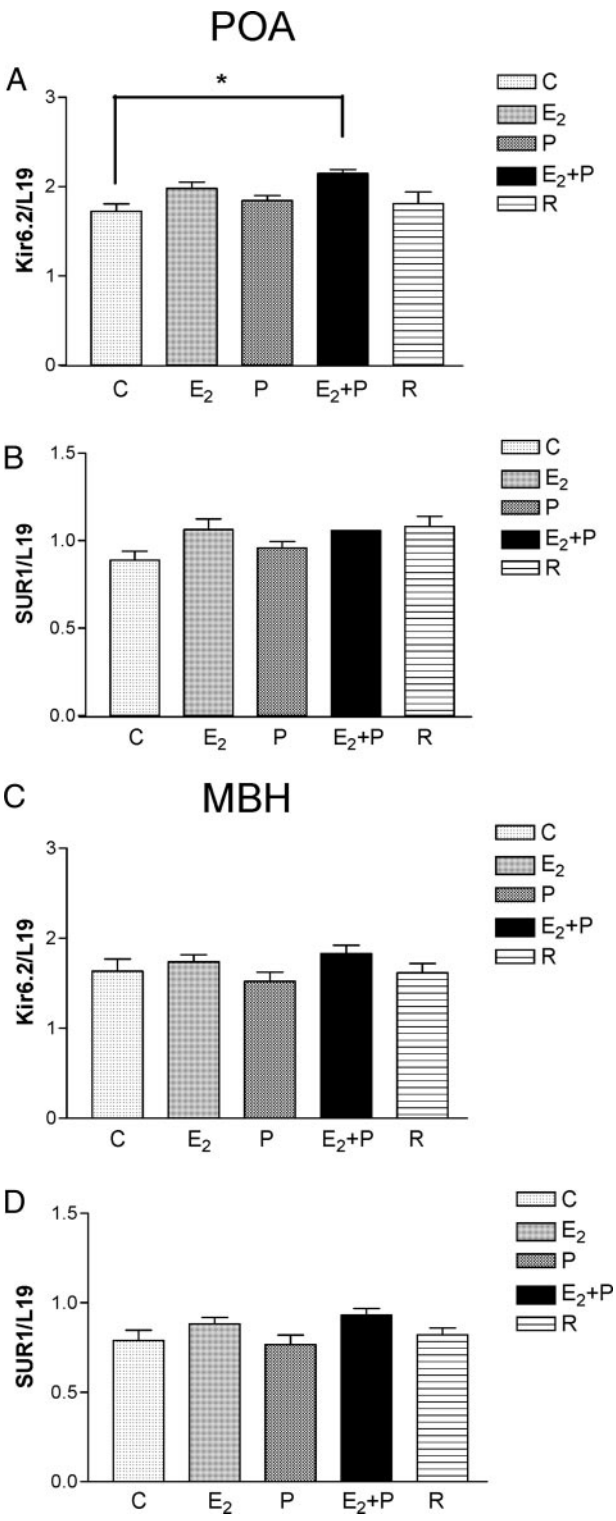

FIG. 6. Comparison of Kir6.2 and SUR1 subunit mRNA expression in the POA (A and B) and MBH (C and D) in OVX, 24-h vehicle- (C), E_2-, P-, E_2+P-, or E_2+P+RU486 (R)-treated rats (n = 5 or 6 for each group). E_2+P significantly up-regulated Kir6.2 mRNA expression in the POA (*, $P < 0.05$), which was reversed by RU486. SUR1 mRNA expression in the POA was not affected by any steroid treatment. Neither subunit in the MBH was affected by estrogen and/or progesterone.

