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Kainate/Estrogen Receptor Involvement in Rapid Estradiol Effects *in Vitro* and Intracellular Signaling Pathways

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Although the interactions between sex steroids and GnRH have been extensively studied, little is known about the mechanism of estradiol (E2) effects on GnRH secretion. In the present study, we used retrochiasmatic hypothalamic explants of 50-d-old male rats, and we observed that E2 significantly increased the glutamate-evoked GnRH secretion *in vitro* within 15 min in a dose-dependent manner. E2 also significantly increased the L-arginine-evoked GnRH secretion. E2 effects were time dependent because the initially ineffective 10^{-9} M concentration became effective after 5 h of incubation. The E2 effects involved the estrogen receptor (ER) α because they were similarly obtained with the specific ERα agonist 1,3,5-tris(4-hydroxyphenyl)-4-propyl-1H-pyrazole. The use of glutamate receptor agonists and antagonists indicated that E2 effects on GnRH secretion evoked by both glutamate and L-arginine involved the 2-amino-3-hydroxy-5-methyl-4-isoxazol propionic acid/kainate receptors. Similar E2 effects on the kainate-evoked secretion were observed throughout development in both sexes. The observation of similar E2 effects using explants containing the median eminence alone indicated that the median eminence was a direct target for E2 rapid effects on the glutamate-evoked GnRH secretion. The signaling pathways involved in E2 effects included an increase in intracellular calcium and the activation of protein kinase A, protein kinase C, and MAPK. It is concluded that E2 can stimulate the glutamate- and nitric oxide-evoked GnRH secretion *in vitro* through a rapid pathway involving the ER and kainate receptor as well as through a slower mechanism responding to lower E2 concentrations. (*Endocrinology* 146: 2313–2323, 2005)

GNRH IS SYNTHESIZED BY hypothalamic neurons located in the preoptic region and released in the portal vascular system in a pulsatile manner (see review in Ref. 1). Glutamate, the major excitatory neurotransmitter in the hypothalamus (2), can elicit GnRH secretion *in vitro* (3–5) and *in vivo* (6) through activation of ionotropic receptors including the *N*-methyl-D-aspartate (NMDA), kainate, and 2-amino-3-hydroxy-5-methyl-4-isoxazol propionic acid (AMPA) subtypes. Furthermore, activation of NMDA receptors can advance the timing of puberty in monkeys and rats (7, 8), whereas kainate receptors have been involved in the preovulatory LH surge (9). Estradiol (E2) is known to exert complex effects on GnRH neuronal function including long term or genomic effects through binding to estrogen receptor (ER) subtypes (10). The common concept that E2 only acts on GnRH neurons through intermediate cells (10) was challenged after the expression of ER in GnRH neurons has been reported and confirmed (11–14). E2 has recently been shown to act directly on GnRH neurons either by increasing cAMP response element-binding protein phosphorylation (13) or intracellular calcium ($[Ca^{2+}]i$) levels (15). In addition, E2 can also exert rapid effects on the GnRH secretion by stimulating the release of nitric oxide (NO) (16) and this latter mediator can also evoke GnRH secretion by itself (17).

In a recent study with rat hypothalamic explants, we have shown that E2 could increase the frequency of pulsatile GnRH secretion *in vitro* in a sex- and age-dependent manner (18). Such *in vitro* E2-mediated effects required the participation of kainate and ERs. Similar effects of E2 on kainate receptor-mediated effects have also been reported in hippocampal (19) or oxytocinergic neurons (20). Although previous attempts to show involvement of AMPA/kainate receptors in the onset of puberty had failed (21), we recently provided some evidence that both kainate receptors and ERs are involved in the occurrence of precocious puberty after *in vivo* administration of E2 (18). To our knowledge, such interactions between E2/ER and kainate receptors in the control of GnRH secretion and the underlying mechanisms had not been described so far. To further investigate these interactions, we studied the effect of E2 on the glutamate-evoked GnRH secretory response using rat hypothalamic explants.

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Abbreviations: AMPA, 2-Amino-3-hydroxy-5-methyl-4-isoxazol propionic acid; AP5, DL-2 aminophosphonopentanoic acid; CA, cyclopiazonic acid; $[Ca^{2+}]i$, intracellular calcium; 5α-DHT, 5α-dihydrotestosterone; DNQX, 6,7-dinitroquinoxaline-2,3-dione; E2, estradiol; ER, estrogen receptor; IP3, inositol triphosphate; L-Arg, L-arginine; ME, median eminence; NMA, *N*-methyl-D,L-aspartate; NMDA, *N*-methyl-D-aspartate; NO, nitric oxide; PKA, protein kinase A; PKC, protein kinase C; POA, preoptic area; PPT, 1,3,5-Tris(4-hydroxyphenyl)-4-propyl-1H-pyrazole; RCH, retrochiasmatic hypothalamus; SERCA, sarco(endo)plasmic reticulum Ca^{2+}-ATPase; SYM 2206, 4-aminophenyl-1,2-dihydro-1-methyl-2-propylcarbamoyl-6,7-methylenedioxyphtalazine.

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Materials and Methods

Animals and hypothalamic explant incubation

Male and female Wistar rats aged 5, 15, 25, and 50 d were purchased at the University of Liège. All experiments were carried out with the

approval of the Belgian Ministry of Agriculture and the Ethical Committee at the University of Liège.

After decapitation, the hypothalamus was rapidly dissected. The limits to obtain the retrochiasmatic hypothalamus (RCH) were the caudal margin of the optic chiasma, the rostral margin of the mammillary bodies, and the lateral hypothalamic sulci (22). To include the preoptic area (POA) in some explants (RCH-POA explants), the anterior cut was at the rostral margin of the optic chiasma. Median eminence (ME) explants were dissected with microscissors under a binocular microscope (TL2, ×4, Olympus, Tokyo, Japan). Each explant was transferred into an individual incubation chamber containing 500 μl of phenol-red free MEM supplemented with glucose, magnesium, glycine, and bacitracin to achieve final concentrations of 25 mM, 1 mM, 10 nM, 20 μM, respectively. The explants were maintained in a static incubator at 37.5 C and in an atmosphere of 95% O^2-5% CO_2. In each experiment, 12–15 explants were studied individually for 4–7.5 h. The medium was collected every 7.5 min and was kept frozen until assayed for GnRH.

GnRH assay

Using 100 μl of the incubation medium, GnRH was measured in duplicate. The RIA procedure has been described in detail previously, with intra- and interassay coefficients of 14 and 18%, respectively (3, 23). The CR11-B81 anti-GnRH antiserum (final dilution 1:80,000) was kindly provided by Dr. V. D. Ramirez (Urbana, IL) (24). The data below the limit of detection (5 pg per 7.5 min) were assigned that value.

Reagents

All reagents, except steroids and R76713, were directly diluted in MEM. The GnRH secretagogues included glutamate (Sigma, Bornem, Belgium), *N*-methyl-D,L-aspartate (NMA, Sigma), kainate (Opika-1 kainic acid, Ocean Produce International, Shelburne, Nova Scotia, Canada), and L-arginine (L-Arg, Sigma). NMDA, AMPA/kainate, or AMPA receptors were respectively blocked by AP5 (DL-2 aminophosphonopentanoic acid, Sigma), DNQX (6,7-dinitroquinoxaline-2,3-dione; Tocris, Illkirch, France) or SYM 2206 (4-aminophenyl-1,2-dihydro-1-methyl-2-propylcarbamoyl-6,7-methylenedioxyphtalazine; Tocris).

