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Long-Term Superfusion of Rat Pituitary Cells: Interaction of 17β-Estradiol with Pulses of Gonadotropin-Releasing Hormone on Luteinizing Hormone Release[1] (41946)

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Abstract. In a series of four experiments, the temporal development of acute inhibitory and delayed stimulatory effects of 17β-estradiol (E) on luteinizing hormone (LH) release by superfused rat anterior pituitary cells pulsed with gonadotropin-releasing hormone (GnRH) was studied. Dispersed anterior pituitary cells from ovariectomized rats were cultured on Bio-Beads for 3 days and then placed in columns and superfused for up to 24 hr. During superfusion, the cells were exposed to GnRH pulses (3×10^{-9} *M*, one 6-min pulse/hr). Cells treated with E (3×10^{-10} *M*) either before (only 24 hr prior to superfusion) or before and during superfusion released significantly ($P < 0.05$) more LH in response to the first few pulses of GnRH than cells treated with diluent. In contrast, cells treated with E only during superfusion initially released less GnRH-induced LH than cells treated with diluent. In a subsequent experiment, the inhibitory effect of E reached a maximum by 1.5 hr ($P < 0.01$), and then gradually disappeared after 4.5 hr. Cells superfused simultaneously with E and fixed "low"-dose GnRH (5×10^{-10} *M*) pulses did not exhibit enhanced LH responses with time to that dose of GnRH. However, E-superfused cells responded more than diluent-superfused cells to subsequent stimulation with a higher-dose GnRH pulse. Superfusion of cells with E for 16.5 hr in the absence of GnRH pulses also did not increase release of LH to low-dose (5×10^{-10} *M*) pulses of GnRH, yet did cause a transitory increase to subsequent high-dose (10^{-8} *M*) GnRH pulses. In conclusion, these results demonstrate the direct biphasic inhibitory then stimulatory effects of E on GnRH-induced LH release by superfused rat anterior pituitary cells. Expression of the stimulatory effect of E is related to the dose of GnRH. © 1984 Society for Experimental Biology and Medicine.

The physiological pattern of luteinizing hormone (LH) secretion during the reproductive cycle is pulsatile (1–4), resulting from episodic release of gonadotropin-releasing hormone (GnRH) from the hypothalamus (5, 6). Estrogen (E) alters pituitary LH secretion by modulating both pituitary responsiveness to GnRH and secretion of hypothalamic GnRH during the estrous cycle of the rat (7, 8). Several *in vivo* studies suggest that E acts directly on the anterior pituitary in a biphasic manner to modulate LH release in response to GnRH (9–12).

Although studies using *in vitro* static cultures of rat pituitary cells have confirmed a direct stimulatory effect of E on LH secretion (13–18), these studies have not consistently revealed an early inhibitory effect of E (13, 17–19). Recent reports on short-term superfusion of pituitary fragments (20, 21) or pituitary cell cultures (18) demonstrated an early transient inhibitory effect of GnRH-induced LH release. The delayed stimulatory effect of E on cell responsiveness to GnRH has not been examined in superfusion studies. Previously, we developed an *in vitro* pituitary cell superfusion system to mimic the *in vivo* pulsatile pattern of LH secretion over a 24-hr period (22). Here we describe the biphasic effect of E on LH release by pituitary cells superfused in the presence of GnRH pulses for periods of up to 24 hr.

Materials and Methods. *Preparation and superfusion of cell-bead cultures.* Preparation and superfusion of cells were performed as described previously (22). Anterior pituitaries were obtained from female Sprague–Dawley rats (Holtzman Co., Madison, Wisc.; 160–180 g body wt) by decapitation 12 days after ovariectomy. Pituitaries were cut into fragments, dispersed by collagenase and hyaluronidase following brief exposure to trypsin,

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and then cultured on Bio-Beads (Bio-Rad Laboratories, Richmond, Calif.) in supplemented medium 199 (Grand Island Biological Co., Grand Island, N.Y.) containing 10% ovariectomized rat serum and 2.5% fetal bovine serum. Both sera were pretreated with dextran–charcoal. Cells were cultured for 62–66 hr before transfer to superfusion columns. Eight columns, each containing approximately 10^7 cells from three to four pituitaries, were superfused simultaneously with a serum-free low-$NaHCO_3$ superfusion medium (22). The effluent from each column was collected into 12- or 15-min fractions.