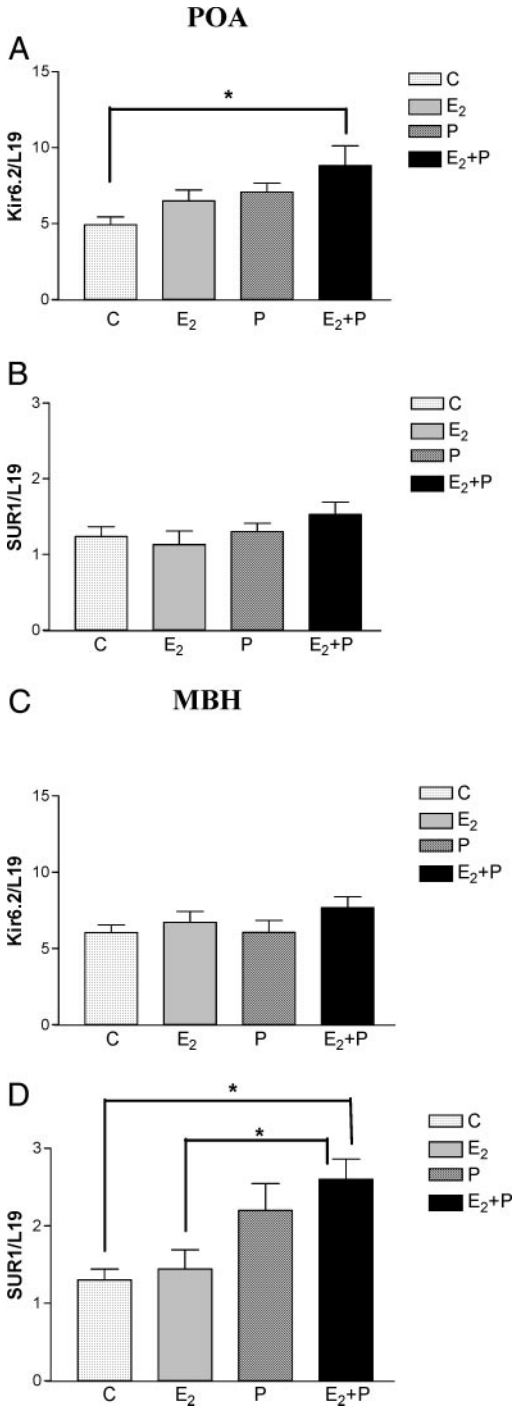

FIG. 7. Comparison of Kir6.2 and SUR1 mRNA expression in the POA (A and B) and MBH (C and D) in OVX, 8-d vehicle- (C), E_2-, P-, or E_2+P-treated rats (n = 8 for each group). In the POA, E_2+P significantly increased Kir6.2 mRNA expression compared with vehicle (*, $P < 0.05$), whereas E_2 or P alone had no significant effects on Kir6.2 mRNA expression. SUR1 subunit expression levels in the POA were not different among different treatment groups. In the MBH, E_2+P significantly increased (*, $P < 0.05$) SUR1 subunit expression compared with vehicle and E_2. Kir6.2 subunit expression in MBH was not altered by any steroid treatment.

icantly increased by E_2+P compared with vehicle and E_2 (1.30 ± 0.14 in C group and 1.44 ± 0.25 in E_2 group *vs.* 2.61 ± 0.26 in E_2+P group; $P < 0.05$; Fig. 7, C and D). The 8-d treatments with E_2 or P alone did not change the expression level of either subunit in both POA and MBH.

Effects of steroid treatments on serum E_2, P, and LH levels

Trunk blood samples from animals in experiments 2 and 3 were assayed for E_2, P, and LH by respective RIAs, and the results of these analyses are provided in Table 1 (E_2 and P) and Fig. 8. The E_2 and P levels in rats receiving E_2 or P treatments for 24 h or 8 d were determined to be in the normal physiological range for these hormones in intact female rats (Table 1). For both the short-term (24 h) and long-term (8-d) treatment groups, the relative LH levels were determined to be highest in the vehicle-treated OVX animals, intermediate in the animals treated with either steroid hormone alone, and lowest in animals treated with the combination of E_2+P. P levels were found to be lower after 8 d *vs.* 24 h of treatment; however, they remained within the physiological range of values for the rat estrous cycle. These measurements confirm that the steroid treatments produced the desired, physiological hormone levels in the steroid-treated animals and that the combined E_2+P treatments effectively provided a negative feedback suppression of LH levels equivalent to that present in ovarian-intact animals (Fig. 8).