All steroids were initially dissolved in absolute ethanol and subsequently diluted in the incubation medium to achieve a final ethanol concentration of 0.01%. 17β-Estradiol, 17α-estradiol, progesterone, testosterone, 5α-dihydrotestosterone (5α-DHT), and the specific ERα agonist PPT (1,3,5-Tris(4-hydroxyphenyl)-4-propyl-1H-pyrazole) were purchased at Sigma. The ER antagonists tamoxifen and ICI 182.780 were obtained from Tocris and used at 10^{-6} and 10^{-7} M, respectively. The aromatase inhibitor, R76713 (racemic Vorozole; Janssen Pharmaceutica, Beerse, Belgium) was used at 10^{-5} M. R76713 was initially diluted in a 20% polyethyleneglycol solution (3.35 mg/ml, VWR International, Leuven, Belgium) and subsequently diluted in the incubation medium to achieve a 10^{-5} M solution containing 0.02% polyethyleneglycol.

To study protein kinase involvement in E2 effects, the following inhibitors were used: staurosporine (nonspecific protein kinase inhibitor, 10^{-7} M; Sigma), adenosine 3′,5′-monophosphorothioate Rp isomer [Rp-cAMP: protein kinase A (PKA) inhibitor (cadenosine 3′,5′-monophosphorothioate Rp isomer), 10^{-5} M; Calbiochem, VWR international], chelerythrine chloride [protein kinase C (PKC)-specific inhibitor, 10^{-5} M; Sigma], or PD98,059 (MAPK inhibitor, 2′-amino-3′-methoxyflavone, 5.10^{-5} M; Calbiochem, VWR International). To inhibit phospholipase C, U73122 was used (Sigma), whereas modulation of calcium levels from intracellular stores was studied by using caffeine (1,3,7-trimethyxanthine) and the sarco(endo)plasmic reticulum Ca^{2+}-ATPase (SERCA) pumps inhibitor, cyclopiazonic acid (both from Sigma).

Study protocols

Effect of E2 on the GnRH secretion evoked by glutamate receptor agonists: dose and time dependency. We previously reported that millimolar concentrations of glutamate or glutamate receptor agonists were required to increase the *in vitro* GnRH secretion in a dose-related manner (3, 25). In the present experiments, the secretory response of GnRH was evoked every 37.5 min by a submaximal concentration (10^{-2} M) of glutamate or the glutamate receptor agonists, NMA or kainate. The explants were stimulated for 7.5 min by the secretagogue (*i.e. black boxes* for kainate in Fig. 1A) every 37.5 min (*i.e.* every fifth fraction of 7.5 min). The explants were first stimulated by the secretagogue alone, and the GnRH secretory

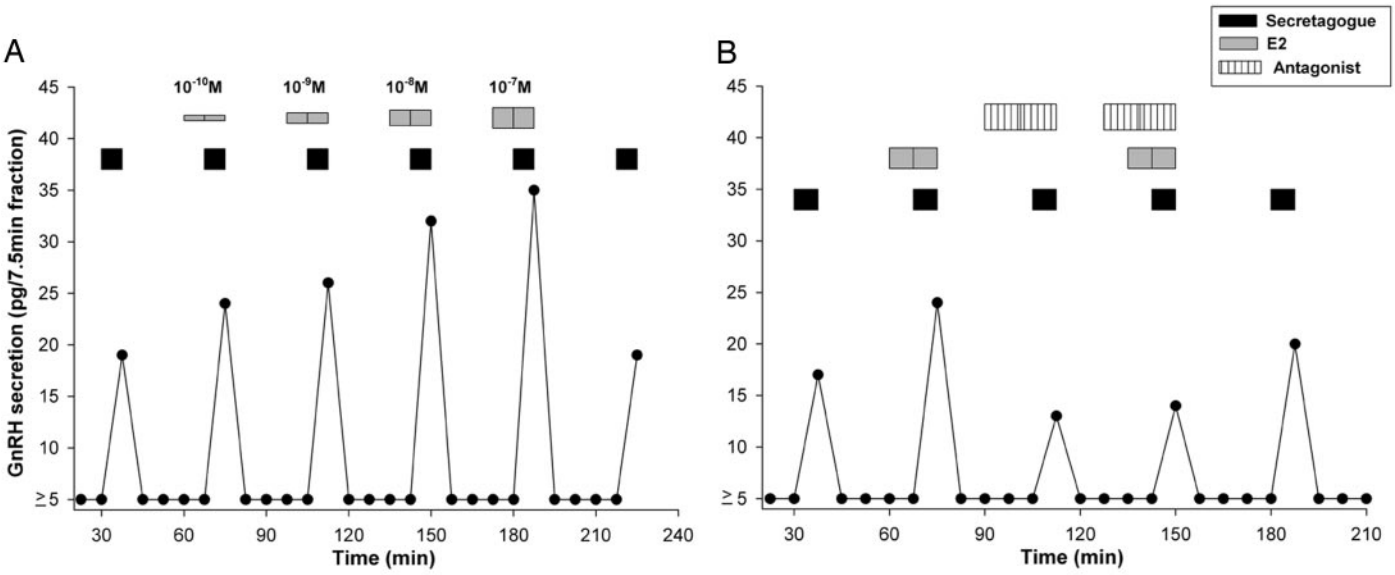

FIG. 1. A, Representative profile of GnRH secretion from a RCH explant obtained from a 50-d-old male rat and stimulated every 37.5 min with 10^{-2} M of kainate as secretagogue (*black boxes*). After a 7.5-min stimulation with kainate alone (control stimulation), increasing concentrations of E2 (*gray boxes*) were added to the medium for two 7.5-min fractions, before and together with kainate. At the end of the experiment, the secretory response to kainate was studied again in control conditions. B, Effects of E2 (10^{-7} M, *gray boxes*) on the GnRH secretory response obtained from a 50-d-old male rat hypothalamic explant. The GnRH secretory response was evoked by glutamate as a secretagogue (*black boxes*) in the presence of SYM 2206 as an antagonist of the AMPA receptors (*hatched bars*). The antagonist was used for two 7.5-min fractions before and one 7.5-min fraction together with the glutamate stimulation, which amounts to 22.5 min of total incubation time with the antagonist. E2 (*gray boxes*) was added to the incubation medium following the same protocol as explained in A. The experiment was ended by a stimulation with the secretagogue alone.

response obtained at that time was considered as the control response. The effects of E2 on the secretagogue-evoked GnRH secretion were studied by addition of increasing concentrations of E2 in the incubation medium 7.5 min before and together with glutamate (*i.e. gray boxes* in Fig. 1A). The explants were stimulated repeatedly with the secretagogue at the same concentration through the entire experiment, and a final stimulation with the secretagogue alone was done to assess the viability of the explants by comparing the final response with that obtained initially (Fig. 1A). Based on the dose-response study, we used a 10^{-7} M concentration of E2 in subsequent experiments except when stated otherwise.

The effect of reducing the incubation time with E2 was investigated by shortening to 7.5 min the length of the period during which the explants were incubated with E2. The effect of increasing the time of incubation with E2 on the potentiation of the glutamate-evoked release of GnRH was studied by using 15-d-old female rat hypothalamic explants, which were stimulated for 7.5 min with 10^{-2} M of glutamate every 37.5 min during an experiment of 7.5 h. After the initial control stimulation in the absence of E2, the RCH explants were continuously incubated in either control medium alone (control) or medium added with 10^{-9}, 10^{-8}, or 10^{-7} M of E2. The explants were incubated in regular medium without E2 during the last five 7.5-min periods, and the explant viability was assessed by a glutamate stimulation at the end of the experiment.