Experiments. *Experiment 1.* We examined both the immediate and "long-term" temporal effects of E on GnRH-induced LH release. Dispersed pituitary cells were first cultured on beads for 40 hr. Then, the cells were cultured with either E (3×10^{-10} *M*) or diluent (0.03% ethanol) for an additional 24 hr. Four columns each were prepared from cell-bead cultures preincubated with either E or diluent. Two columns from each of the E or diluent incubated groups were superfused with medium containing E (3×10^{-10} *M*), while the other two columns from each group were superfused with medium containing diluent. Two hours after beginning the superfusion, GnRH (Beckman Instruments, Palo Alto, Calif.) (3×10^{-9} *M*), with or without E, was introduced into corresponding columns as one 6-min pulse/hr for the next 22 hr. Thus, there were four treatments: E before GnRH, E before and during GnRH, E during GnRH, and no E (control).

Experiment 2. We examined more closely (i) the possible transitory inhibitory effect of E on LH release induced by pulses of GnRH, and (ii) the possible interaction of E and dose of GnRH on release of LH. Eight superfusion columns were prepared from the cell-bead cultures. Four columns were superfused with medium containing E (3×10^{-10} *M*) while the other four columns were superfused with medium containing the diluent. Two hours after beginning the superfusion, GnRH (3×10^{-9} *M*), with or without E, was introduced into corresponding columns as one 6-min pulse/hr. After 23 hr of E superfusion (i.e., GnRH pulse 22), the superfusion pumps were turned off and the concentration of GnRH in each column was immediately raised to approximately 10^{-7} *M* by direct injection of a 100-μl bolus of 10^{-6} *M* GnRH into each column. After 6 min of incubation, the columns were superfused again with GnRH-free medium for 1 hr.

Experiment 3. We modified Experiment 2 to determine the effect of an abrupt increase of E concentration on LH release induced by GnRH pulses. We used low-amplitude GnRH pulses in order to maintain cell responsiveness to GnRH (22). Near the end of the superfusion, we increased pulse duration and amplitude to further examine the possible interaction of E and GnRH pulse parameters on LH release after prolonged exposure to E. Eight columns of cells were superfused without E for 2 hr. Then, the first pulse of GnRH (5×10^{-10} *M*) was administered in the absence of E. At 0.5 hr prior to the second pulse of GnRH, four of the eight columns were superfused with medium containing E (6×10^{-10} *M*). GnRH was then applied as one 6-min pulse/hr for 21 pulses. At the 22nd pulse, GnRH pulse duration was increased to 30 min followed by a 30-min superfusion without GnRH. Then the amplitude of pulse 23 was increased by raising the concentration of GnRH to 10^{-8} *M* for 30 min followed by a 30-min superfusion without GnRH.

Experiment 4. We examined the effects of E and GnRH-pulse amplitude on LH release after a period of superfusion with E in the absence of GnRH pulses. Eight columns of cells were superfused with E-free medium for 2.5 hr. Starting at 2.5 hr, four of the columns were superfused with medium containing E (6×10^{-10} *M*), while the other four columns were superfused with medium containing diluent. After 16.5 hr of E or diluent exposure, all columns were exposed to four hourly 6-min pulses of 5×10^{-10} *M* GnRH, with or without E. This was followed by three additional hourly 6-min pulses of 10^{-8} *M* GnRH (given at 20.5 to 22.5 hr after the start of E exposure).

Measurement of cellular and medium LH. Both LH secreted by the cells during superfusion and content of LH remaining in the cells after superfusion were measured by radioimmunoassay as described previously (22). NIH-oLH-S20 was the reference standard.

Data presentation and analysis. Data are presented as net LH release/pulse of GnRH (22). All experiments were analyzed as split-plots in time using analysis of variance for repeated measures (24). Significant treatment effects were further examined by the Newman–Keuls test. Student's *t* test was used to determine differences in cellular LH content and response to the bolus challenge of 10^{-7} *M* GnRH.