Discussion

The K_{ATP} channels function as metabolic sensors, playing critically important roles in protective responses to metabolic stresses such as hypoglycemia (36), ischemia (37), and hypoxia (27). In hypothalamic glucose-responsive neuronal populations, they mediate neurophysiological responses to hypoglycemia, such as increases in feeding behavior and sympathetic activation of pancreatic glucagon secretion (38). However, recent studies have demonstrated that K_{ATP} channels also mediate hypothalamic responses to hormonal signals under normal physiological conditions, such as those conveyed by insulin (16) and leptin (17). In these experiments, we have assessed the roles that K_{ATP} channels may play in the actions of gonadal steroid hormones on neuroendocrine circuitries that govern GnRH release. Our studies were prompted by the observations that both K_{ATP} channels and steroid hormone receptors are expressed in subsets of POMC (20, 21, 39), NPY (20, 40), and GABAergic (8, 20, 41) neurons and that these same cell groups may mediate the feedback actions of ovarian steroids on GnRH neurosecretion (31, 32, 42). Our results reveal that treatment of OVX rats with physiological levels of E_2 and P confers responsiveness of the GnRH pulse generator to K_{ATP} channel modulation, whereas it also stimulates expression of mRNAs encoding K_{ATP} channel subunit Kir6.2 in the POA. These data are consistent with the hypothesis that the suppression of GnRH pulsatility by ovarian hormones is mediated, in part, via the stimulation of K_{ATP} channel expression and/or activation.

TABLE 1. Hormone levels after steroid treatments

Hormone levels	Control[a]	24-h E_2 treatment	8-d E_2 treatment
E_2 (pg/ml)	5.60 ± 0.81	8.15 ± 1.14	29.2 ± 4.59
P (ng/ml)	5.73 ± 0.68	23.36 ± 1.36	10.23 ± 0.99

[a] Combined values for 24-h and 8-d control treatments.

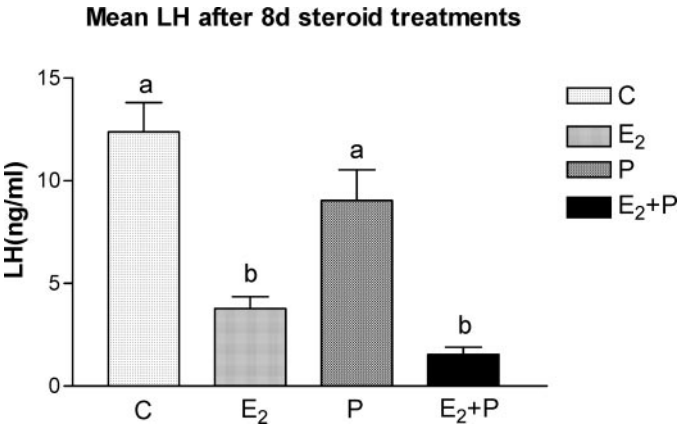

FIG. 8. LH levels after 8-d steroid treatments. E_2 and E_2+P treatment significantly reduced LH levels compared with vehicle and P treatment. Groups with *different letters* are significantly different from each other. $P < 0.05$, E_2 *vs.* P; $P < 0.001$, C *vs.* E_2, C *vs.* E_2+P, and P *vs.* E_2+P.

The expression of Kir6.2 mRNA in the POA is most enhanced in response to combined treatment with E_2 and P, compared with responses to either hormone administered alone. Similarly, the blockade of K_{ATP} channels by tolbutamide results in an enhancement in the frequency of pulsatile LH secretion, and hence the frequency of pulsatile GnRH release, only in rats treated with a combination of the two steroids. The specificity of tolbutamide effects was corroborated by the ability of the channel opener diazoxide to block the enhancement of LH pulse frequency. In addition, diazoxide alone rapidly and strongly suppressed LH pulse amplitude and mean levels, further supporting a role for these channels in the regulation of GnRH/LH secretion. The presence of estrogen is important for the full manifestation of P's negative feedback effects on gonadotropin secretion in female rats (4), and many reports have confirmed that E_2 treatments induce expression of nuclear PRs in the POA and MBH (43, 44). Taken together, these observations suggest that the negative feedback actions of P are transduced by the activation of E_2-induced PRs and that these feedback effects are manifest in part via PR-mediated up-regulation of K_{ATP} channel expression. This idea is further reinforced by the finding that nuclear PR antagonism with RU486 blocks the ability of the combined E_2 and P treatment regimen to increase Kir6.2 mRNA expression (present study) and to suppress LH secretion (6).