Involvement of glutamate receptor subtypes and NO in E2 effects on the GnRH secretion. The specific involvement of each ionotropic glutamate receptor subtype was studied through the use of specific glutamate receptor antagonists. NMDA, AMPA/kainate, or AMPA receptors were respectively blocked by AP5, DNQX, or SYM 2206. Each antagonist was added to the incubation medium for two 7.5-min fractions before and one 7.5-min fraction during the secretagogue stimulation (Fig. 1B), which amounts to 22.5 min of total incubation time with the antagonist.

We used L-Arg as a NO precursor to evoke GnRH secretion. Based on a dose-response study in comparison with 10^{-2} M of glutamate, a 2×10^{-2} M concentration of L-Arg was used to evoke a GnRH secretory response from hypothalamic explants. The effects of E2 on the GnRH secretory response evoked by L-Arg as a secretagogue was studied by using the same experimental design as that shown in Fig. 1A. DNQX was used in the same way as explained above (see Fig. 1B), to examine the involvement of AMPA/kainate receptor in E2 effect on the L-Arg-evoked GnRH secretion.

Steroid and receptor specificity of E2 effects. The specificity of E2 effects on the glutamate-evoked GnRH secretion was investigated with different steroids. The explants were first stimulated by glutamate alone (control stimulation) and then in the presence of 10^{-7} M of E2 (as a positive control). The same experimental protocol was used to study the effects of 17α-estradiol, progesterone, and testosterone on the glutamate-evoked GnRH secretion. These steroids were used at the same concentration as E2 (10^{-7} M) except E2-BSA, which was also used at 10^{-6} M.

The involvement of classical ERs in E2 amplification of the glutamate-evoked GnRH secretion was investigated by the use of the ER antagonists tamoxifen and ICI 182.780 and the specific ERα agonist PPT. The ER antagonists were used in the same way as the glutamate receptor antagonists. After a control stimulation with glutamate alone (control stimulation) and glutamate in the presence of E2 (positive control), increasing concentrations of PPT were tested according to the experimental pattern shown in Fig. 1A.

The aromatase inhibitor R76713 was used at 10^{-5} M based on dose-response studies (data not shown). The aromatase inhibitor and 5α-DHT were used to verify that the testosterone effect on the glutamate-evoked GnRH secretion required its prior aromatization into E2. In this experiment, the explants were first stimulated by glutamate alone (control stimulation) and then by glutamate in the presence of testosterone (positive control). The aromatase inhibitor was then used for 22.5 min such as described in the case of the glutamate receptor antagonists (Fig. 1B), whereas the effect of 5α-DHT was studied through its addition to the medium for two 7.5-min fractions before and together with the glutamate stimulation (same design as Fig. 1A).

E2 effects on GnRH-evoked secretion obtained from RCH-POA, RCH, or ME explants. After dissection and transfer to the incubator, the three different types of explants were first stimulated with the secretagogue alone as a control response (glutamate, NMA, or kainate) and then with the secretagogue in the presence of E2 (same design as in Fig. 1A).

E2 effects in relation to age and sex. The effects of age and sex on the kainate-evoked GnRH secretion were studied by using RCH hypothalamic explants from male and female rats at 5, 15, 25, or 50 d. Hypothalamic explants were subjected to the same protocol as in Fig. 1A with kainate as the stimulatory secretagogue.

Intracellular signaling pathways involved in E2 effects. To study protein kinase involvement in E2 effects, the following protein kinase inhibitors were used: staurosporine, Rp-cAMP, chelerythrine chloride, or PD98,059. To inhibit phospholipase C, U73122 was used, whereas the participation of $[Ca^{2+}]i$ was studied using caffeine and cyclopiazonic acid.

Analysis of the GnRH secretory response evoked by a secretagogue

At least four explants were used in each experimental group. Each explant was used as its own control. The secretory response (picogram per 7.5-min fraction) was calculated as the difference between the levels in the 7.5-min fractions obtained immediately before and during exposure to the secretagogue. To compare the results displayed in different graphs and tables, data were expressed as percentages of the control response. Such a transformation did not affect the statistical analysis because identical significance levels were obtained using data expressed as absolute levels (picogram per 7.5 min) or percentages relative to controls. The GnRH values (in picograms) for the secretagogue stimulation alone (control stimulation) are also mentioned in the tables or graph legends.

Data were analyzed by one-way ANOVA with correction for repeated measures except for comparisons of E2 effect between males and females (two individual experiments) in which unpaired one-way ANOVA was used. Each test was followed by Newman-Keuls posttest when the threshold for significance of differences ($P < 0.05$) was reached (GraphPad Prism software; GraphPad, San Diego, CA).

Results

Involvement of the kainate receptor subtype in E2 effects on GnRH secretion evoked by glutamate receptor agonists or NO

Figure 2 shows the effect of increasing concentrations of E2 on the GnRH secretory response evoked by glutamate, kainate, or NMA used as secretagogues. In each case, the stimulation of RCH explants (obtained from 50-d-old male rats) with the secretagogue alone elicited a GnRH secretory response that was considered as the control response. The baseline GnRH secretory level was usually undetectable and did not increase during incubation with E2 alone (data not shown). A dose-related increase in the GnRH secretory response was caused by E2 and was significant from a concentration of 10^{-8} or 10^{-7} M in E2 when the explants were stimulated by kainate (Figs. 1A and 2A) or glutamate (Fig. 2A), respectively. No effect of E2 was observed when GnRH secretion was evoked by NMA (Fig 2A). The kainate receptor subtype was specifically involved because the AMPA/kainate receptor antagonist DNQX completely prevented E2 effect, whereas E2 was still able to increase the glutamate-evoked secretion in the presence of the NMDA or AMPA receptor antagonists AP5 or SYM 2206, respectively (Fig. 2B). In the absence of E2, a decrease in the GnRH secretory response was caused by each of the three glutamate receptor subtypes antagonists used separately, indicating that they all participated in the glutamate-evoked GnRH secretion.