Results. *Experiment 1.* Analysis of variance of the LH responses to GnRH pulses revealed significant effects of both time and E treatment as well as a significant interaction between time and E treatment ($P < 0.01$, Fig. 1). Preincubation of pituitary cells with 17β-estradiol (E, 3×10^{-10} *M*) for 24 hr potentiated release of LH by cells subsequently exposed to pulses of GnRH. The response to the first three GnRH pulses by all E-preincubated cells (E_B, E_{BA}) was 170 to 230% ($P < 0.05$) of release by control cells (C, preincubated and superfused without E). Thereafter, the stimulatory effect of E declined and disappeared. Cells preincubated with E only before superfusion (E_B) exhibited a temporal response pattern similar to that of cells exposed to E both before and during superfusion (E_{BA}). Cells preincubated with diluent and exposed to E only during the superfusion (E_A) appeared to release less LH initially than the control cells. However, the difference was statistically insignificant ($P > 0.05$).

Experiment 2. Results of this experiment illustrated the acute inhibitory and delayed stimulatory effects of E superfusion on LH release by diluent-preincubated cells pulsed with GnRH (Fig. 2). E superfusion significantly reduced ($P < 0.05$) net LH release in response to the initial three GnRH pulses given 2 to 4 hr after the onset of exposure to E. Thereafter, control and E-superfused cells exhibited similar responses to 3×10^{-9} *M* GnRH pulses. The main effects of E, time, and the interaction between E and time were significant ($P < 0.01$). E treatment significantly ($P < 0.01$) increased LH release in response to the bolus challenge of 10^{-7} *M*

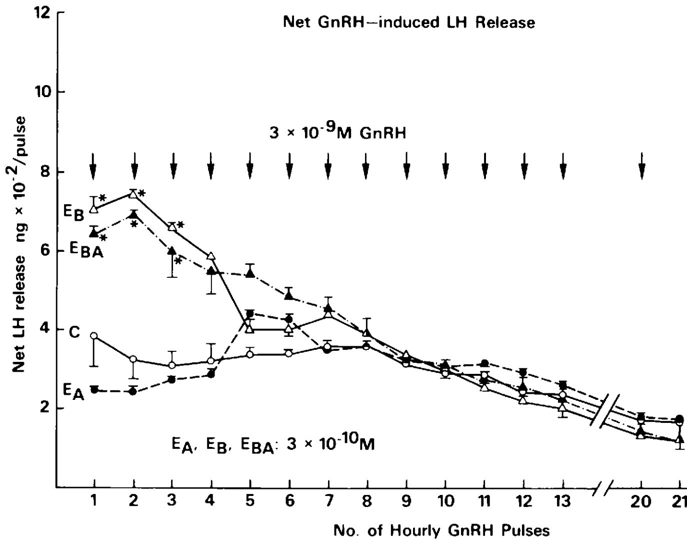

FIG. 1. Effects of 17β-estradiol (E) treatments before (E_B), before and after (E_{BA}), or after (E_A) the start of superfusion on GnRH-induced LH release by superfused pituitary cells. Pituitary cell-bead cultures were preincubated for 24 hr with or without E (3×10^{-10} *M*), then packed in eight columns (9.7×10^6 cells per column) and superfused with media containing E or diluent for 2 hr before the first GnRH pulse (3×10^{-9} *M*, one 6-min pulse every hr). See details in Materials and Methods (Experiment 1). C, control cells (neither preincubated nor superfused with E); * $P < 0.05$ vs corresponding control cells at the same GnRH-pulse number. Each point represents the mean ± SEM of two replicate columns. SEM is not shown when it is too small to be legibly graphed. Individual samples were not collected between pulse 14 and pulse 19, although GnRH pulses were administered.