Previous studies have also provided evidence for steroid dependency of K_{ATP} channel subunit expression or activity in nonneural tissues. Estrogen has been shown to reduce myocardial injury in ischemia-reperfusion through the activation of K_{ATP} channels (23). In heart-derived H9c2 cells, E_2 increases K_{ATP} channel formation and thereby protects cardiac cells from hypoxia-reoxygenation damage (45). In a canine model of myocardial infarction, E_2 was found to exert an antiarrhythmic effect mediated by activation of sarcolemmal K_{ATP} channels (46). More relevant to the present studies would be information on the effects of P on K_{ATP} channel function; however, we are not aware of any studies before the present ones to have addressed this issue. Nevertheless, the P-dominated hormonal milieu of pregnancy has been shown to be associated with enhanced K_{ATP} channel activity in uterine, cerebral, and renal vascular beds (47). Moreover, it was recently reported that Kir6.2 expression levels in the aorta and kidney are increased in pregnant *vs.* nonpregnant Wistar rats (48) and that K_{ATP} channel blockade reverses the decrease in systolic blood pressure that normally occurs in pregnant animals but does not alter systolic blood pressure in nonpregnant animals (48). These findings suggest that increases in circulating P during pregnancy are associated with an up-regulation of K_{ATP} channel expression and activity, although a causal relationship remains to be demonstrated in these peripheral tissues.

We have found that ovarian steroids alter LH responses to a K_{ATP} channel blocker, tolbutamide, administered in the third cerebroventricle. It is reasonable to assume that these drug effects are exerted within the brain and not on pituitary gonadotropes, because it is generally accepted that modulation of LH pulse frequency, as was observed in these experiments, reflects an antecedent alteration in the frequency of GnRH pulse generation. The locus of tolbutamide's effects within preoptico-hypothalamic tissues, however, cannot be determined from these experiments. Both the 24-h and 8-d E_2 and P treatments stimulated Kir6.2 mRNA expression in the POA but not in the MBH, and increased Kir6.2 expression has been shown in other studies to mediate increased responses to K_{ATP} channel modulators (49). The effects of E_2 and P may therefore be mediated by an induction of Kir6.2 expression in the POA. It also remains possible, however, that steroid-induced alterations in K_{ATP} channel subunit expression are dissociable from those that alter responses to K_{ATP} channel modulation, and thus an MBH action cannot be ruled out.

Thus, the effects of ovarian steroids may be mediated by their actions either in the preoptic cell groups that form afferent circuitries that govern GnRH secretion or in GnRH neurons themselves. The latter possibility is a viable one given the recent demonstration that ERα associated with the membranes of GT1-7 cells (50) and the immunohistochemical demonstration of ERs in a subset of GnRH neurons from adult animals (10). It is also a strong possibility that estrogen and P exert their effects on preoptic GABAergic and/or glutamatergic neurons. Recent studies have implicated GABAergic and glutamatergic neurons as estrogen-sensitive afferents to GnRH neurons that regulate their episodic firing patterns (12). Both neuronal types have been shown to co-express ovarian steroid receptors (8), and their activity is regulated by these steroids (51). GABAergic cells in several brain regions have been demonstrated to express Kir6.2 (20) and exhibit altered electrical activity and GABA release in response to K_{ATP} channel modulators (52). NPY and POMC neurons in the MBH are also candidates as targets of these steroid actions, because they have both been implicated in mediating inhibitory effects on GnRH neurosecretion (31, 32, 53), and subsets of both types of peptidergic neurons express steroid receptors (39, 40) and K_{ATP} channels (20, 21).