Because E2 was reported to increase the basal GnRH se-

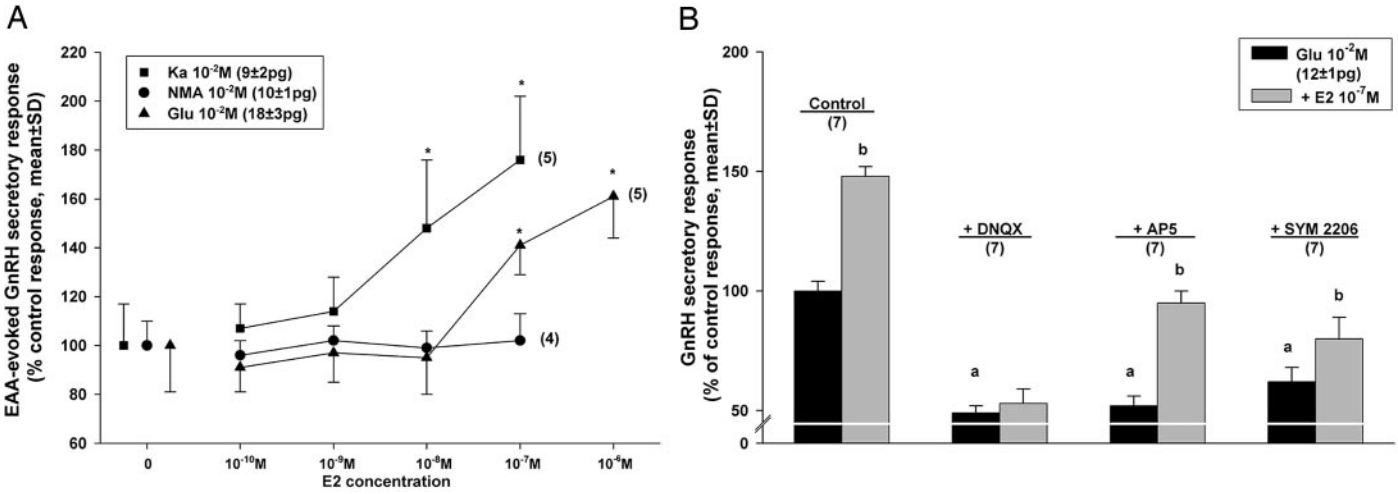

FIG. 2. A, Effects of increasing concentrations of E2 on the GnRH secretory response evoked by different excitatory amino acids (EAAs) including glutamate (Glu), kainate (Ka), and NMA. The results (mean ± SD) are expressed in percentage of the control response (10^{-2} M of the EAA used alone). The design is similar to the experiment illustrated in Fig. 1A. The number of explants (n) for each experiment is mentioned on the graph and the values (mean ± SD in picograms) for the control GnRH secretory response evoked by each of the three different secretagogues are displayed in the legend box. The experiments used RCH hypothalamic explants obtained from 50-d-old male rats. *, $P < 0.05$, E2 together with EAA *vs.* controls with EAA alone (before and after exposure to E2). B, Effects of antagonists of glutamate receptor subtypes on the E2 stimulation of the glutamate-evoked GnRH secretion obtained from 50-d-old male rat hypothalamic explants. Antagonists of AMPA/kainate (DNQX), NMDA (AP5), and AMPA (SYM 2206) receptors were used at a 10^{-6} M concentration. The value for the GnRH secretory response evoked by glutamate alone (control stimulation) is 12 ± 1 pg, and the number of explants (n) for each experiment is mentioned on the graph. a, $P < 0.05$, glutamate+glutamate receptor antagonist *vs.* control glutamate stimulation; b, $P < 0.05$, E2-treated *vs.* control glutamate stimulation.

cretion in a NO-dependent manner (16), we investigated the effect of E2 on the GnRH secretory response evoked by L-Arg, a NO precursor. Based on the dose-response effect of L-Arg on GnRH secretion in comparison with glutamate (Table 1), a 2×10^{-2} M concentration of L-Arg was used. Using increasing concentrations of E2, the L-Arg-evoked GnRH secretory response was significantly increased by E2 at concentrations of 10^{-8} M or higher (Fig. 3A). Such an effect involved the AMPA/kainate receptor subtype because the significant increase in L-Arg-evoked GnRH secretory response induced by 10^{-7} M of E2 (L-Arg: 9 ± 1 pg or 100 ± 9%; L-Arg+E2: 143 ± 9%, $P < 0.05$; L-Arg *vs.* L-Arg+E2) was prevented by the AMPA/kainate receptor antagonist DNQX at 10^{-6} M (L-Arg+DNQX: 87 ± 8%; L-Arg+E2+DNQX: 93 ± 6%; $P < 0.05$, L-Arg+E2 *vs.* L-Arg+E2+DNQX; $P > 0.05$, L-Arg+DNQX *vs.* L-Arg+E2+DNQX). When used alone, DNQX also significantly decreased the L-Arg-evoked GnRH secretion ($P < 0.05$, L-Arg *vs.* L-Arg +DNQX).

TABLE 1. Mean (±SD) GnRH secretory response from RCH hypothalamic explants (n = 4) obtained from 50-d-old male rats

Secretagogue	Concentration (M)	GnRH secretory response (mean ± SD)	
		pg/7.5 min	% of Glu stimulation
Glu	10^{-2}	33 ± 4	100 ± 13
L-Arg	10^{-3}	*a*	*a*
L-Arg	5×10^{-3}	9 ± 3	26 ± 8
L-Arg	10^{-2}	15 ± 3	44 ± 10
L-Arg	5×10^{-2}	55 ± 8	163 ± 24

The explants were stimulated by glutamate or increasing concentrations of L-Arg.

[a] Value below the detection level.

Time dependency of E2 effects

When E2 was used for a total of 15 min (*i.e.* the 7.5-min fractions before and during exposure to glutamate), a significant stimulatory effect was observed using 10^{-7} M of E2 (Fig. 2, Table 2). When the steroid was used only for 7.5 min together with glutamate, a significant stimulation could be observed, but a 10^{-6} M concentration of E2 was required

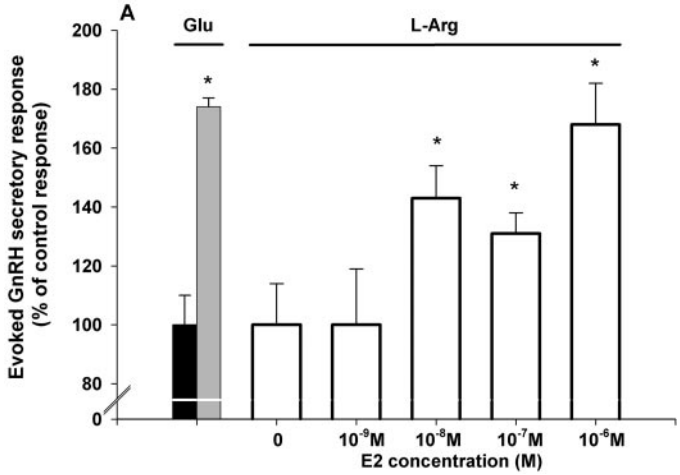

FIG. 3. Effects of increasing concentrations of E2 on the L-Arg-evoked GnRH secretion (*open bars*) in comparison with E2 (10^{-7} M, *gray bar*) effects on the glutamate (Glu)-evoked GnRH secretion (control response, *black bar*). RCH hypothalamic explants were obtained from 50-d-old male rats (n = 4). The GnRH secretory response evoked by glutamate was 14 ± 2 pg, and the response evoked by L-Arg was 9 ± 1 pg. *, $P < 0.05$, E2+secretagogue (glu or L-Arg) *vs.* secretagogue alone.

TABLE 2. Mean (±SD) GnRH secretory response evoked by glutamate expressed as percentage of control response (glutamate alone, 10^{-2} M), in the presence of different steroids and steroid antagonists

n	Steroid	Antagonist (for 22.5 min)	Steroid concentration (for 15 min)		
			0 (pg)	0 (Control in %)	10^{-7} M
4	E2		15 ± 4	100 ± 26	150 ± 28[a]
	E2	Tamoxifen		90 ± 12	84 ± 17
4	E2		13 ± 2	100 ± 15	168 ± 14[a]
	E2	ICI 182.780		93 ± 11	83 ± 13
4	17α-E2		36 ± 8	100 ± 22	103 ± 25
4	P				93 ± 34
4	T		22 ± 2	100 ± 10	140 ± 12[a]
4	5α-DHT				106 ± 10
4	T	R76713	22 ± 2	104 ± 11	100 ± 7

In each experiment, four to five RCH explants from 50-d-old male rats were used. The ER antagonists tamoxifen and ICI were used at 10^{-6} M and 10^{-7} M, respectively. The aromatase inhibitor (R76713) was used at 10^{-5} M. P, Progesterone; T, testosterone.

[a] $P < 0.05$, E2 *vs.* control.