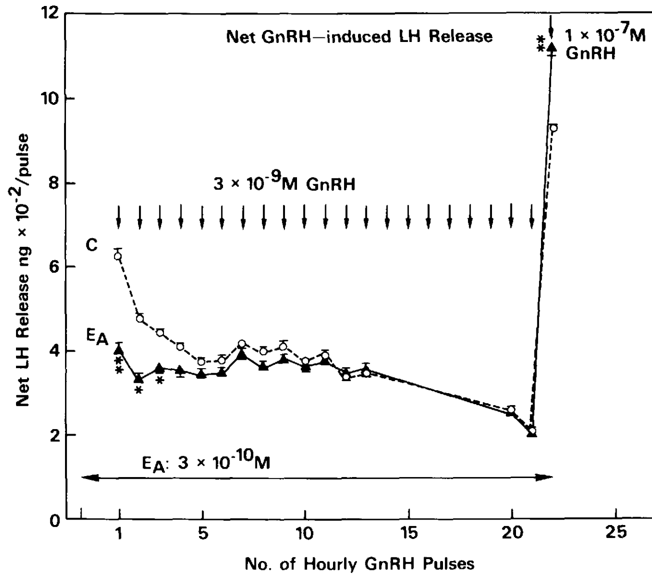

FIG. 2. Biphasic effect of E superfusion on cell responsiveness to GnRH pulses. Eight columns of pituitary cell-bead cultures (9.9×10^6 cells per column) were superfused with or without E (3×10^{-10} *M*) for 2 hr before the first pulse of GnRH (3×10^{-9} *M*, one 6-min pulse every hr). Each point represents the mean ± SEM of four replicate columns. E_A, cells treated with E after the start of superfusion; C, control cells; * $P < 0.05$; ** $P < 0.01$ vs corresponding control cells at the same GnRH pulse number. Individual samples were not collected between pulse 14 and pulse 19.

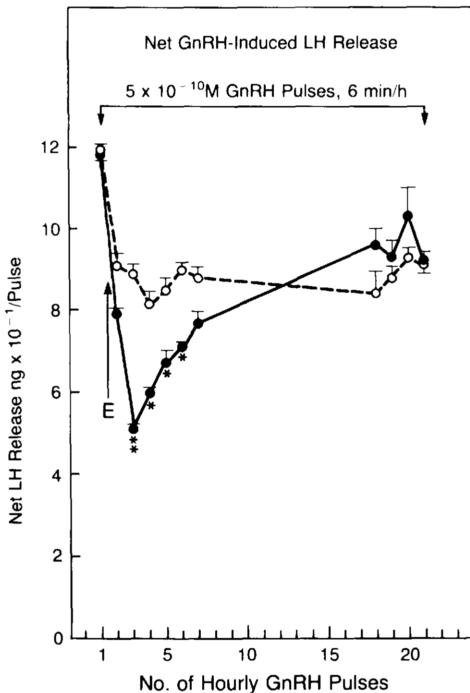

FIG. 3. Time course of the inhibitory effect of E on cell responsiveness to GnRH pulses. Eight columns of pituitary cell-bead cultures (9.4×10^6 cells per column) were superfused without E for 2 hr before the first pulse of GnRH (5×10^{-10} *M*, one 6-min pulse per hr). Then, half of the eight columns were exposed to E (6×10^{10} *M*) 0.5 hr before the second pulse of GnRH containing E (solid line), while the other half (control cells, broken line) were superfused without E and pulsed with GnRH containing no E. Each point represents the mean ± SEM of four replicate columns. * $P < 0.05$; ** $P < 0.01$ vs corresponding control cells. Individual samples were not collected between pulse 8 and pulse 17.

GnRH given after 23 hr of E exposure (pulse 22). At the end of superfusion, control and E-superfused cells contained similar ($P > 0.05$) amounts of LH (18.7 ± 0.3 and 17.9 ± 0.6 $\mu g/10^7$ cells, respectively).

Experiment 3. In this experiment, we demonstrated the rapid onset of the inhibitory effect of E superfusion on cell responsiveness to repeated GnRH pulses (5×10^{-10} *M*, 6 min/hr) (Fig. 3). Maximum E inhibition ($P < 0.01$) was observed 1.5 hr after E (GnRH pulse 3). Thereafter, responsiveness of E-superfused cells to subsequent GnRH pulses (pulses 4 to 6) increased, but was still lower

than that of control cells ($P < 0.05$). Beginning at 5.5 hr (pulse 7), E-superfused and control cells responded similarly ($P > 0.05$) to repeated GnRH pulses up to 19.5 hr of E exposure (pulse 21). Increasing pulse duration of GnRH (5×10^{-10} *M*) from 6 to 30 min after 20.5 hr of E exposure yielded identical GnRH-induced LH release by E-superfused and control cells (Fig. 4). However, when pulse amplitude was increased by increasing the concentration of GnRH to 10^{-8} *M*, E-treated cells released significantly ($P < 0.01$) more LH than control cells (Fig. 4). At the end of superfusion cellular LH contents were not significantly ($P > 0.05$) different for control and E-superfused cells (21.2 ± 0.2 vs 21.5 ± 0.2 μg/10^7 cells, respectively).