We observed a modest increase in Kir6.2 mRNA levels at 24 h after steroid treatments and a robust enhancement after 8 d of treatment. Although expression of the predominant SUR subtype in the brain, SUR1, tended to be higher in the POA of steroid-treated rats, this trend did not reach statistical significance. Expression of SUR1 mRNA was significantly increased

by the 8-d E_2 and P treatment in the MBH. It is possible that our measurements reflect an underestimate of the net effects of steroids on channel subunit expression, because only a subset of neurons expressing Kir6.2 and SUR1 in the POA and MBH also express steroid receptors and would be responsive to the steroid treatments. It is also possible that the stronger effect of E_2 and P is exerted on the expression of Kir6.2 and that the pool of SUR1 is not limiting in the formation of the channel complex. In cardiac tissue, the opposite appears to be true, because E_2 stimulates formation of new channels by stimulating expression of SUR2A in the context of a nonlimiting pool of Kir6.2. However, overexpression of Kir6.2 alone in the forebrain results in increased protection against hypoxic-ischemic injury, suggesting that the total pool of SURx available for coupling is not limiting (37).

Ovarian steroids may additionally exert nongenomic effects that increase the activation of K_{ATP} channels. Apart from their role in glucose sensing, neuronal K_{ATP} channels are known to be activated by circulating metabolic hormones, specifically insulin (16) and leptin (17). They have therefore been suggested to act as integrators of physiological signals that impact energy homeostasis. The actions of insulin, and to a lesser extent leptin, are mediated in part by the activation of phosphoinoside 3-kinase (PI3K), production of phosphatidylinositol triphosphate, and consequent activation of K_{ATP} channels (54). Recent studies have demonstrated that estrogen and other steroid hormones can activate plasma membrane-associated receptors and thereby initiate intracellular signaling pathways that culminate in relatively rapid, nongenomic effects on neuronal function (55). Several of these effects have also been shown to be mediated by the stimulation of PI3K activity (56). It is therefore possible that the ability of E_2 and P to up-regulate LH responsiveness to K_{ATP} channel modulation may be mediated, in part, by the activation of PI3K in target neurons.

In most mammals, sustained activity of the GnRH pulse generator appears to be dependent upon the availability of oxidizable metabolic fuels, thereby coupling fertility to energy reserves that are required for reproductive success. Thus, food restriction, excessive energy expenditure, or other states of negative energy balance produce varying degrees of inhibition of GnRH pulsatility (57). It is likely that this adaptive mechanism is collectively mediated by a number of hormones, neuropeptides, and metabolic signals that exert integrated actions via metabolic sensors in the periphery, brainstem, and preoptic-hypothalamic tissues (57). There is some evidence to suggest that at least some of these pathways conduct signals that converge with steroid hormone feedback mechanisms that regulate GnRH neurosecretion. It has been demonstrated, for example, that food restriction increases the efficacy of estrogen's negative feedback effects on LH secretion (58). In the present studies, we have provided evidence that the expression and/or activation of K_{ATP} channels in the POA may contribute to the negative feedback actions of ovarian steroids on GnRH pulsatility. It is therefore possible that K_{ATP} channel activation may represent one common pathway by which ovarian steroids and hypoglycemia, and perhaps also sustained negative energy balance, may suppress GnRH pulsatility.

In summary, we have demonstrated that combined treatment with E_2 and P stimulates expression of the mRNA encoding the K_{ATP} channel subunit Kir6.2 and confers responsiveness of the GnRH pulse generator to stimulation by tolbutamide, a K_{ATP} channel blocker. Our observations are consistent with the hypothesis that ovarian steroids can exert negative feedback effects on GnRH pulsatility by stimulating the expression of K_{ATP} channels and/or modulating their activity. This idea is more generally consistent with the previous findings that steroids can exert suppressive effects on GnRH neurons, or on the circuitries controlling GnRH neurosecretion, by modulating the activity of other members of the inwardly rectifying K^+ channel superfamily (14). It remains to be determined whether the regulation of K^+ channel gene expression is a common physiological mechanism by which other steroid hormones may regulate hormone-sensitive neurons throughout the brain.

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