(control: 27 ± 4 pg per 7.5 min or 100 ± 15%; E2 10^{-7} M: 141 ± 32%; E2 10^{-6} M: 178 ± 47%; $P < 0.05$ control *vs.* E2 10^{-6} M).

When explants were continuously incubated with different concentrations of E2 for a prolonged period of 7 h (Fig. 4), a significant increase in the glutamate-evoked GnRH secretion was observed at lower concentrations of E2 (10^{-9} and 10^{-8} M) that were previously found to be ineffective. After 4 h of incubation, a 10^{-8} M concentration of E2 became as effective as 10^{-7} M, and after 5.5 h, 10^{-9} M of E2 started to be significantly active. Noteworthy, in the control group, there was no significant difference in the secretory responses of GnRH evoked by glutamate alone during the whole experiment, whereas after the explants were incubated with E2 for 7 h, the GnRH secretory response to the control stimulation at the end of the experiment was significantly greater than the initial control response evoked by glutamate alone.

Steroid and receptor specificity of E2 effects

To confirm the specificity of E2 effects, we tested the effects of progesterone and 17α-estradiol on the glutamate-evoked GnRH secretion. Both steroids had no effect on the glutamate-evoked GnRH secretion (Table 2). Using 10^{-7} M of testosterone, the glutamate-evoked GnRH secretion was significantly increased in an aromatase-dependent manner because the aromatase inhibitor R76713 prevented that effect, whereas the nonaromatizable androgen 5α-DHT had no effect (Table 2). Two different ER antagonists, ICI 182.780 and tamoxifen, totally prevented the stimulating effect of E2 on GnRH secretion evoked by glutamate (Table 2). The involvement of ERα was further investigated through the use of PPT, a specific ERα agonist that was recently described (26). PPT resulted in a significant dose-dependent stimulation (from 10^{-6} M) of the glutamate-evoked GnRH secretion (Table 3).

ME as a site of E2 effects on GnRH-evoked secretion

As mentioned before, the cell bodies of GnRH neurons are located in the POA of the rat hypothalamus. The RCH explants do not contain the POA, and then no or very few GnRH cell bodies can be found (27). In the aforementioned experiments, E2 effects were studied using RCH hypothalamic explants that contain only GnRH axons and the terminals located in the ME. We then studied E2 effects on the

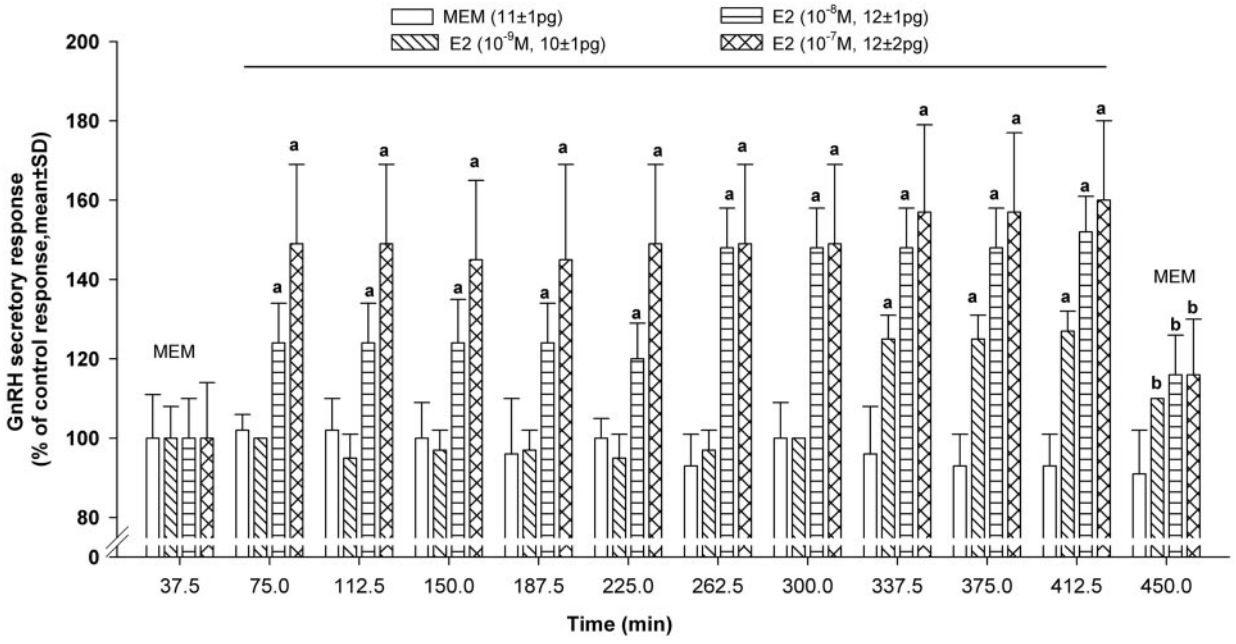

FIG. 4. Effects of different concentrations of E2 (10^{-9}, 10^{-8}, and 10^{-7} M) on the GnRH secretory response evoked by glutamate (10^{-2} M) every 37.5 min (five 7.5-min fractions). The RCH explants, obtained from 15-d-old female rats, were first incubated in MEM alone until the first glutamate stimulation (control stimulation) and then one group (n = 4) was further incubated with MEM alone (MEM), whereas the three other groups (n = 4 in each group) were continuously incubated with different concentrations of E2. At the end of the experiment, the explants were incubated with MEM alone during 30 min (four 7.5-min fractions) before the last glutamate control stimulation. The values for the GnRH secretory response evoked by the glutamate alone (control stimulation in picograms) are shown in the legend. a, $P < 0.05$, glutamate+E2 *vs.* first glutamate stimulation (control); b, $P < 0.05$: first glutamate stimulation *vs.* last glutamate stimulation.

TABLE 3. Mean (±SD) GnRH secretory response evoked by glutamate (10^{-2} M) in the presence of increasing concentrations of the ERα-specific agonist PPT

Steroid	Concentration (M)	
None		100 ± 12
E2	10^{-7}	148 ± 15[a]
PPT	10^{-8}	106 ± 9
PPT	10^{-7}	102 ± 11
PPT	10^{-6}	116 ± 11[a]
PPT	10^{-5}	133 ± 15[a]

The results are expressed as percentage of the control stimulation (glutamate alone, 34 ± 4 pg). GnRH secretory response was obtained from RCH hypothalamus (n = 4) of 50-d-old male rats.

[a] $P < 0.05$, E2 *vs.* control.

GnRH secretory response in RCH explants containing the POA (RCH+POA, Table 4). In addition, the ME, which contains GnRH terminals only and the neighboring afferent axons, astroglial cells, and tanycytes as well as endothelial cells, was also used to study E2 effects. E2 (10^{-7} M) increased the GnRH secretion evoked by glutamate or kainate, but not NMA, in a similar manner using the three types of explants (Table 4). These data suggest that ME is a direct target of kainate receptor-mediated stimulatory effects of E2 on GnRH secretion.

Sex and age independence of E2 effects

Using RCH explants from male and female rats aged 5, 15, 25, and 50 d, GnRH secretion evoked by kainate was significantly increased by E2 in a dose-dependent manner (10^{-8} to 10^{-6} M, Fig. 5). At the highest E2 concentration studied (10^{-6} M), a significant sex difference was observed. This sex difference was variable with age because E2 effects were greater in males than females at 5 and 50 d, whereas females showed higher responses at 15 and 25 d. The GnRH control response evoked by glutamate alone was higher in females than in males at the four studied ages.