Experiment 4. In the absence of GnRH pulses, cells exposed to E only during superfusion developed a transitory increase in response to subsequent challenge with GnRH (Fig. 5). The expression of an effect of E appeared to depend on the dose of GnRH. Estrogen-superfused and control cells responded similarly ($P > 0.05$) to the first four low-amplitude GnRH pulses (5×10^{-10} *M*, 6 min/hr) given after 16.5 to 19.5 hr of E exposure. E had no significant main effect ($P > 0.05$) on LH release in response to the three 10^{-8} *M* GnRH pulses given after 20.5 to 22.5 hr of E exposure. However, the time effect ($P < 0.01$) and interaction ($P < 0.05$) between time and E were significant. Analysis of simple effects showed that E increased ($P < 0.05$) the response to the first pulse of 10^{-8} *M* GnRH but not the response to subsequent pulses. Neither group of cells showed a temporal decline in responsiveness to either 5×10^{-10} or 10^{-8} *M* GnRH pulses. The tem-

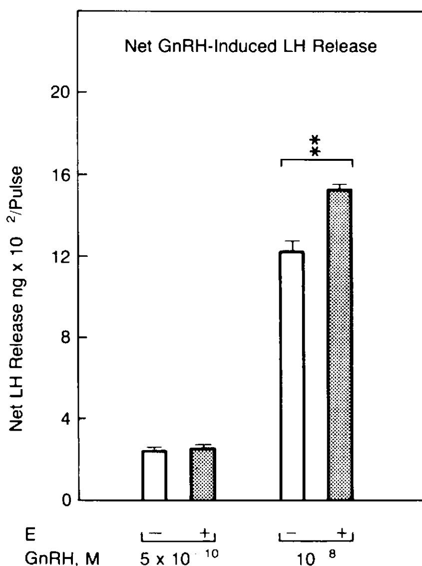

FIG. 4. Effect of E superfusion on cell responsiveness to 30-min GnRH pulses of two amplitudes. After the 21st pulse of GnRH at 5×10^{-10} *M* for 6 min/hr (Fig. 3), cells were exposed to 5×10^{-10} *M* GnRH for 30 min, followed by a 30-min superfusion without GnRH. Then cells were exposed to 10^{-8} *M* GnRH for 30 min followed by a 30-min superfusion without GnRH. Each bar represents the mean ± SEM of four replicate columns. ** $P < 0.01$.

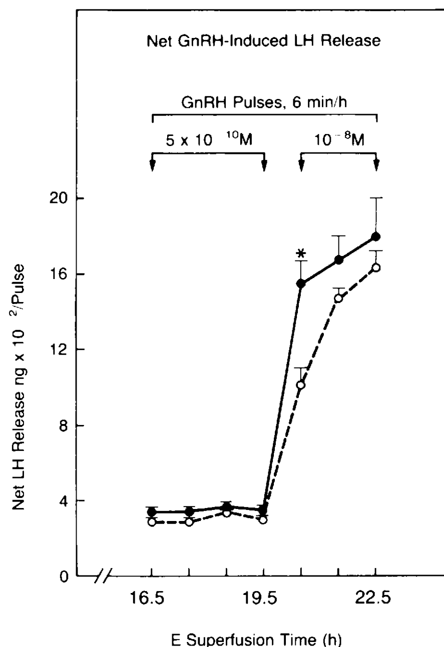

FIG. 5. Effect of E superfusion without concomitant GnRH pulses on cell responsiveness to two challenge doses of GnRH. Eight columns of pituitary cell-bead cultures (9.3×10^6 cells per column) were superfused without E for 2.5 hr then half of the eight columns were superfused with E (6×10^{-10} *M*) while the other half were superfused without E for 16.5 hr. No GnRH pulses were given during the pretreatment period. Then, four GnRH pulses (5×10^{-10} *M*, 6 min/hr) with or without E were given to corresponding cells at 16.5 to 19.5 hr of E exposure. Another three GnRH pulses (10^{-8} *M*, 6 min/hr) were given at 20.5 to 22.5 hr of E exposure. Each point represents the mean ± SEM of four replicate columns. Broken line: control cells not superfused with E; solid line: E-superfused cells. *$P < 0.05$ vs corresponding control cells.

poral increase in response to the three pulses of 10^{-8} M GnRH contrasted with the declining responses that occurred when GnRH pulses were given 2 to 3 hr after the start of superfusion (22) (Figs. 2, 3). At the end of superfusion, control and E-superfused cells contained similar ($P > 0.05$) amounts of LH (32.3 ± 1.6 vs 31.7 ± 1.1 μg LH/10^7 cells, respectively).

Discussion. These results demonstrate that 17β-estradiol has a direct biphasic effect on the response of rat anterior pituitary cells to pulses of GnRH—first inhibitory, then stimulatory. The onset of inhibition was rapid, reached a maximum by 1.5 hr, and disappeared after 4.5 hr of exposure to E. The stimulatory effect then developed gradually. Demonstration of the stimulatory effect appeared to depend on the specific conditions under which exposure to E occurred and on the dose of GnRH.

The temporal pattern of the inhibitory effect of E on pituitary responsiveness to GnRH confirms previous *in vivo* (10, 11) and *in vitro* (18–21) studies. Although demonstrable under a variety of conditions, the physiological significance of this rapid onset, transitory, inhibitory effect of E on GnRH-induced LH release is not known.

In contrast to the inhibitory effect, the stimulatory effect requires 8–12 hr to develop either *in vivo* (10, 11) or *in vitro* (13, 17). Results of studies using static cultures suggest that the increased responsiveness plateaus between 16 and 22 hr. In our experiments, pituitary cells were exposed to E for at least 16.5 hr, a period sufficient to induce maximal responsiveness in static cultures (13, 17).

Development of the stimulatory effect of E on cells *in vitro* appears to depend on specific culture conditions. This effect is readily demonstrable when cells are exposed to E under static culture conditions in the absence of GnRH (13–18) (this study, Fig. 1). However, we found that expression of this effect by superfused cells was strongly influenced by dose and pattern of GnRH.

In the first experiment (Fig. 1), cells superfused simultaneously with E and fixed high-dose GnRH pulses did not show an increased response to that dose of GnRH with time. Instead, the cells were desensitized to GnRH. Thus, even if E were exerting an effect, it probably could not be expressed. Our previous studies (22) suggest that desensitization resulted from the relatively high dose of GnRH. Another perspective on these results is that E will not prevent desensitization of gonadotrophs to high doses of GnRH.

In Experiments 2 and 3, we again did not observe a temporal increase in LH release in the presence of E and constant-dose GnRH pulses, even though in Experiment 3 we used a dose of GnRH which previously showed no indication of desensitizing the cells. However, in both experiments, E-superfused cells responded more than diluent-superfused cells to subsequent stimulation with a higher dose of GnRH. These results, which agree with the *in vivo* studies of Schuiling and Gnodde (25) and van Dieten and van Rees (26) suggested that the sensitizing effect of E cannot be demonstrated in the presence of GnRH pulses of the same amplitude. Thus, in Experiment 4, we exposed the cells to E on the column for 16.5 hr in the absence of GnRH. Surprisingly, they did not show an increased response to subsequent low doses of GnRH; however, they showed a greater response to the first pulse of high-dose GnRH than the E untreated cells. The greater responsiveness, however, was transitory. Overall, these results suggest that demonstration of the sensitizing effect of E on GnRH-induced LH release appears to depend on both specific cell culture conditions and dose of GnRH.

An ancillary observation made in Experiment 4 was that the responses to the second and third pulses of the high dose of GnRH were greater than the response to the first pulse. This pattern of temporally increased responses to subsequent pulses contrasted to that of cells which had not been exposed to GnRH for prolonged periods. (22) (this study, Fig. 1) In the latter situation, we consistently observed that the responses to the first pulse were greater than the responses to subsequent pulses. The explanation for the different temporal pattern in responses is not known. However, one possibility is that prolonged absence of exposure to GnRH results in a transient hypersensitivity of the gonadotroph to the first GnRH pulse.

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