Intracellular signaling pathways involved in E2 effects

The involvement of $[Ca^{2+}]$i levels in E2 effects on the GnRH secretory response was also studied (Table 5). The depletion of ryanodine receptor- and inositol triphosphate (IP3) receptor-dependent $[Ca^{2+}]$i stores by caffeine did not prevent E2 effects. Cyclopiazonic acid (CA), an inhibitor of SERCA pumps, prevented the replenishment of the $[Ca^{2+}]$i stores after stimulation, thus maintaining a higher concentration of $[Ca^{2+}]$i in the cell. In the presence of CA, the control GnRH secretory response to glutamate was significantly increased, whereas the E2-potentiating effects on the glutamate-evoked GnRH secretory response were not prevented (Table 5). The above results indicate that an increase in $[Ca^{2+}]$i from intracellular stores is not involved in E2 effect. On the contrary, calcium from extracellular environment appears to be involved in E2 effects because nickel, an inhibitor of calcium entry (28), and nifedipine, an inhibitor of L-type voltage-gated calcium channels, prevented E2 effects (Table 5).

Because an increase in $[Ca^{2+}]$i can activate protein kinases, we investigated the involvement of different protein kinases in E2 effect (Table 5). U73122, which is a phospholipase C inhibitor, prevents the formation of IP3 and DAG and then inhibits the subsequent release of IP3 receptor-dependent $[Ca^{2+}]$i and the phosphorylation of PKC. U73122 had no effect on the control glutamate stimulation, whereas it prevented E2 effects. From the above results using caffeine, we hypothesized that this inhibitory effect resulted from the inhibition of PKC rather than the IP3 pathway. Such an involvement of PKC in E2 effect was confirmed by the use of chelerythrine chloride, a PKC inhibitor, which prevented E2 effect. Other inhibitors of protein kinases including staurosporine (PKA and PKC inhibitor), Rp-cAMP isomer (specific PKA inhibitor) and PD98,056 (MAPK inhibitor) were used, and each of them prevented E2 effects. When used alone, the specific PKA and PKC inhibitors slightly decreased GnRH secretory response to glutamate stimulation. However, this decrease was significant only when PKA and PKC were both inhibited by staurosporine.

Discussion

Using rat hypothalamic explants, we report here that E2 rapidly increased (within 7.5–15 min) the GnRH secretion evoked by glutamate in a dose-dependent manner through a pathway involving ERs and the kainate receptor subtype. Using rat hypothalamic slices, Drouva *et al.* (29) reported that GnRH secretion evoked by a potassium depolarization was increased by E2 within 6 min, and similar effects have been found in the quail (30). To our knowledge, it is the first time that E2 and kainate receptors are reported to interact in a mechanism resulting in an increase of the GnRH secretory response evoked by glutamate. In previous experiments (18), we showed that, in the rat, E2 could increase the frequency of the pulsatile GnRH secretion *in vitro* and that such an effect was correlated to the occurrence of sexual precocity *in vivo*.

TABLE 4. E2 effect on glutamate-receptor-mediated secretion of GnRH using hypothalamic explants (n) obtained from 50-d-old male rats

Explants	RCH + POA		RCH		ME	
	Control	+ E2	Control	+ E2	Control	+ E2
Glutamate	100 ± 11 (22 ± 3 pg)	133 ± 11[a]	100 ± 14 (7 ± 1)	130 ± 14[a]	100 ± 9 (7 ± 1)	123 ± 13[a]
Kainate	100 ± 14 (23 ± 3 pg)	132 ± 8[a]	100 ± 10 (6 ± 1)	143 ± 10[a]	100 ± 14 (7 ± 1)	124 ± 13[a]
NMA	100 ± 5 (21 ± 1 pg)	113 ± 1	100 ± 10 (7 ± 1)	103 ± 12	100 ± 14 (7 ± 1)	114 ± 9

The explants included both the POA and the RCH or the RCH only or the ME. The control GnRH secretory response was obtained by stimulating each explant with one of the secretagogues alone (10^{-2} M, control response) and the results are expressed as percentage of the control response (mean ± SD, values in picograms are also given in *parentheses.*). E2 concentration was 10^{-7} M.

[a] $P < 0.05$, estradiol *vs.* control.

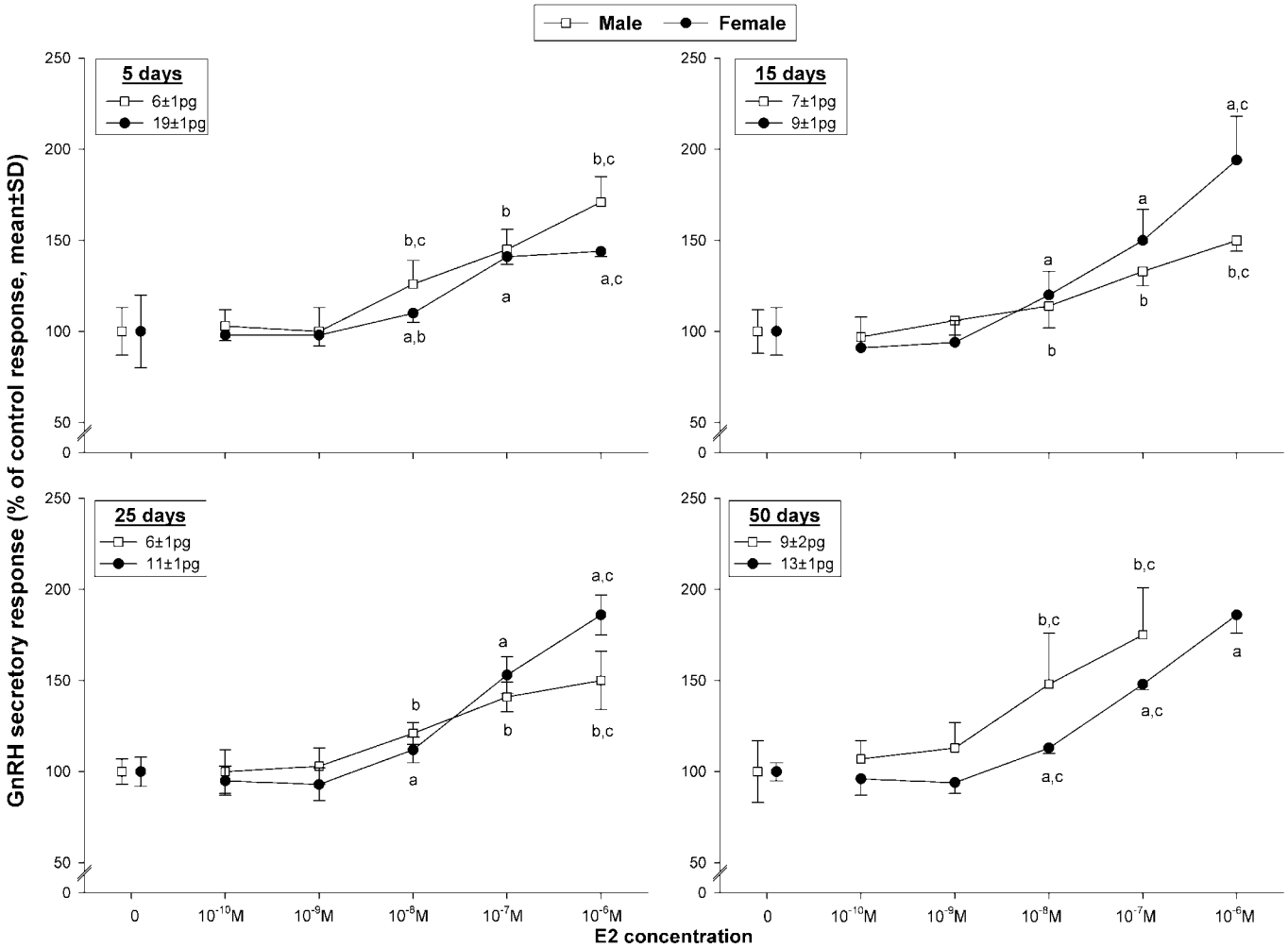

FIG. 5. Effects of increasing concentrations of E2 (10^{-10} to 10^{-6} M) on the kainate-evoked GnRH secretion *in vitro* using RCH hypothalamic explants obtained in male and female rats at 5, 15, 25, and 50 d of postnatal age. The results (mean ± SD) are expressed in percentage of the control response (kainate alone, 10^{-2} M) and E2 was added for two 7.5-min fractions, *i.e.* before and together with glutamate. The values for the GnRH secretory response evoked by the glutamate alone (control stimulation in picograms) are shown in the legend. a, $P < 0.05$, E2 *vs.* control in female; b, $P < 0.05$, E2 *vs.* control in male; c, $P < 0.05$, male *vs.* female.

Although the parameters of GnRH secretion studied here are different, the similarities in the requirement of ER and kainate receptors are striking.

Although the validity of the data obtained using our paradigm has been addressed previously, the possibility of excitotoxic effects due to the high glutamate concentrations used and the significance of glutamate-evoked *vs.* spontaneous GnRH secretion warrant some discussion. To our knowledge, in all the published studies using glutamate or NMDA/NMA to stimulate GnRH secretion from hypothalamic explants (4, 31), the required concentrations are in the 10- to 50-mM range, which is similar to those required in our paradigm, whereas the effective concentrations on cultured immortalized GnRH neurons are slightly lower (between 0.1 and 10 mM) (32). This discrepancy suggests that the bioavailability of glutamate receptor agonists is reduced in the explant incubation system when compared with a cell culture system. In our paradigm, we showed that the GnRH secretory response to a depolarization was preserved as long as NMA concentrations less than 100 mM were used (23). As shown in the present study, the sustained capacity of the explants to respond for several hours to repeated glutamate stimulation and the recovery of the initial response after several hours of incubation was consistent with an absence of excitotoxic effects of short-term glutamate stimulations. Another technical issue is whether endogenous secretory pulses could affect the response to a secretagogue added in the medium. As a matter of fact, a secretory episode evoked by veratridine depolarization (33) or glutamate receptor stimulation (34) has previously been shown to be followed by a period of refractoriness to endogenous or exogenous secretagogues. Such refractoriness results from an inhibitory autofeedback of the GnRH secretion involving the $GnRH_{(1–5)}$ subproduct, which is present in the medium after endopeptidase degradation of the decapeptide (34). The duration of that refractory period is age dependent and related to the developmental changes in the frequency of the pulsatile GnRH secretion (33, 34). According to those studies, the hypothalamic explants obtained from rats at different ages were stimulated every 37.5 min, which is outside the refractory period.

TABLE 5. Mean (±SD) GnRH secretory response evoked by glutamate (Glu), in the presence of E2 and modulators of intracellular calcium release ($[Ca^{2+}]i$, caffeine, cyclopiazonic acid), L-type calcium channel antagonist (nifedipine), or different protein kinase (PK) antagonists

n	Glu (10^{-2} M) + antagonist	Antagonist activity	Control response (pg)	E2 concentration (for 15 min)	
				0 (Control)	10^{-7} M
4	Caffeine (5×10^{-3} M)	$[Ca^{2+}]i$ depletor	14 ± 2	100 ± 12	178 ± 21[a]
				86 ± 6	161 ± 24[a]
4	Cyclopiazonic Ac (10^{-5} M)	SERCA inhibitor	15 ± 2	100 ± 11	185 ± 19[a]
				145 ± 3[c]	263 ± 34[a,b]
4	Nickel	Inhibitor of Ca^{2+} entry	11 ± 1	100 ± 8	155 ± 30[a]
				74 ± 8[c]	76 ± 0[b,d]
5	Nifedipine (2×10^{-6} M)	L-type calcium channel antagonist	10 ± 2	100 ± 15	147 ± 22[a]
				81 ± 14[c]	75 ± 15[b]
9	U73122 (2×10^{-6} M)	PLC inhibitor	14 ± 3	100 ± 22	150 ± 22[a]
				91 ± 26	93 ± 29[b]
4	Staurosporine (10^{-7} M)	PK inhibitor	12 ± 1	100 ± 7	150 ± 4[a]
				90 ± 4[c]	95 ± 4
5	Rp cAMP (10^{-5} M)	PKA inhibitor	35 ± 5	100 ± 15	168 ± 14[a]
				95 ± 15	95 ± 16[b]
5	Chel Chloride (10^{-5} M)	PKC inhibitor	12 ± 1	100 ± 7	150 ± 4[a]
				94 ± 4	90 ± 4[b]
5	PD 98056 (5×10^{-5} M)	MAPK (ERK1/2) inhibitor	11 ± 1	100 ± 7	155 ± 13[a]
				100 ± 12	102 ± 12[b]

The RCH explants (n) were obtained from 50-d-old male rats. The data are expressed in percentage of the control response (in picograms) obtained after stimulating the explants with glutamate alone.

[a] $P < 0.05$, control *vs.* E2 (either without or with the antagonist).

[b] $P < 0.05$, glutamate/E2/antagonist *vs.* glutamate/E2.

[c] $P < 0.05$, control/antagonist *vs.* control.

[d] The calculated secretory response for each explant gave the same result, and SD was thus zero.

Our present study shows that E2 increased the glutamate-evoked GnRH secretion in a dose-dependent manner. E2 was effective at concentrations between 10^{-8} and 10^{-6} M that were similar to those required to increase the frequency of pulsatile GnRH secretion *in vitro* (18). Although these concentrations were in the supraphysiological range, they are consistent with the concentrations that are reported by others to affect GnRH secretion *in vitro* (16), $[Ca^{2+}]i$ levels in GnRH neurons (15) or kainate-evoked currents in hippocampal (19) and oxytocinergic neurons (20). In addition, we also showed that lower concentrations of E2 (10^{-8} and 10^{-9} M) significantly increased the glutamate-evoked GnRH secretion when the explants were incubated with E2 for several hours. From these results, it can be inferred that the dose dependency of E2 effects is influenced by the exposure length of the explants to E2 and that effects such as those described here could require lower E2 concentrations in the *in vivo* conditions. Whereas the rapid E2 effects disappeared immediately when the steroid was no longer present, it seemed that the continuous and prolonged incubation of the explants with E2 initiated additional mechanisms that appeared to be activated within 3–4 h and persisted after removal of E2 from the medium. These two time-related components of the response to E2 could be consistent with an involvement of both rapid, nongenomic, and slow or genomic mechanisms.

The E2 effects on the glutamate-evoked GnRH secretion were steroid and stereo specific, and similar effects could be observed with testosterone. The effects of testosterone were prevented by an aromatase inhibitor, which indicates that testosterone was effective after its aromatization into E2. These data are in agreement with the localization of the aromatase enzyme (35) and mRNA (36) in the arcuate and ventromedial hypothalamic nuclei.

It has been recently shown that GnRH neurons express ERβ (11). Our experiments indicate that E2 increased the glutamate-evoked GnRH secretory response through an ER-mediated mechanism because the ER antagonists tamoxifen and ICI 182.780 prevented E2 effects. However, our data using the specific ERα agonist PPT (26) suggest that the ERα subtype is involved in E2 effects. The higher effective concentrations of PPT ($\geq 10^{-6}$ M) were consistent with the concentrations 10–100 times higher than E2 that are needed to achieve effects similar to E2 either *in vitro* (38) or *in vivo* (39). Further studies using specific ERα antagonists and/or ERβ agonists will be needed to verify that ERα is the only ER subtype involved in E2 effects.

E2 can rapidly act in various biological systems through the activation of secondary effectors such as $[Ca^{2+}]i$, cAMP, and/or protein kinases (for review see Ref. 40). Because it was shown that E2 cannot directly induce kainate receptor opening as it does at the NMDA receptors in hippocampal neurons (41), the E2 effects could possibly involve an indirect effect through activation of phosphorylation processes that would subsequently activate kainate receptors. Here E2 was found to increase the glutamate-evoked GnRH secretion through activation of protein kinases as E2 effects were prevented using specific inhibitors of PKA, PKC, and MAPK. A slight inhibitory effect of the protein kinase inhibitor staurosporine on the glutamate-evoked GnRH secretion was also observed, which is agreement with the involvement of PKA and PKC in the GnRH secretory process that has been described in GnRH cell lines (42). The upstream effectors to the protein kinase cascade could include the entry of calcium from the extracellular environment and a subsequent increase in $[Ca^{2+}]i$ level because blocking of the L-type calcium channel by nifedipine or the inhibition of extracellular Ca^{2+}

entry (by nickel) prevented E2 effects on the GnRH secretory response to glutamate. In addition, the inhibitor of SERCA pumps, CA (28), significantly increased the GnRH secretory response evoked by glutamate alone as well as the E2-potentiating effect on that response. After a glutamate stimulation, the $[Ca^{2+}]$i levels would return to basal levels through the action of Ca^{2+} pumps, such as the SERCA, that will reconstitute the $[Ca^{2+}]$i stores. In the presence of a SERCA inhibitor, there will be a sustained increase in $[Ca^{2+}]$i levels that would account for the increased GnRH secretory response to glutamate that was observed. However, the effect of CA was additive to E2 effect, which indicates that the increase in $[Ca^{2+}]$i levels responsible for E2 effects on the glutamate-evoked GnRH secretion would mainly be of extracellular origin.

In addition to the effects on the glutamate-evoked GnRH secretion, we also found that E2 could increase the L-Arg-evoked GnRH secretory response. This effect could be due to an increase in NO release because E2 has been reported to stimulate NO synthase (43). This hypothesis is supported by the study of Prevot *et al.* (16) reporting that nanomolar concentration of E2 increased the basal GnRH secretion *in vitro* from ME through an increase in NO release. We reported here that the E2-induced increase in the NO-mediated GnRH secretion was dependent on kainate receptor function, suggesting an indirect interaction between E2 and NO. Thus, our data suggest the existence of a still-unidentified link between the intracellular E2-ER complex and the kainate receptors that could mediate NO effects as well.

A possible cross-talk point between E2 and kainate receptor could involve the MAPK pathway because MAPK phosphorylation after E2 or kainate stimulation has been reported in neurons (44), glial cells (45, 46), or endothelial cells (43). Such effects require the activation of PKC (44) or phospholipase C (45) that were also involved in E2 potentiation of glutamate-evoked GnRH secretion. Recently ER subtypes have been identified in GnRH neurons (for review, see Ref. 14), and direct effects of E2 on GnRH neurons were reported (13, 15). However, in our conditions, a direct action of E2 on GnRH cell bodies is unlikely because E2 increased the glutamate-evoked GnRH secretion obtained from retrochiasmatic explants in which GnRH cell bodies are virtually absent (27). In addition, E2 was found to be effective in ME explants. Similar effect of E2 on GnRH secretion at the ME was also reported by others (16), which supports the idea that E2 can act at the level of the ME with possible involvement of glial or endothelial cells. Consistent with this latter hypothesis, the kainate receptor subunits GluR7 mRNA (47) and GluR5–6 protein (47, 48) have been reported in glial cells of the ME (48). The detection of ER in glial cells of the ME (49) and the fact that, in our experiments, ERα is involved further supports the concept of glial cells as mediators of E2 effects on the glutamate-evoked GnRH secretion. Moreover, GnRH secretion can be modulated by glial cells (50), and E2 can increase GnRH secretion *in vitro* through the astrocytic release of growth factors such as TGFβ (51). In our conditions, the involvement of ERα in E2 effects suggests that they are more likely to be mediated through intermediate cells because there is increasing evidence that ERβ are the receptors mediating direct effects of E2 on GnRH neurons (14, 15, 52).

We recently described that the frequency of pulsatile GnRH secretion from RCH explants could be stimulated by E2 through a kainate- and ER-dependent mechanism (18). Importantly, such a mechanism did operate in the female only before sexual maturation (5 and 15 d), in contrast to the E2 amplification of the glutamate-evoked secretion of GnRH that occurred in both sexes and at every age studied. In our conditions, pulsatile GnRH secretion cannot be endogenously generated from isolated ME explants and requires either RCH neurons impinging on GnRH axons/terminals or GnRH cell bodies (27). We therefore hypothesized to a dual E2- and kainate receptor-mediated effect on GnRH secretion. One is located in the ME and involves the terminal GnRH secretory processes, irrespective of sex and developmental stage. Another one is located in the RCH and modulates the frequency of the GnRH pulsatile secretion in a sex- and developmental-dependent manner. We could then hypothesize that a possible sexual dimorphism in the response to the glutamate stimulation is blunted by the supraphysiological concentrations of E2 that were used to study E2 rapid effects. This hypothesis is consistent with the fact that E2 can act at lower concentrations if the incubation period is increased. Further studies are warranted to investigate the duality of E2 effects on the glutamate-evoked GnRH secretion.

Glutamate is a major excitatory amino acid in the neuroendocrine functions (2) and has been reported to increase GnRH secretion either *in vivo* (6) or *in vitro* (3–5). Glutamate has also an important role in the estrus cycle because glutamate receptor antagonists can block the preovulatory LH surge when administered *in vivo* (53–55). Because we reported a terminal effect of E2 on the ME, we could hypothesize that the principal cell type involved in E2 effect could be the tanycytes and/or endothelial cells that both have been involved in the occurrence of the preovulatory surge (56, 57). In the case of tanycytes, their involvement in E2 effect on the glutamate-evoked GnRH secretion is supported with the localization of ERs (37) at the level of the ME, together with the colocalization of kainate receptor subunits with tanycytes markers (48). These observations are in favor of an E2/ER/NO/kainate receptor-mediated pathway stimulating the release of GnRH at the ME level, with a possible relevance to the occurrence of a preovulatory surge. According to our data, it is, however, too early to elaborate a complete mechanistic scheme.

In conclusion, using rat hypothalamic explants, E2 rapidly increased the glutamate-evoked GnRH secretion in a dose- and steroid-dependent manner. This E2 effect involved kainate and ERs and required protein kinase activation. Through such a mechanism, E2 rapidly modulated GnRH secretion in both sexes and would likely imply interactions between GnRH terminals and interneurons and glial or endothelial cells located in the ME. Further studies will aim at elucidating the intracellular mechanisms linking the E2-ER complex and kainate receptor stimulation as well as the localization of these effects.

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